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Corentin Louis

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L'UNIVERSITE DE RENNES

ECOLE DOCTORALE N° 637
Sciences de la Vie et de la Santé
Spécialité : Cancérologie

Par

Corentin LOUIS

**Les ARN circulaires régulés par le TGF β dans le cholangiocarcinome
intrahépatique : mécanismes et biomarqueurs**

Thèse présentée et soutenue à Rennes, le 20 mars 2023

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Liste des publications

Les articles en premier auteur sont disponibles dans la section Annexes.

I- Revues

1°) **Louis, C.***, Leclerc*, D., and Coulouarn, C. (2022). Emerging roles of circular RNAs in liver cancer. *JHEP Rep* 4, 100413. 10.1016/j.jhepr.2021.100413.

2°) **Louis, C.**, Edeline, J., and Coulouarn, C. (2021). Targeting the tumor microenvironment in cholangiocarcinoma: implications for therapy. *Expert Opin Ther Targets* 25, 153-162. 10.1080/14728222.2021.1882998.

3°) **Louis, C.***, Papoutsoglou, P.*, and Coulouarn, C. (2020). Molecular classification of cholangiocarcinoma. *Curr Opin Gastroenterol* 36, 57-62. 10.1097/MOG.0000000000000611.

4°) Papoutsoglou, P.*, **Louis, C.***, and Coulouarn, C. (2019). Transforming Growth Factor-Beta (TGFbeta) Signaling Pathway in Cholangiocarcinoma. *Cells* 8. 10.3390/cells8090960.

II- Commentaires

5°) **Louis, C.**, and Coulouarn, C. (2022). One stone, two birds: circACTN4, a nexus for a coordinated activation of Hippo and Wnt/beta-catenin pathways in cholangiocarcinoma. *J Hepatol* 76, 8-10. 10.1016/j.jhep.2021.10.002.

6°) **Louis, C.**, Desoteux, M., and Coulouarn, C. (2019). Exosomal circRNAs: new players in the field of cholangiocarcinoma. *Clin Sci (Lond)* 133, 2239-2244. 10.1042/CS20190940.

III- Travaux collaboratifs

7°) Bevant, K., Desoteux, M., Angenard, G., Pineau, R., Caruso, S., **Louis, C.**, Papoutsoglou, P., Sulpice, L., Gilot, D., Zucman-Rossi, J., and Coulouarn, C. (2022). TGFbeta-induced FOXS1 controls epithelial-mesenchymal transition and predicts a poor prognosis in liver cancer. *Hepatol Commun* 6, 1157-1171. 10.1002/hep4.1866.

8°) Desoteux, M.*, **Louis, C.***, Bevant, K., Glaise, D., and Coulouarn, C. (2021). A Minimal Subset of Seven Genes Associated with Tumor Hepatocyte Differentiation Predicts a Poor Prognosis in Human Hepatocellular Carcinoma. *Cancers (Basel)* 13. 10.3390/cancers13225624.

9°) Lobe, C., Vallette, M., Arbelaiz, A., Gonzalez-Sanchez, E., Izquierdo, L., Pellat, A., Guedj, N., **Louis, C.**, Paradis, V., Banales, J.M., et al. (2021). Zinc Finger E-Box Binding Homeobox 1 Promotes Cholangiocarcinoma Progression Through Tumor Dedifferentiation and Tumor-Stroma Paracrine Signaling. *Hepatology* 74, 3194-3212. 10.1002/hep.32069.

10°) Angenard, G., Merdrignac, A., **Louis, C.**, Edeline, J., and Coulouarn, C. (2019). Expression of long non-coding RNA ANRIL predicts a poor prognosis in intrahepatic cholangiocarcinoma. *Dig Liver Dis* 51, 1337-1343. 10.1016/j.dld.2019.03.019.

IV- Soumis

11°) **Louis, C.**, Pineau, R., Papoutsoglou, P., Desotteux, M., Leclerc, D., Leroux, R., Rocio, M., Edeline, J., Coulouarn, C. (2023). TGFβ-induced circLTBP2 predicts a poor prognosis in intrahepatic cholangiocarcinoma and mediates gemcitabine resistance by sponging miR-338-3p. *J Hepatol*.

V- Pour soumission

12°) Robin, F. *, **Louis, C.***, Cros, J., Bachet J B., Coulouarn, C.*, Sulpice, L*. Distal bile duct cancer and pancreatic ductal adenocarcinoma: Call for a new definition of periampullary tumors.

13°) Papoutsoglou, P., **Louis, C.**, Pineau, R., L'Haridon, A., Banales, JM., Gilot, D., Aubry, M. and Coulouarn, C. TGF β -induced long non-coding RNA LINC00313 activates Wnt signalling and promotes cholangiocarcinoma.

14°) Desoteux, M., Maillot, B., Bévant, K., Angenard, G., **Louis, C.**, Ferlier, T., Raffaële Leroux, Sulpice, L., Boudjema, K. and Coulouarn, C*. Clinical relevance of distinct TGF β pathway inhibition signatures in patients with liver cancer.

Liste des abréviations

5-FU : 5-fluorouracile	CSC : cellules souches cancéreuses
AA : acides aminés	CSH : cellules stellaires hépatiques
ACE : antigène carcino-embryonnaire	CSP : cholangite sclérosante primitive
ASC : active symptom control	CTGF : connective tissue growth factor
AFP : alpha-fœtoprotéine	DAPK1 : death-associated protein kinase 1
ALDH1 : aldéhyde déshydrogénase 1	E-ARNcirc : ARNcirc exonique
α-SMA : α-smooth muscle actin	EI-ARNcirc : ARNcirc exonique-intronique
ARNcirc : ARN circulaires	FAC : fibroblastes associés au cancer
ARNInc : ARN longs non codants	FGFR2 : fibroblast growth factor receptor 2
ARNnc : ARN non codant	FDA : food and drug administration
ARNm : ARN messenger	GEO : gene expression omnibus
α-SMA : alpha-smooth muscle actin	GSK3β : glycogen synthase kinase
ASO : oligonucléotides antisens	IL-6 : interleukine-6
CA19-9 : antigène carbohydate 19-9	ID : intraductal
CCA : cholangiocarcinome	IDH1/2 : isocitrate déshydrogénase 1/2
CCAe : cholangiocarcinome extrahépatique	IL-6 : interleukine-6
CCAd : cholangiocarcinome distal	INCa : institut national du cancer
CCAi : cholangiocarcinome intrahépatique	IRES : sites d'entrée internes des ribosomes
CCAp : cholangiocarcinome périhilaire	LAP : latency-associated peptide
CCND1 : cycline D1	LTBP : protéines de liaison au TGFβ latentes
CD : cellules dendritiques	MAPK : MAP kinase
CDH1 : E-cadhérine	MAT : macrophages associés aux tumeurs
CDH2 : N-cadhérine	MEC : matrice extracellulaire
CDK : kinase dépendante de la cycline	MDSC : cellules myéloïdes suppressives
CEL : cellules endothéliales lymphatiques	miARN : micro ARN
CHC : carcinome hépatocellulaire	MMP9 : métalloprotéinase 9
CK19 : cytokératine 19	
CML : calcium-modulating ligand	

MT : micro-environnement tumoral

NAFLD : non-alcoholic fatty liver disease

NASH : non-alcoholic steatohepatitis

NK : natural killer

OR : odd ratio

OS : survie globale

PAF : protéine d'activation des fibroblastes

PD : periductal

PD-1 : programmed-death-1

PDGFR β : platelet-derived growth factor receptor beta

PD-L1 : programmed-death ligand 1

PDK1 : phosphoinositide-dependent kinase

PI3K : phosphatidyl-inositol-3' kinase

QKI : quaking

RBP : protéines de liaison à l'ARN

RFS : survie sans rechute

shRNA : small hairpin RNA

TACE : chimio-embolisation transartérielle

TARE : radio-embolisation transartérielle

TAZ : transcriptional co-activator with PDZ-binding motif

TEM : transition épithélio-mésenchymateuse

TGF β : transforming growth factor β

TLR : thérapies locorégionales

TIEG1 : TGF β inducible early growth response protein 1

TIL : lymphocytes infiltrant la tumeur

TSB : target site blockers

TNM : tumeur-node metastasis

VE : vésicules extracellulaires

VHB : virus de l'hépatite B

VHC : virus de l'hépatite C

VIM : vimentine

YAP : yes-associated protein

YY1 : ying yang 1

Chapitre 1 : Introduction

I. Le cholangiocarcinome (CCA)

1. Classification des CCA

A- Classification anatomique

Le terme cholangiocarcinome (CCA) désigne un groupe de tumeurs qui résultent de la transformation maligne des cholangiocytes situés le long de l'arbre biliaire, allant du bas cholédoque jusqu'aux petits canaux biliaires intrahépatiques. Les CCA présentent une grande hétérogénéité anatomique, histologique et moléculaire. Les recommandations actuelles indiquent que les CCA doivent être classés en 3 sous-classes en fonction de leur localisation anatomique et de leurs caractéristiques histologiques. La première classe regroupe les CCA intrahépatiques (CCAi) qui se développent aux dépens des voies biliaires intrahépatiques du second ordre. Les CCA extrahépatiques (CCAe) sont eux divisés en deux sous-classes : les cholangiocarcinomes périhilaires (CCAp) qui émergent du gros canal biliaire hilare hépatique et les CCA distaux (CCAd) qui se développent sur le cholédoque en aval de l'insertion du canal cystique (Figure 1A) (Blechacz et al., 2011; Ebata et al., 2014).

Cependant, il est important de prendre en compte la variabilité phénotypique et génotypique des tumeurs au sein d'un même sous-type. Cela limite la pertinence de cette classification et nécessite une approche plus prudente. Notamment, elle ne prend pas en compte les différences entre les petits et les grands canaux intrahépatiques qui présentent pourtant des caractéristiques et une prise en charge spécifiques. De plus, les CCAp et les CCAi provenant de voies biliaires plus larges présentent des phénotypes moléculaires similaires. Par conséquent, la distinction entre ces deux formes de CCA peut être remise en question. Une autre limite de cette classification repose sur le fait que les petits canaux et les ductules biliaires sont tous deux présents dans le parenchyme hépatique. Les CCAp peuvent donc provenir soit de ces petits canaux, soit des canaux hilaires. Il est souvent difficile de distinguer ces deux entités sur la base de leur morphologie macroscopique, ce qui conduit à des erreurs de diagnostic (Bragazzi et al., 2018).

D'un point de vue histologique, les CCAe sont principalement des tumeurs productrices de mucine, caractérisées par l'expression de marqueurs de surface tels que MUC1, MUC2 et MUC5A5 (Sasaki and Nakanuma, 1996; Sasaki et al., 1998). Les CCAi, bien que partageant certains traits histologiques et moléculaires, présentent un phénotype et un comportement clinique pouvant varier considérablement d'un patient à l'autre. Les adénocarcinomes qui se

développent sur les canaux biliaires de second ordre sont principalement présents sous forme de masses tumorales (MF) infiltrant le parenchyme hépatique par le système porte et les vaisseaux lymphatiques. Les CCAi provenant des grands canaux biliaires présentent plutôt un schéma de croissance similaire aux CCAp et aux CCAd. Ils peuvent se développer sous forme de tumeurs MF, périductales (PD) ou intraductales (IC) (Figure 1B) (Kendall et al., 2019).

B- Classification moléculaire

Les progrès considérables des approches -omiques et l'intérêt croissant de la communauté pour le CCA, ont permis d'établir des profils génétiques et génomiques caractéristiques pour les différents sous-types de CCA. Les analyses moléculaires intégratives ont notamment apporté des classifications morpho-moléculaires pertinentes. Ces classifications complémentaires présentent de nombreux avantages, allant d'une meilleure prise en compte de l'hétérogénéité des CCA, à l'identification de nouveaux sous-groupes de CCA et des cibles thérapeutiques associées.

Plusieurs études ont identifié des altérations génétiques spécifiques aux CCAi ou aux CCAe. Par exemple, Putra *et al.* ont démontré que les CCAi présentent principalement des mutations des *Isocitrate Déshydrogénase 1/2 (IDH1/2)* et de *NRAS*, tandis que des mutations de la *Tumor Protein 53 (TP53)* (75%), de *KRAS* (50%), et de *BRAF* (12.5%) sont plutôt associées aux CCAe (Putra et al., 2015). Une analyse s'appuyant sur une cohorte de 137 CCAi et 74 CCAe a également révélé des altérations génétiques spécifiques aux CCAi, telles que des mutations de *IDH1/2*, de la *BRCA1 associated protein (BAP1)* et des fusions du gène du récepteur 2 du facteur de croissance des fibroblastes (*FGFR2*). De même, quelques altérations ont été exclusivement retrouvées dans les CCAe (*e.g.* les mutations de la *Protein Kinase CAMP-Activated Catalytic Subunit Alpha - PRKACA* et du *AT-Rich Interaction Domain 1B - ARID1B*). Des mutations étaient communes aux deux sous-types (*e.g.* les mutations de *KRAS*, de la *Guanine Nucleotide binding protein - GNAS* et de *TP53*) (Figure 1C). Cette étude a également permis d'identifier des voies de signalisation propagées par des facteurs de croissance spécifiques au CCAi ou au CCAe, offrant ainsi des perspectives pour des thérapies personnalisées (Nakamura et al., 2015).

Une étude plus récente s'appuyant sur une grande cohorte de 496 patients atteints de CCAi s'est basée sur trois altérations génomiques (*IDH1/2*, *KRAS* et *TP53*) pour différencier des sous-groupes de patients. Ainsi, cette classification moléculaire a permis d'identifier des

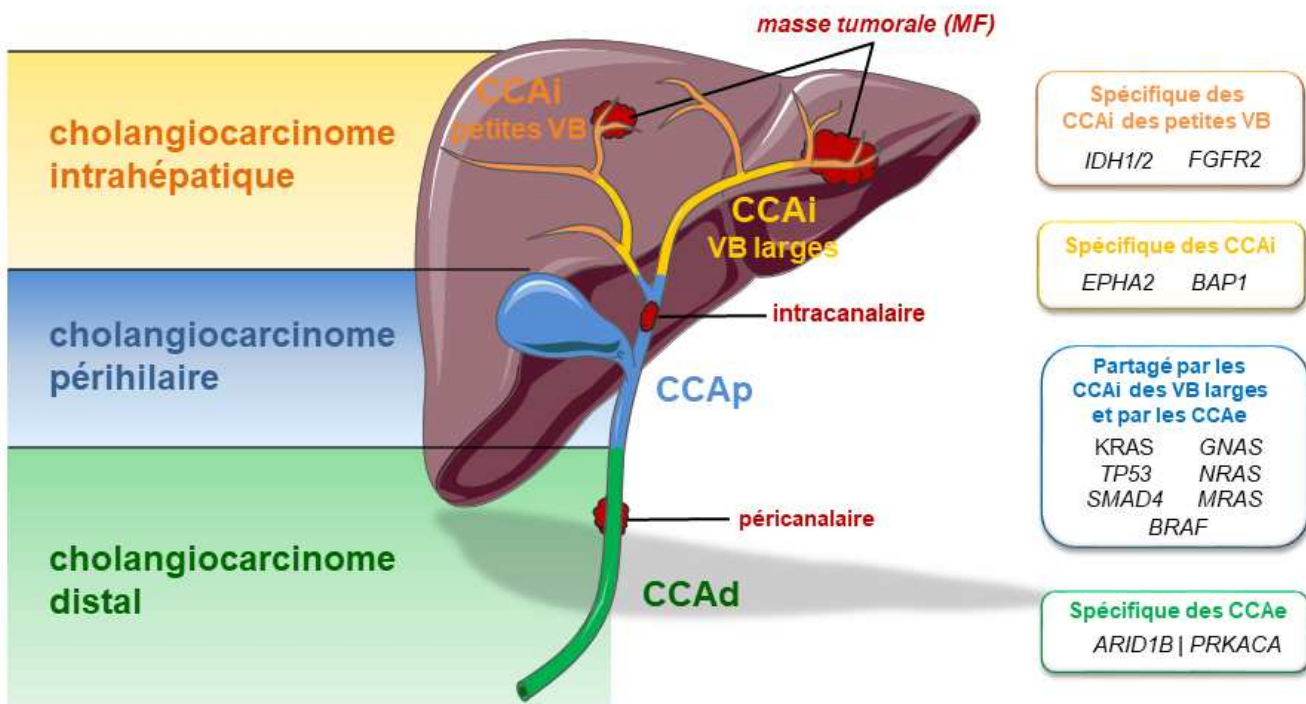
signatures mutationnelles uniques, des profils co-mutationnels et une activité anormale de certaines voies de signalisation pour chaque sous-groupe. Une telle stratification des CCAi pourrait notamment aider à prédire des réponses thérapeutiques dans le cadre de protocoles de repositionnement de médicaments (Nepal et al., 2018).

Certaines études ont également soulevé l'idée que les classifications reposant sur les profils mutationnels pourraient être associées aux différentes classes morphologiques de CCA. Ainsi, deux sous-groupes de CCAi peuvent être établis : les CCAi des voies biliaires larges, avec une grande fréquence de mutation pour *KRAS* (15-30 %) et *TP53* (10-40 %), et les CCAi des petites voies biliaires (typiquement MF) qui montrent plutôt un enrichissement en mutations *IDH1/2* et en gène de fusion *FGFR2* (Figure 1C) (Kendall et al., 2019). Le *FGFR2* est un récepteur transmembranaire, avec une activité tyrosine kinase, qui est un composant principal de la voie de signalisation des facteurs de croissance des fibroblastes. Cette voie est fréquemment activée dans le CCA où elle peut favoriser la migration et l'angiogenèse (Ang, 2015). La fusion du gène *FGFR2* avec un autre gène (e.g. *BICC1*, *AHCYL1*, *TACC1*, *MGEA5*, *PPHLN*) est un événement souvent associé à la survenue de CCAi. En outre, *FGFR2* fusionne avec *PRKACA* et *PRKACB*, respectivement dans les CCAi et les CCAe. La formation de ces gènes chimériques entraîne l'activation constitutive de *FGFR2* et de ses fonctions pro-tumorales. Bien que le *FGFR2* soit un des gènes de fusion les plus fréquemment associés au CCAi, de nombreux gènes sont également affectés dans ce type de néoplasie (e.g. *RABGAP1L-NTRK1*) (Valle et al., 2017).

A classification anatomique

B classification histo-morphogique

C classification moléculaire



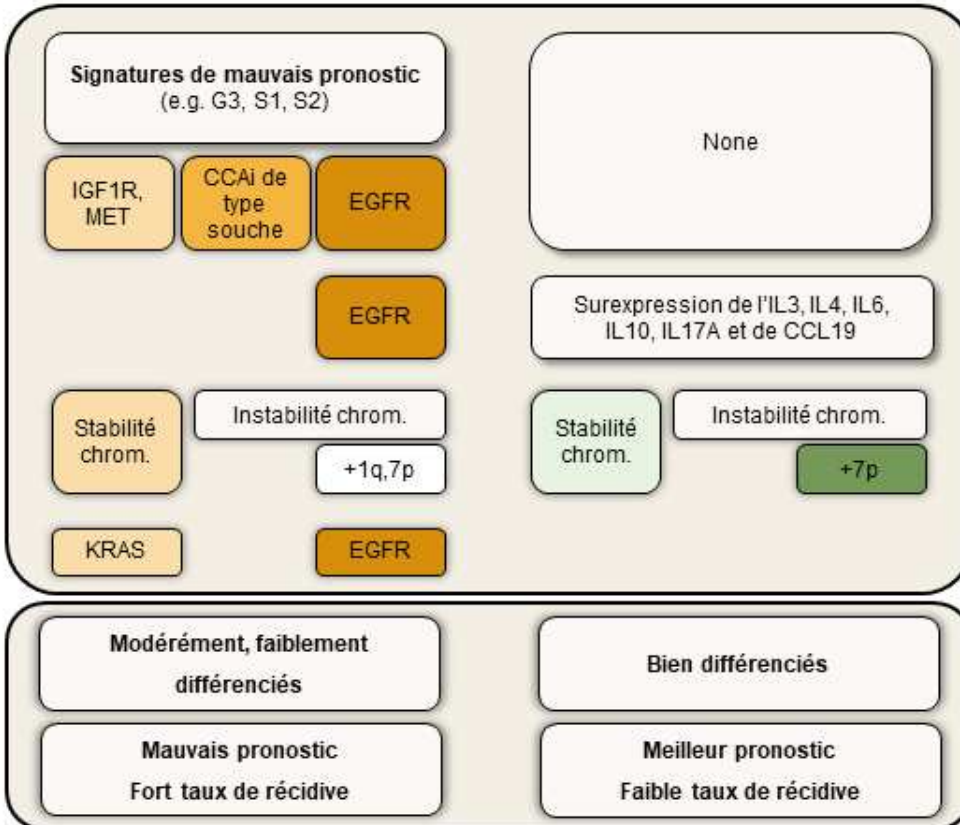
(A) Les cholangiocarcinomes (CCA) sont classés en trois catégories en fonction de leur localisation anatomique : les CCA intrahépatiques (CCAI) se développent à l'intérieur du foie à partir des canaux biliaires (CB) de deuxième ordre et des CB intrahépatiques proximaux. Les CCA extrahépatiques (CCAe) regroupent les CCA périhilaires (CCAp) qui se développent à partir des canaux hépatiques gauche et droit, et les CCA distaux (CCAd) qui se trouvent plus bas dans l'arbre biliaire (CCAd), proche de l'ampoule de Vater. (B) Trois principaux modèles de croissance des CCA ont été observés : masse tumorale (MF), intraductale (ID) et périductale (PD). Les CCAi sont principalement présents sous forme MF, tandis que les CCAe sont principalement des tumeurs mucineuses avec une croissance ID et PD. (C) L'identification d'altérations spécifiques aux niveaux génétique, épigénétique et génomique a permis de révéler des sous-types moléculaires de CCA associés au pronostic de survie des patients, ouvrant ainsi la voie au développement de thérapies ciblées.

C- Classification par génomique intégrative

L'une des premières caractérisations moléculaires effectuées sur une large cohorte de CCA (n=104) a permis l'identification de sous-classes de patients aux caractéristiques cliniques distinctes. Cette étude associe notamment les mutations *KRAS* à une mauvaise survie globale (OS) (Andersen et al., 2012).

Une autre étude a regroupé des données de l'expression génomique, des aberrations chromosomiques et de l'activation de certaines voies de signalisation dans une grande cohorte de 149 CCAi. Deux sous-groupes moléculaires ont ainsi été identifiés (Figure 2) (Sia et al., 2013). La classe dite « inflammatoire » (38 % des CCAi) se caractérise par l'activation des voies de signalisation inflammatoires et par une expression particulièrement importante de cytokines Th2 (IL-10 et IL-4). Une seconde classe dite « proliférative » (62 % des CCAi) est quant à elle caractérisée par l'activation des voies de signalisation pro-tumorales MET ou RAS/RAF/MAPK et par des mutations de *KRAS*. Il est important de noter que les tumeurs incluses dans cette seconde classe sont de nature très agressive et sont corrélées à une faible survie et à un taux de récurrence élevé (Figure 2) (Sia et al., 2013). Ces deux études fondamentales ont ainsi mis en évidence le potentiel des approches de génomique intégrative pour affiner la classification des CCA, ouvrant ainsi la voie pour les approches thérapeutiques personnalisées (Andersen et al., 2012; Sia et al., 2013).

Sous-classe moléculaire de CCAi



Caractéristiques moléculaires

Enrichissement dans une signature génomique

Expression génomique

Variations du nombre de copies

Mutations

Caractéristiques cliniques

Une étude plus récente a également utilisé des approches de génomique intégrative pour identifier des sous-classes de CCAi caractérisées par la composition et l'activité de leur micro-environnement tumoral (MT). Afin d'établir cette classification, une méthode computationnelle basée sur l'utilisation de signatures transcriptomiques spécifiques du type cellulaire a été utilisée. De plus, un ensemble de 14 signatures géniques représentant les principales populations de cellules infiltrées dans le MT, et de 8 signatures correspondant à des populations typiquement retrouvées dans le MT, ont ainsi permis de classer les patients de différentes cohortes de CCAi. Quatre sous-types immunitaires ont ainsi été identifiés et associés à des caractéristiques immunitaires distinctes : une immunité inexistante, une immunité exclue, une immunité active et une immunité exaltée. Le premier sous-type est caractérisé par un manque de cellules immunitaires dans l'environnement tumoral et comprend les patients qui présentent le pronostic le plus défavorable. Le sous-type d'immunité exclue est caractérisé par la présence de cellules immunitaires, mais qui ne sont pas en mesure de s'infiltrer dans la tumeur. Le sous-type d'immunité active est caractérisé par la présence de cellules immunitaires capables de s'infiltrer dans la tumeur, mais qui ne sont pas en mesure d'y mener une réponse immunitaire efficace. Le sous-type d'immunité exaltée est caractérisé par la présence de cellules immunitaires capables de s'infiltrer dans la tumeur et d'initier une réponse immunitaire efficace. Cette étude a également montré que le sous-type qui présentait une activité immunitaire inexistante avait le plus haut niveau d'expression du *programmed-death Ligand 1* (PD-L1), qui est une protéine qui supprime la réponse immunitaire. Ils ont également constaté que les sous-types d'immunité exclue et d'immunité active avaient les plus hauts niveaux d'expression de TGF β , qui est également une protéine impliquée dans la suppression de la réponse immunitaire. Cette nouvelle classification met ainsi en évidence l'importance du système immunitaire dans le développement et la progression du CCAi et suggère que cibler celui-ci pourrait être une approche prometteuse pour traiter les patients présents dans ces sous-groupes (Job et al., 2020).

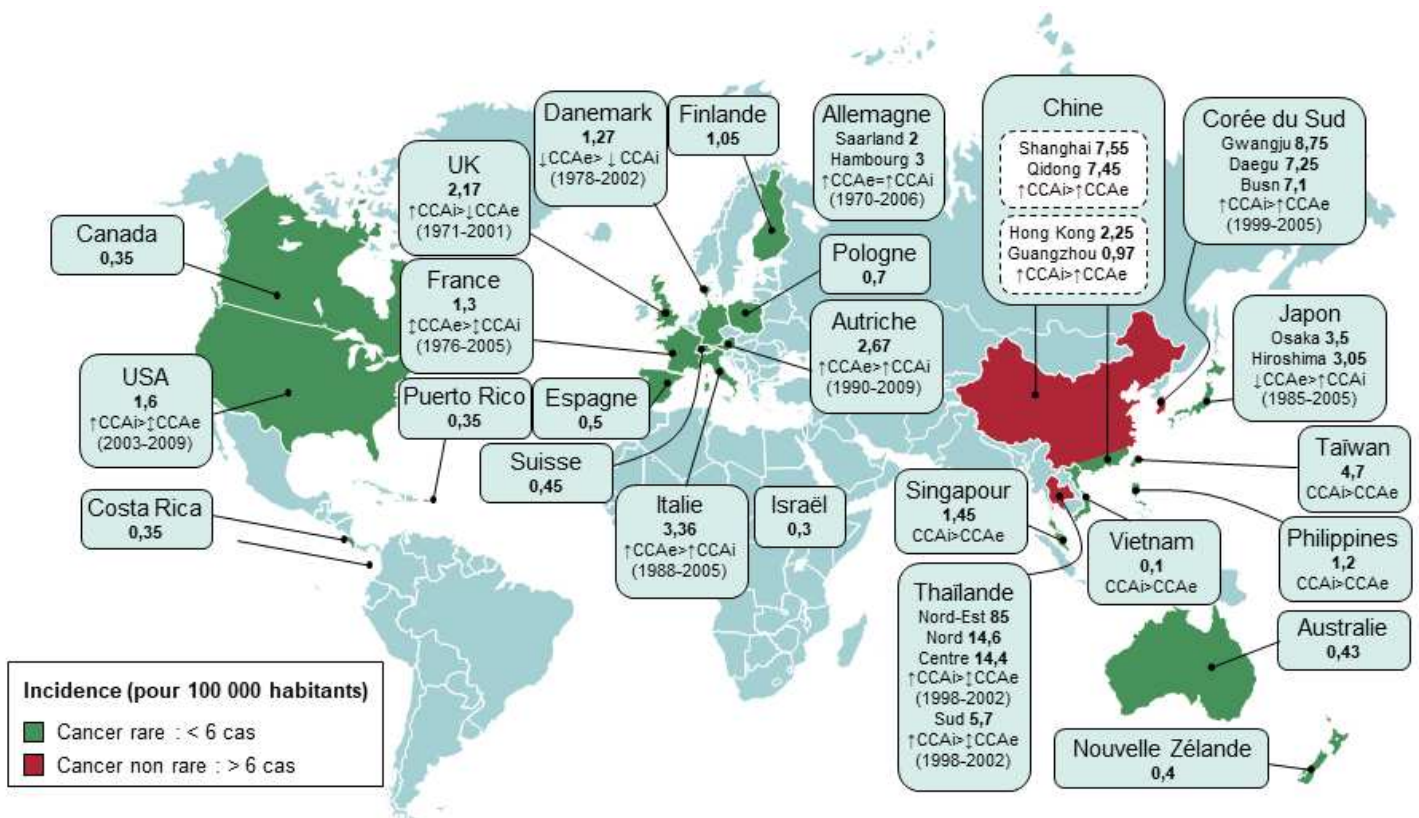
2. Épidémiologie et facteurs de risque

Bien que les nombreux défis pour classer des tumeurs si hétérogènes conduisent à un calcul approximatif de leur incidence, une augmentation progressive du nombre de cas a été enregistrée au niveau mondial au cours des dernières décennies (Bertuccio et al., 2019). Des estimations récentes chiffrent une incidence entre 0.35 et 2/100 000 cas dans les pays

d'Occident, où le CCA est considéré comme un cancer rare. En revanche, l'incidence dans les pays d'Asie du Sud-Est est 40 fois plus élevée du fait de facteurs de risque endémiques (jusqu'à 85/100000 en Thaïlande) (Figure 3) (Banales *et al.*, 2016).

Les dernières avancées visant à affiner la classification des CCA ont permis de souligner les disparités d'incidence entre les différents sous-types. En effet, alors que l'incidence des CCAe varie peu, celle des CCAi est en constante augmentation au cours de la dernière décennie. Cette variation soudaine d'incidence peut être associée aux progrès effectués pour diagnostiquer les CCAi. En effet, certains cas de cancers secondaires primitifs ont récemment été reclassifiés en CCAi, ce qui peut expliquer cette hausse de l'incidence. Aux États-Unis, elle a par exemple bondi de 2,3 % chaque année au cours des 40 dernières années, passant de 0,44 à 1,18 pour 100 000 habitants. Il est d'ailleurs commun de dire que le CCAi est la deuxième tumeur maligne hépatique la plus fréquente après le carcinome hépatocellulaire (CHC). La tendance est similaire dans la plupart des pays occidentaux où le taux de mortalité spécifique des CCAi ne cesse d'augmenter.

Ces données d'incidence ont été prises en compte dans la stratégie décennale 2021-2030 de l'Institut National du Cancer (INCa) qui encourage les projets de recherche sur les cancers de mauvais pronostic, notamment sur les cancers du foie et des voies biliaires. C'est en suivant cette feuille de route que nous avons concentré nos efforts sur le CCA sous sa forme intrahépatique.



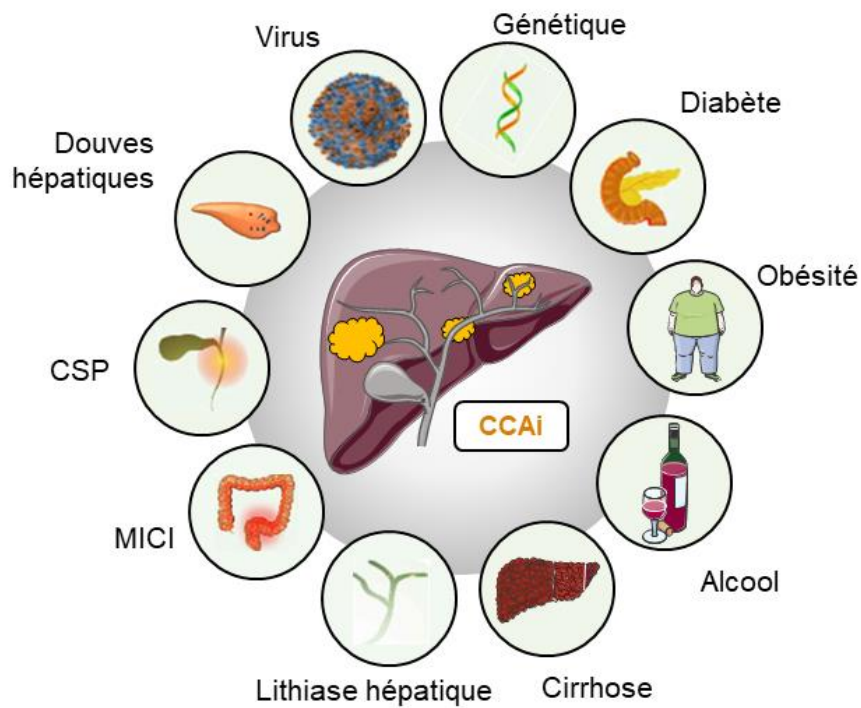
Les disparités géographiques dans l'incidence du CCA sont associées à des facteurs de risque bien établis. L'incidence importante relevée en Asie du Sud-Est est notamment due à des infections et réinfections d'une partie de la population par deux parasites de la classe des trématodes : *Clonorchis sinensis* et *Opisthorchis viverrini*. Ces douves hépatobiliaires sont endémiques en Corée du Sud, dans le sud-est de la Chine et à Taïwan pour *Clonorchis sinensis* et en Thaïlande, au Laos et au Cambodge pour *Opisthorchis viverrini*. Elles se transmettent par l'ingestion de poisson cru ou peu cuit, à la suite de quoi les parasites adultes pondent leurs œufs au niveau des voies biliaires intrahépatiques, provoquant ainsi une inflammation chronique et persistante du foie, qui peut ensuite entraîner un remodelage tissulaire, une fibrose extensive et une cicatrisation, conduisant finalement à une transformation maligne. Il est important de noter que la présence d'autres co-facteurs tels que la cirrhose, les dommages oxydatifs et les réponses immunitaires chroniques anti-douve

peuvent également jouer un rôle important dans le développement de tumeurs au niveau des voies biliaires (Saijuntha et al., 2019).

Dans les pays occidentaux où les CCAi sont des tumeurs moins fréquentes, les facteurs de risque sont principalement liés à une inflammation chronique des voies biliaires intrahépatiques. Cette inflammation chronique peut être de cause infectieuse. Les virus de l'hépatite B et C (VHB et VHC) ont notamment été identifiés comme des facteurs de risque reconnus pour les CCA, en particulier pour les CCAi. Ici encore, des disparités existent entre les pays occidentaux et asiatiques. En effet, alors que dans les populations occidentales, le CCAi est plus fortement associé au VHC, dans les populations asiatiques, ce type de tumeur est majoritairement associé au VHB (Matsumoto et al., 2014).

L'inflammation chronique à l'origine du développement de CCAi peut également être d'origine inflammatoire, avec en première ligne la cholangite sclérosante primitive (CSP) qui est une maladie inflammatoire chronique des voies biliaires pouvant évoluer en fibrose et potentiellement en CCAi. Des études menées dans des pays occidentaux ont rapporté que les patients atteints de CSP sont davantage sujets au développement de CCA avec une prévalence allant de 5 % à 15 % et un taux de développement annuel variant de 0,5 % à 1,5 %. La classification histologique peut également être corrélée avec l'étiologie des tumeurs. Par exemple, les CCAi MF sont souvent associés à une maladie hépatique chronique telle que la cirrhose ou l'hépatite virale, tandis que les CCAi mucineux sont plus souvent liés à une CSP (Lee et al., 2017).

Plus récemment, des études épidémiologiques ont révélé que des facteurs de risque connus du CHC sont également associés au développement de CCAi. Ces études associent la survenue de CCAi à des troubles du métabolisme tels que l'obésité, le diabète ou encore les maladies hépatiques dysmétaboliques d'origine non alcoolique (*Non-Alcoholic Fatty Liver Disease*, NAFLD) qui englobent un éventail de maladies hépatiques allant de la stéatohépatite non alcoolique (*Non-Alcoholic Steatohepatitis*, NASH) à la cirrhose (Shaib et al., 2005). Par exemple, une étude a rapporté que la NAFLD était associée à un risque accru de développer un CCAi (OR = 2,22, IC 95 % 1,52-3,24) (Wongjarupong et al., 2017). Ces facteurs de risque partagés suggèrent par ailleurs une carcinogenèse commune aux deux tumeurs. Il est parfois suggéré que l'augmentation du nombre de cas de syndrome métabolique est corrélée à la tendance haussière de l'incidence des tumeurs hépatobiliaires (Da Fonseca et al., 2022).



3. Prise en charge du CCAi

A- Présentation clinique et diagnostic

Le premier défi rencontré avec la prise en charge du CCAi est sans nul doute son diagnostic. En effet, c'est un cancer agressif et de nature silencieuse qui est souvent diagnostiqué à des stades trop tardifs. Lorsqu'il est symptomatique, le CCAi se manifeste par une cachexie, des douleurs abdominales, des sueurs nocturnes, une fatigue et/ou un ictère. Le diagnostic du CCAi est toutefois souvent fortuit (20 à 25 % des cas), notamment lors de dépistage de maladies chroniques du foie (Razumilava and Gores, 2014).

B- Histologie

En cas de suspicion de CCAi, la réalisation d'une biopsie est obligatoire, notamment pour le différencier d'un CHC ou d'une métastase provenant d'un adénocarcinome. En effet, ces tumeurs présentent de nombreuses similitudes et les distinguer avec certitude, uniquement à partir de leur aspect histologique, peut engendrer des erreurs de diagnostic.

Les CCAi sont caractérisés sur le plan histologique par des structures tubulaires et/ou papillaires et un stroma fibreux. Il est également possible de confirmer l'origine biliaire intrahépatique grâce à des marqueurs spécifiques. Des immunomarquages tissulaires de la cytokératine 7 (corrélée positivement) et de la cytokératine 20 (corrélée négativement) sont notamment utilisés dans ce but (Rullier et al., 2000). Toutefois, ces marqueurs ne sont pas spécifiques du CCAi et l'analyse histologique doit souvent être complétée par une analyse anatomo-pathologique et radiologique.

C- Imagerie

Plusieurs techniques d'imagerie sont utilisées pour le dépistage de masses hépatiques. Les examens morphologiques utilisés sont généralement l'échographie, puis la tomodensitométrie (CT), sans et avec produit de contraste, l'IRM et parfois le PET-Scan. Les CCAi sont des tumeurs particulièrement fibreuses, ce qui permet de les différencier des CHC par imagerie. Cette particularité des CCAi est notamment visible au scanner et se traduit par un aspect ferme et hypovasculaire. Ainsi, le CCAi peut se manifester de plusieurs manières sur un CT avec produit de contraste. Ils sont notamment caractérisés par un rehaussement de contraste en forme de bord périphérique pendant la phase artérielle ou portal, avec un centre plus atténué pendant la phase retardée, quelques minutes après la perfusion du produit de contraste (Ciresa et al., 2015).

La tomographie par émission de positons au fluorodéoxyglucose (TEP-FDG) peut également être une option pour la détection de CCAi. Les petits CCAi formant des masses de petite taille (< 1cm) peuvent être détectés en utilisant cette méthode, avec une sensibilité rapportée de 85-95 %. L'utilité clinique de la TEP pour le diagnostic des CCAi est néanmoins limitée puisque la tomographie ou l'IRM apportent plus de précision (Anderson et al., 2004).

D- Marqueurs tumoraux sériques

Afin de confirmer ou d'infirmer un diagnostic complexe, il est possible de s'appuyer sur la quantification de plusieurs marqueurs sériques. Les taux sanguins de l'antigène carcino-

embryonnaire (ACE) et de l'antigène carbohydre 19-9 (CA19-9) sont notamment souvent élevés chez les patients atteints de CCAi. Ces marqueurs sont néanmoins trop peu spécifiques et sensibles pour être utilisés à des fins de dépistage, et doivent être uniquement utilisés en complément des techniques de diagnostic par imagerie (Moro et al., 2020).

E- Systèmes de stadification

La phase suivante de la prise en charge est de déterminer si la tumeur est résécable chirurgicalement. À cette fin, des stadifications tumorales ont été développées pour identifier les patients pouvant bénéficier de la chirurgie. Pendant des années, la stadification du CCAi s'est faite en utilisant le même système TNM (*tumour-node metastasis*) que celui du CHC. En 2010, la 7^{ème} édition du manuel de stadification de l'AJCC/UICC a proposé un système de stadification spécifique au CCAi (Edge and Compton, 2010). Ce système se base sur des critères spécifiques comprenant le nombre de tumeurs, l'invasion vasculaire, l'invasion des structures adjacentes, l'histologie et les métastases ganglionnaires. Par ailleurs, la taille de la tumeur a été retirée des facteurs pronostiques associés aux CCAi.

Cette classification a néanmoins été amendée en 2017 par une 8^{ème} version. L'un des principaux changements a été la distinction entre les CCAi et les CCAe dans la classification TNM, du fait de leurs caractéristiques cliniques, biologiques et pronostiques différentes. Auparavant, ces deux types de CCA étaient classés ensemble. De plus, la nouvelle classification prend en compte l'extension tumorale dans les différents segments hépatiques et les branches biliaires, ainsi que la présence de métastases ganglionnaires et à distance. Elle inclut également des facteurs pronostiques tels que l'âge, l'état de performance, la bilirubine sérique, la présence d'une tumeur multifocale et la réponse aux chimiothérapies (Amin et al., 2017).

4. Options thérapeutiques

A- Chirurgie

La chirurgie reste à ce jour le traitement à intention curative de référence pour le CCAi. Néanmoins, la majorité des patients n'y est pas éligible du fait de la présence d'une tumeur localement avancée ou métastatique au moment du diagnostic. Moins d'un tiers des patients diagnostiqués sont éligibles à la chirurgie (Tan et al., 2008). La survie après résection dépend principalement de la présence de marges de résection saines, de l'absence d'invasion vasculaire et de métastases ganglionnaires. Une méta-analyse de 2014 donne des chiffres

de survie post-résection à visée curative avec une OS à 5 ans de 21-35 %, et une survie globale médiane d'environ 25 mois (Mavros et al. 2014).

Dans le cas où le chirurgien est contraint de laisser des résidus tumoraux visibles au microscope ou que l'invasion ganglionnaire est trop importante (R1/N1), des traitements adjuvants peuvent être envisagés pour améliorer la qualité de vie des patients (Figure 8). Une étude rétrospective incluant 2751 patients avec des CCAi, dont 35.8 % avaient reçu un traitement adjuvant, a rapporté un vrai bénéfice de cette stratégie sur la survie des patients N1 (OS médiane : 19,8 vs. 10,7 mois ; $p= 0,001$). En revanche, ces bénéfices restaient limités pour les patients N0 (OS médiane : 29,4 vs. 29 mois ; $p= 0,33$) (Miura et al., 2015). L'essai randomisé de phase III Prodigé-12, dont l'objectif était de démontrer l'intérêt d'un traitement GEMOX (*gemcitabine* et *oxiplatine*) post-opératoire comparé à une surveillance simple, n'est pas parvenu à démontrer d'impact sur la survie sans rechute (RFS). Cette essai mélange néanmoins différents types de cancer biliaires (CCAi, CCAe et carcinomes de la vésiculaire biliaire). Il est important de prendre en compte l'hétérogénéité phénotypique et génotypique des cancers biliaires, ainsi que les limites associées à l'étude de ces cancers rares, lors de l'interprétation des résultats d'études cliniques. Ces résultats doivent donc être considérés avec prudence et confirmés par d'autres études. (Edeline et al., 2019).

B- Thérapies locorégionales (TLR)

Dans le cas de tumeurs non résécables (TNM2) dont l'envahissement reste limité au foie, des thérapies locorégionales, telles que la chimio-embolisation transartérielle (TACE) et la radio-embolisation transartérielle (TARE), sont de plus en plus envisagées pour améliorer le pronostic des patients (Figure 8). La combinaison d'une TACE et d'un traitement cisplatine a notamment permis d'améliorer le pronostic de patients inéligibles à la chirurgie, avec une amélioration de la OS (12,2 vs 3,3 mois) (Park et al., 2011). Une étude multicentrique incluant 200 patients avec des CCAi a conclu que cette approche permettait une stabilisation de la progression de la tumeur dans 62 % des cas, et une réponse partielle ou complète dans 26 % des cas (Hyder et al., 2013).

La TARE est une technique de radiothérapie interne qui utilise des microsphères chargées d'Yttrium-90 (Y90) pour délivrer une dose de radiation sur une tumeur hépatique. Les microsphères sont injectées dans l'artère hépatique qui nourrit la tumeur, permettant ainsi une délivrance ciblée et localisée de la dose de radiation à la tumeur. L'efficacité de la TARE en combinaison avec une chimiothérapie systémique (*cisplatine-gemcitabine*, CisGem) a

également été évaluée au cours de l'essai clinique de Phase II MISPHEC incluant 41 patients avec des CCAi localement avancés qui n'étaient pas éligibles à la chirurgie. Cette étude rapporte un meilleur taux de réponse à 3 mois pour ces patients (39 %) et une amélioration de leur pronostic illustrée par une OS de 22 mois et une survie sans progression de 14 mois. Parmi ces patients, 9 (soit 22%) ont ensuite pu bénéficier d'une chirurgie et 8 (soit 20%) ont bénéficié d'une résection chirurgicale R0. Ces résultats suggèrent un futur prometteur pour l'utilisation de la TARE en combinaison avec une chimiothérapie comme traitement de première ligne des CCAi (Edeline et al., 2020).

L'ablation par radiofréquence (ARF) semble aussi être une approche pouvant bénéficier aux patients diagnostiqués pour un CCAi. Quelques études suggèrent que cette approche pourrait être bénéfique pour le traitement de petites lésions, non résécables et sans signes de propagation extrahépatique. L'ARF de petites tumeurs (< 3cm) a ainsi conduit à la nécrose précoce de 90 à 100 % d'entre elles (Kim et al., 2011a; Kim et al., 2011b). Chez les patients présentant une récurrence locale ou une tumeur résiduelle après intervention chirurgicale, l'ARF a permis d'atteindre une OS de 27,4 à 51 mois, ce qui suggère une amélioration du pronostic comparé à celui obtenu avec d'autres traitements palliatifs (Kamphues et al., 2010).

Le potentiel des TLR a été mis en évidence par une revue systématique de la littérature regroupant 101 cohortes pour un total de 1200 patients atteints de CCAi et traités par des TLR, incluant la TACE, la TARE et l'ARF. Cette revue révèle que la OS et la RFS de ces patients étaient significativement meilleures que celles des patients n'ayant reçu aucune thérapie ou une thérapie systémique seule (Edeline et al., 2021). Néanmoins, bien que les résultats des différents types de TLR soient prometteurs, cette revue de la littérature était principalement composée de d'études rétrospectives à bras unique. De plus, la qualité des rapports ainsi que leur hétérogénéité ne permettent pas de conclure sur l'efficacité de ces traitements.

C- Thérapies systémiques

De nombreux essais cliniques ont été conduits au cours des trente dernières années dans le but d'identifier les chimiothérapies systémiques les plus bénéfiques pour le traitement du CCAi. Le traitement standard de première intention actuellement recommandé pour les maladies non résécables, localement avancées ou métastatiques est l'association de *cisplatine* et de *gemcitabine* (CisGem) (Figure 8). Cette association est devenue le standard thérapeutique dans le traitement des cancers des voies biliaires à la suite des études ABC-

01 (Phase II) et ABC-02 (Phase III). La première étude rapporte notamment une meilleure survie sans progression à 6 mois pour les patients ayant reçu la combinaison CisGem comparée à ceux ayant uniquement reçu la *gemcitabine* (57,1 vs. 45,5 %) (Valle et al., 2010). Une phase III a alors été conduite sur une cohorte de 410 patients montrant cette fois les bénéfices sur la OS de la combinaison GemCis par rapport au traitement *gemcitabine* seul (11,7 vs 8,1 mois) (Valle et al., 2010). Les bénéfices des chimiothérapies de première ligne restent néanmoins modérés, avec une OS atteignant difficilement un an.

Concernant le traitement de seconde ligne, l'essai de phase III ABC-06 mené chez des patients britanniques a montré un bénéfice clinique du traitement de seconde ligne FOLFOX (*folinic acid, fluorouracil, and oxaliplatine*) (Figure 8). Cette étude démontre que l'ajout de FOLFOX à l'*Active Symptom Control* (ASC) améliore la OS médiane chez des patients atteints d'un cancer avancé des voies biliaires (CCA, carcinome de la vésicule biliaire et carcinome de l'ampoule de Vater), avec une augmentation significative des taux de survie globale à 6 et 12 mois (Lamarca et al., 2021).

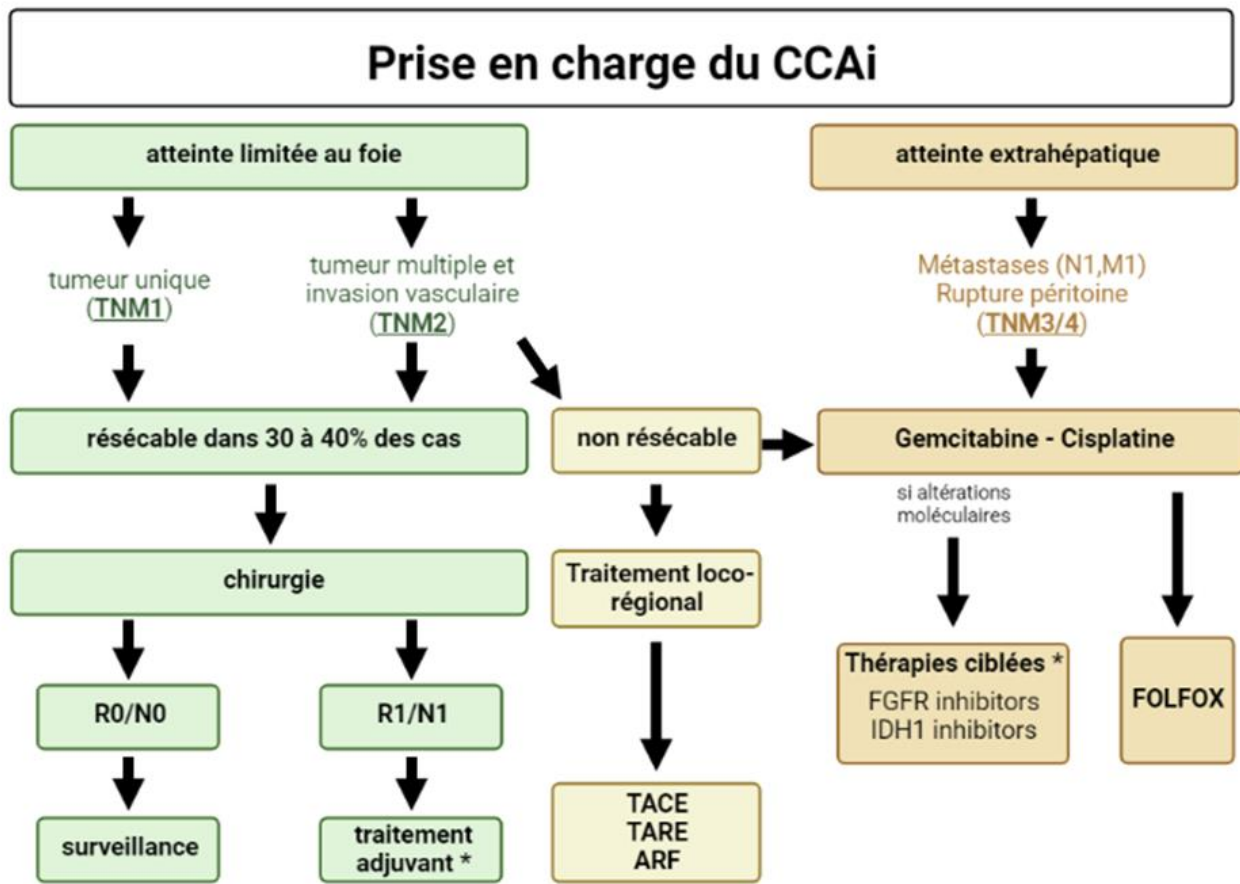
D- Thérapies ciblées

La place des thérapies ciblées dans le traitement du CCAi est encore mal définie. Plusieurs essais cliniques évaluent l'effet d'agents moléculaires spécifiques ciblant diverses voies de signalisation impliquées dans la survenue de CCA (Figure 8). Les inhibiteurs de tyrosine kinase (e.g. *erlotinib, bevacizumab, cetuximab, panitimumab* et *lapatinib*) ont par exemple été envisagés. Néanmoins, l'utilisation de ces inhibiteurs n'ont démontré que de très modestes avantages en termes de survie lorsqu'ils ont été testés sur des populations sélectionnées sans tenir compte des anomalies moléculaires. Des études cliniques de plus grande envergure, ainsi qu'une meilleure sélection des patients en fonction de leurs altérations moléculaires seraient donc nécessaires pour obtenir des résultats plus concluants (Poddubskaya et al., 2018).

L'identification de mutations spécifiques aux CCAi permet également le développement de thérapies ciblées. Les inhibiteurs de l'IDH1, IDH2 et de FGFR sont en cours d'évaluation. Les premiers résultats suggèrent néanmoins que ces inhibiteurs pourraient améliorer significativement la survie des patients. Par exemple, l'essai ClarIDHy, a visé à démontrer l'efficacité de l'*ivosidenib* (un inhibiteur de l'IDH1 mutant) par rapport au placebo chez les patients atteints de CCA non résécable ou métastatique avec mutation de l'IDH1. Cet essai clinique démontre le bénéfice clinique de l'*ivosidenib* pour les patients atteints de CCA

avancés portant une mutation de l'IDH1. La OS médiane était de 2-7 mois avec l'*ivosidenib* contre 1-4 mois avec le placebo. Ces résultats ont néanmoins été améliorés par le cross-over prévu dans l'étude (Abou-Alfa et al., 2020; Saborowski et al., 2020; Zhu et al., 2021).

L'immunothérapie apparaît également comme une stratégie de traitement prometteuse visant à concentrer et renforcer l'activité antitumorale du système immunitaire. Notamment, des essais cliniques utilisant des anticorps monoclonaux ciblant les régulateurs des points de contrôle immunitaire, tels que le *Programmed-death-1* (PD-1) ou son principal ligand PD-L1, ont donné des résultats prometteurs dans divers cancers hématologiques et solides (Pardoll, 2012). En outre, ces approches d'immunothérapie peuvent être combinées à une inhibition de la voie TGF β également impliquée dans la carcinogenèse du CCAi. L'utilisation du *Bintrafusp alfa* (M7824) a notamment été envisagée pour le traitement de cancers des voies biliaires. Cette protéine de fusion bifonctionnelle, la première de sa catégorie, est composée du domaine extracellulaire du récepteur TGF β humain II (leurre pour le TGF β) fusionné à l'extrémité C-terminale de chaînes lourdes d'un anticorps IgG1 bloquant le PD-L1. L'utilisation du *Bintrafusp alfa* en seconde ligne a notamment montré des résultats prometteurs dans des essais de Phase I de faible effectif (NCT02699515) sur une cohorte de patients avec des tumeurs des voies biliaires, avec un taux de réponse complète de 20 % (Yoo et al., 2020). Son développement a néanmoins été stoppé du fait de résultats négatifs dans des essais randomisés.



Bien que des progrès significatifs aient été réalisés dans la compréhension des bases moléculaires de la pathogenèse du CCAi, il n'existe pas de thérapie moléculaire ciblée qui améliore significativement la survie des patients. Ainsi, la conception de stratégies thérapeutiques et l'identification de biomarqueurs innovants passe par une meilleure caractérisation moléculaire de ces tumeurs hétérogènes (Bridgewater *et al.*, 2014).

5. Voies de signalisation impliquées dans la cancérogenèse du CCAi

La pathogenèse du CCA est associée à des altérations génétiques et épigénétiques dans les cellules tumorales, ainsi qu'à d'importants changements dans le MT. Ces modifications participent collectivement à l'activation de nombreuses voies de signalisation responsables de l'apparition de la tumeur et de son développement. Ces voies sont notamment liées au contrôle de la prolifération, de la survie et de la mort cellulaire.

A- Récepteurs à activité tyrosine kinase

L'activation de voies de signalisation médiée par des récepteurs à activité tyrosine kinase (RTK) est une caractéristique partagée par tous les sous-types de CCAi. À cet égard, une expression aberrante de RTK tels que le récepteur de l'*epithelial growth factor* (EGFR), le récepteur ERBB2 et le récepteur du facteur de croissance des hépatocytes (MET) a été relevée dans différentes sous-classes de CCA et associée à un mauvais pronostic. Elle conduit principalement à l'activation des voies RAS-MAPK et PI3K-AKT-mTOR qui sont responsables de la régulation du cycle cellulaire et de la prolifération (Figure 6A) (Banales et al., 2020).

L'activation de la voie RAS-MAPK peut également être due à des mutations activatrices de KRAS ou de BRAF fréquemment associées aux CCAi. Une étude a ainsi rapporté une perturbation de la voie RAS-MAPK associée à des mutations de RAS ou de BRAF dans 62% des CCA (n=42/69), faisant d'elle l'altération la plus fréquente dans cette cohorte (Tannapfel et al., 2003).

L'activité aberrante des voies RAS-MAPK et PI3K-AKT-mTOR peuvent également être associées aux réarrangements chromosomiques conduisant à la fusion de gènes impliquant le RTK FGFR2. Cette altération moléculaire spécifique des CCAi conduit à la formation de protéines de fusion aux actions pro-oncogéniques, notamment *via* l'activation de la voie MEK1/2 (Figure 6A) (Cristinziano et al., 2021).

B- Voies du développement

Les voies du développement, notamment les voies de signalisation Notch, WNT et TGF β sont décrites dans les processus de réparation du foie associés à un contexte inflammatoire. L'inflammation chronique à l'origine du développement de CCAi conduit donc souvent à une activation anormale de ces voies dans le CCAi (Xue et al., 2015).

Par exemple, la voie Notch est impliquée dans la réparation, la croissance, la tubulogenèse, la fibrose et le maintien de la niche de cellules souches des voies biliaires. Il existe quatre récepteurs NOTCH (NOTCH1-4) et cinq ligands *Jagged* (JAG1, 2) et *Delta-like* (DLL1, 3 et 4). La voie Notch est activée par contact avec les cellules adjacentes qui sécrètent ce type de ligand. Ainsi activée, une succession de clivages protéolytiques (d'abord grâce à ADAM-10, puis grâce à la sécrétase- γ) conduisent à la libération du domaine intracellulaire de NOTCH de la membrane. Celui-ci est alors transloqué dans le noyau où il active des gènes

cibles responsables de la régulation du sort des cellules (Figure 6B). Le dysfonctionnement de cette cascade de signalisation peut être impliqué dans certaines pathologies, dont le CCAi. Une surexpression des récepteurs Notch a notamment été signalée dans les CCAi et associée au développement de tumeurs primaires hépatiques (Geisler and Strazzabosco, 2015). Dans le CCAi, NOTCH1 a également été associé à l'augmentation de la prolifération et de la survie des cellules tumorales, à la régulation positive de MCL-1 et de BCLXL80, ainsi qu'à une migration cellulaire induite par l'activation de RAC1 et l'induction de la transition épithélio-mésenchymateuse (TEM) (Zhou et al., 2013).

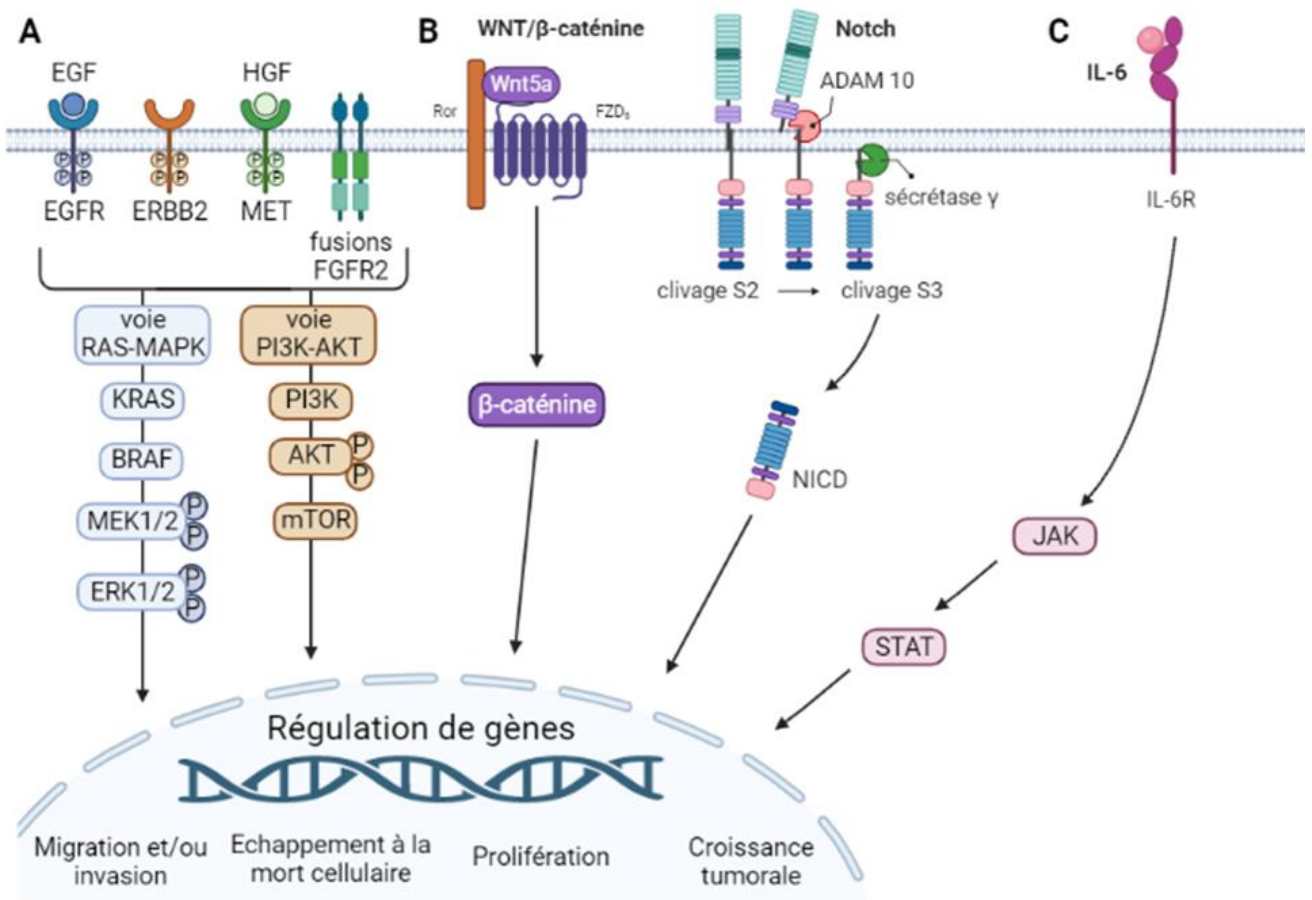
Le rôle de cette voie dans l'initiation du CCAi a notamment été mis en évidence par le développement d'un modèle de souris transgénique dans lequel le domaine intracellulaire de Notch 1 (NICD) est surexprimé spécifiquement dans le foie. Ce modèle a été croisé avec des souris Alb-Cre pour générer un modèle de souris exprimant spécifiquement dans le foie une forme activée du récepteur NOTCH1 (souris Notch1C:AlbCre). Ainsi, des cellules épithéliales avec des caractéristiques de différenciation hépatocytaire et cholangiocytaire ont été observées dans le foie de ces souris après 7 mois. Des changements de la morphologie nucléaire ont été notés dans les tissus hépatiques des souris Notch1C:AlbCre âgées de 8 mois. La xénogreffe de ces cellules dans les flancs de souris immunodéficientes a conduit à la formation de tumeurs portant des caractéristiques histopathologiques du CCAi humain, y compris un stroma abondant et l'expression des marqueurs CK7 et CK17 (Loeuillard et al., 2019).

La voie de signalisation Wnt/ β -caténine régule quant à elle le développement hépatobiliaire et favorise la survie cellulaire dans le CCA (Tokumoto et al., 2005). La fonction de la β -caténine est centrale dans la cascade de signalisation canonique de la voie Wnt qui comprend une grande famille de ligands Wnt et de récepteurs lipoprotéiques *Frizzled*. Dans les cellules épithéliales normales, la β -caténine est principalement liée au niveau de CDH1 engagés dans les jonctions intercellulaires. Dans un contexte pathogène, lorsque l'épithélium est transformé, la perte de CDH1 favorise l'accumulation de la β -caténine dans le noyau. La β -caténine nucléaire s'associe au facteur de transcription LEF/TCF pour réguler l'expression de gènes cibles impliqués dans la prolifération, la différenciation, la migration et l'apoptose des cellules (e.g. *CCND2*, *CDKN2A*, *BIRC5*) (Boulter et al., 2015; Perugorria et al., 2019).

C- Médiateurs inflammatoires

L'inflammation chronique et la fibrose associées au CCAi entraînent la génération de nombreux ligands extracellulaires, tels que l'interleukine-6 (IL-6) et le TGF β , qui modulent les différentes voies de signalisation participant à la progression tumorale.

En particulier, l'IL-6 est abondamment sécrétée lors de la réponse de phase aiguë provoquée par des lésions hépatiques et l'inflammation systémique tout au long de la progression du CCAi. L'IL-6 est sécrétée à la fois par les cellules composant le micro-environnement tumoral (MT) et par les cellules de CCAi, favorisant le maintien de la prolifération et la résistance à l'apoptose des cellules tumorales. L'IL-6 agit notamment en induisant l'expression de la protéine anti-apoptotique MCL-1 dans les cellules de CCA, *via* la phosphorylation (l'activation) du facteur de transcription des transducteurs de signal et des activateurs de transcription 3 (STAT-3). L'expression accrue de MCL-1 confère ainsi aux cellules tumorales une grande résistance au ligand inducteur d'apoptose lié au facteur de nécrose tumorale (TRAIL) (Figure 6C) (Isomoto et al., 2007).



6. Altérations du métabolisme

IDH1 et *IDH2* sont des isocitrate déshydrogénases sont des enzymes oxydoréductases clés du métabolisme cellulaire, qui jouent un rôle important dans la production d'énergie et la synthèse de macromolécules. Ces enzymes jouent un rôle important dans le cycle de Krebs (ou cycle de l'acide citrique), qui est une voie métabolique centrale pour la production d'énergie dans les cellules. Plus précisément, les isocitrate déshydrogénases sont responsables de la troisième étape du cycle de Krebs, au cours de laquelle l'isocitrate est converti en alpha-cétoglutarate avec la production simultanée de dioxyde de carbone (CO₂), de NADH et d'ATP. C'est une étape clé de la production d'énergie dans les cellules, car elle fournit un métabolite intermédiaire essentiel pour la suite du cycle de Krebs. Les mutations de ces enzymes sont fréquemment retrouvées dans plusieurs types de cancer, y compris le CCAi (Gabriel et al., 1986). Les mutations *IDH1* et *IDH2* conduisent à une perte de la fonction catalytique normale de ces enzymes qui convertissent alors l'alpha-cétoglutarate en 2-hydroxyglutarate (2-HG), un métabolite aux propriétés oncogéniques. Cette accumulation de 2-HG peut interférer avec la méthylation de l'ADN et des histones en inhibant l'activité de plusieurs enzymes impliquées dans ces processus, tels que les histones déméthylases et les enzymes de la méthylation de l'ADN. Cette perturbation de la méthylation peut conduire à une expression anormale de gènes et à la perturbation de plusieurs processus cellulaires, tels que la différenciation cellulaire, l'apoptose et la prolifération cellulaire. De plus, le 2-HG peut stabiliser des facteurs de transcription tels que les facteurs HIF-1 α et NF- κ B, qui sont impliqués dans la survie cellulaire, la prolifération et l'angiogenèse. Cette stabilisation anormale peut conduire à une régulation anormale de la transcription de gènes et contribuer à la survie et à la prolifération des cellules cancéreuses (Wang et al., 2014). Les mutations *IDH1* et *IDH2* entraînent également l'inhibition du processus de reprogrammation métabolique, qui se traduit par une baisse de la consommation cellulaire en oxygène et qui contribue à la survie et à la prolifération des cellules cancéreuses (Salati et al., 2020).

Les mutations *IDH1* et *IDH2* ont été trouvées dans environ 15-20 % des CCAi. Ces mutations sont généralement observées conjointement avec des mutations de *TP53* et sont associées à un pronostic plus favorable. Plusieurs études ont montré que les mutations de *IDH1/2* pourraient être considérées comme des facteurs pronostiques du CCAi (Ma et al., 2020).

7. Altérations épigénétiques

Les modifications épigénétiques, telles que la méthylation de l'ADN ou les modifications des histones, jouent également un rôle important dans le développement et la progression du CCAi. Les gènes *ARID1A* et *BAP1* ont notamment été identifiés comme des acteurs importants de ces modifications.

ARID1A et *BAP1* sont respectivement impliqués dans le remodelage de la chromatine SWI/SNF et dans la réponse aux dommages de l'ADN. Les mutations de perte de fonction de ces gènes ont été associées à des changements dans le paysage épigénétique des cellules, notamment à des altérations de la modification des histones et à des changements dans l'expression de gènes impliqués dans la prolifération et la différenciation cellulaire (O'Rourke et al., 2018).

Ces altérations épigénétiques peuvent par exemple modifier des gènes tels que *CCND1* (*cycline D1*), *MYC* et *KRAS* contribuant ainsi à une prolifération accrue, ou encore des gènes tels que *CDH1* (*E-cadherin*) et *CDH2* (*N-cadherin*) associés à une perte de différenciation cellulaire. Quelques études ont cherché à estimer la valeur pronostique de ces mutations, mais il existe encore de nombreuses données contradictoires ne permettant pas de tirer des conclusions sur ce point (Rizzo et al., 2022).

8. Micro-environnement tumoral

A- Un trait caractéristique du CCAi

La recherche en cancérologie a longtemps porté sur la cellule cancéreuse uniquement. Néanmoins, de nombreuses études mettent en lumière que la progression tumorale est non seulement liée au génotype des cellules tumorales, mais aussi aux interactions complexes avec leur environnement, encore nommé stroma (Bremnes et al., 2011).

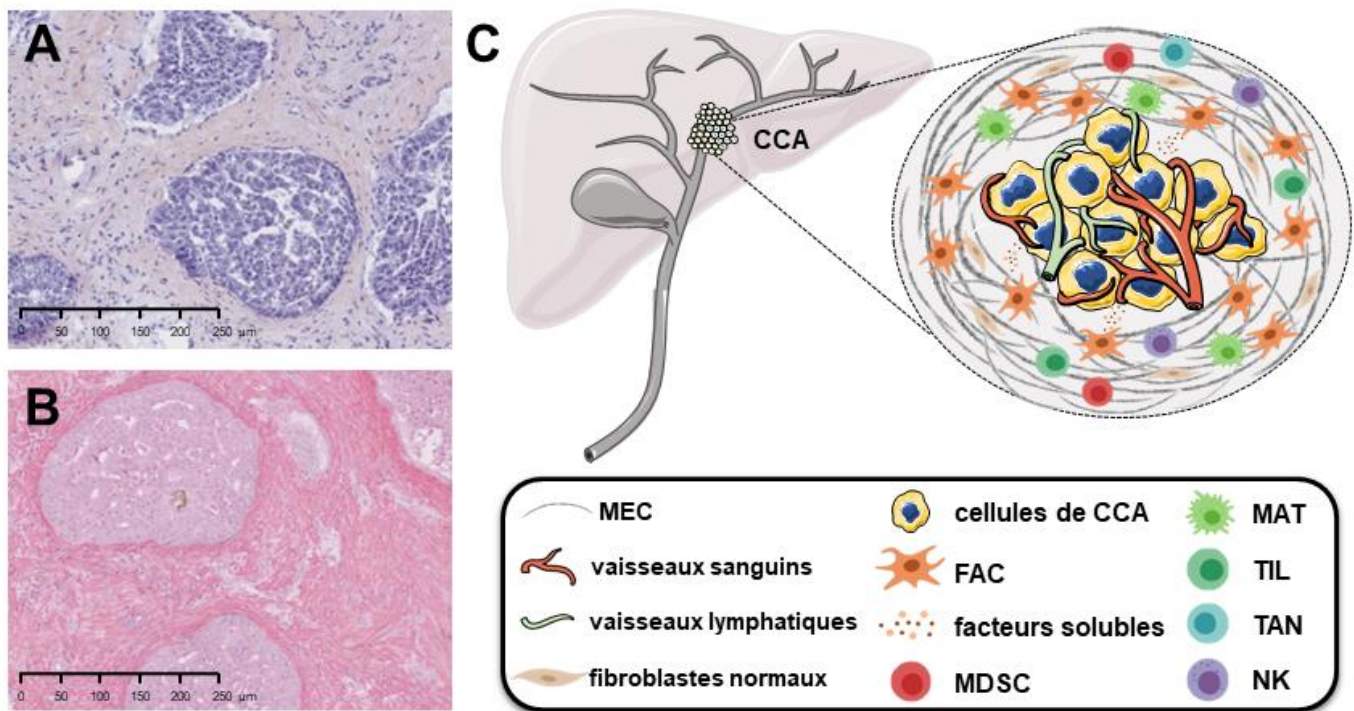
Le CCAi ne fait pas figure d'exception et la présence d'un stroma desmoplastique remanié est une caractéristique histologique distinctive de ce type de tumeur (Figure 7 A-B). Le MT est composé d'un écosystème complexe comprenant des cellules non tumorales qui interagissent avec les composants de la matrice extracellulaire (MEC) et différents facteurs solubles (Figure 7C). Le MT est peuplé d'une grande variété de cellules qui comprend notamment des cellules endothéliales, des péricytes, des fibroblastes associés au cancer (FAC) et des cellules immunitaires (Fabris et al., 2021). Les échanges dynamiques existant

entre le MT et les cellules tumorales de CCAi modulent plusieurs processus associés au développement tumoral. Ils sont notamment impliqués dans la régulation de la mort et de la prolifération cellulaire, de l'angiogenèse, de l'invasion, des métastases et de la chimiorésistance (Hanahan and Coussens, 2012). Les facteurs solubles tels que les cytokines, les chimiokines, les facteurs de croissance et les vésicules extracellulaires (VE) jouent un rôle clé dans l'orchestration de ces interactions (Fabris et al., 2019).

B- Intérêt clinique pour le MT

L'intérêt clinique du stroma a été souligné pour plusieurs tumeurs solides, incluant le cancer du sein, le cancer colorectal et le CCA (Guedj et al., 2021; Zhao et al., 2020a). Ces études suggèrent que le stroma peut avoir une fonction protectrice ou aggravante selon le type de cancer. Dans le cas du CCAi, le rapport entre le stroma et la surface tumorale est inversement corrélé. En effet, les CCAi qui présentent des caractéristiques plus fibreuses sont associés à un meilleur pronostic. Les tumeurs qui présentent une surface stromale élevée sont associées à une meilleure survie sans récurrence (RFS). Ce constat suggère que la présence d'un stroma desmoplastique important aurait des vertus protectrices limitant la progression du CCAi. Le stroma aurait en effet la capacité de freiner l'invasion tumorale en formant une barrière limitant sa diffusion (Guedj *et al.*, 2021). En revanche, un indice élevé de réticulation du collagène (défini comme le nombre de ramifications des fibres de collagène sur toute la longueur du réseau de collagène) est associé à une mauvaise survie globale

(OS) (Guedj *et al.*, 2021). Ainsi, la valeur pronostique du stroma serait influencée non seulement par son abondance, mais aussi par sa réticulation et sa rigidité.

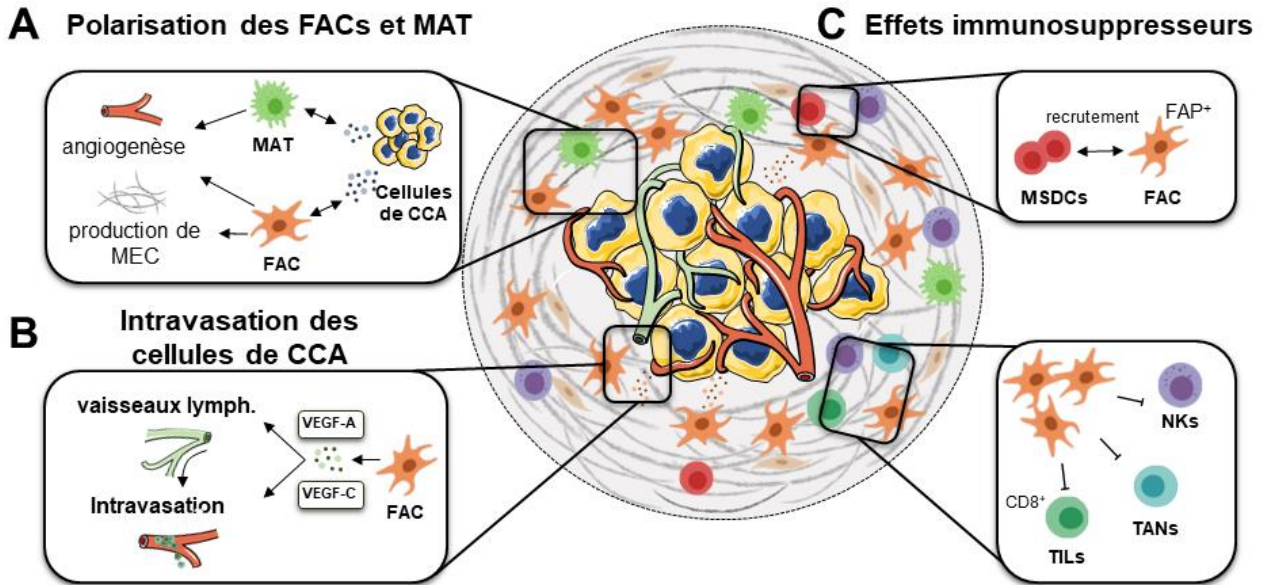


C- Les Fibroblastes Associés au Cancer (FAC)

Une fois le processus desmoplastique bien engagé, les cellules prédominantes dans le stroma sont des cellules de type fibroblastique appelées FAC. Les FAC forment un groupe hétérogène de cellules caractérisées par l'expression de marqueurs phénotypiques, dont l'*alpha-smooth muscle actin* (α -SMA) et le *Platelet-derived growth factor receptor beta* (PDGFR β) (Cadamuro *et al.*, 2019). L'origine des FAC reste ambiguë dans le CCA. Les FAC peuvent dériver d'une grande variété de cellules du foie, telles que les fibroblastes portaux, les cellules stellaires hépatiques (CSH) ou encore les cellules précurseurs

mésenchymateuses dérivées de la moelle osseuse (Affo et al., 2017). Les FAC sont d'abord recrutés par les cellules tumorales de CCAi qui maintiennent ensuite leur activité dans le temps. Une fois activées, les FAC favorisent la progression du CCAi par la sécrétion de divers facteurs solubles, tels que le TGF β , le *Stromal cell Derived Factor 1* (SDF1), le facteur de croissance des hépatocytes (HGF), le facteur de croissance du tissu conjonctif (CTGF) et le facteur de croissance épidermique (EGF) sont abondamment sécrétés par ces cellules (Figure 8A) (Gentilini et al., 2018).

Les FAC sont des protagonistes centraux dans la MT (Figure 8). Ils sont impliqués dans des interactions multidimensionnelles et influencent l'activité d'une grande variété de cellules, telles que les cellules immunitaires, les cellules endothéliales lymphatiques (CEL) et les cellules tumorales de CCAi. Par exemple, Cadamuro *et al.* ont décrit une boucle de régulation paracrine initiée par PDGFD qui intervient dans le stroma du CCAi. Ce mécanisme implique les cellules tumorales de CCAi, les FAC et les CEL (Muniga et al., 2020). En sécrétant du PDGFD, les cellules tumorales induisent la libération de facteurs de croissance de l'endothélium vasculaire (VEGFC et VEGFA) par les FAC, ce qui entraîne l'expansion de la vascularisation lymphatique et l'intravasation des cellules tumorales (Figure 8B). Cette étude suggère que les FAC pourraient être des cibles thérapeutiques potentielles pour le traitement du CCAi. En effet, on peut imaginer qu'une façon de limiter la lymphangiogenèse et l'invasion des cellules de CCAi serait d'induire l'apoptose des FAC ou de cibler l'axe induit par le PDGFD en aval (Muniga *et al.*, 2020). Les FAC sont également connus pour interagir avec les cellules immunitaires. Le sous-ensemble de fibroblastes qui exprime la protéine d'activation des fibroblastes (PAF) présente notamment un phénotype pro-inflammatoire. De plus en plus de données suggèrent que les fibroblastes PAF⁺ pourraient être des médiateurs d'effets inflammatoires et immunosuppresseurs favorisant la progression tumorale (Figure 8C). Plus précisément, les FAC PAF⁺ favorisent le recrutement de cellules myéloïdes suppressives (MDSC) en activant la voie de signalisation de STAT3 (Fabris et al., 2020; Yang et al., 2016).



D- Le système immunitaire

Dans le MT, les réactions inflammatoires jouent un rôle clé dans la carcinogenèse et la progression tumorale. Les caractéristiques inflammatoires et immunitaires du MT ont notamment une importance clinique. Une meilleure compréhension des types de cellules immunitaires et de leurs mécanismes d'action semblent primordiales pour mieux interpréter le pronostic associé aux CCAi (Kitano et al., 2018). En ce qui concerne le système immunitaire adaptatif, les lymphocytes sont les principaux effecteurs des réponses immunitaires antitumorales. Le rôle des lymphocytes infiltrant la tumeur (TIL) a notamment été décrit dans le CCA et serait corrélé au pronostic des patients (Goepfert et al., 2013). Plus précisément, la présence de lymphocytes CD8+ semble être associée à un meilleur pronostic (Asahi et al., 2020). En ce qui concerne le système immunitaire inné, les cellules *Natural Killer* (NK) sont des éléments importants de la surveillance tumorale (Chiossone et al., 2018). Des données épidémiologiques suggèrent que les polymorphismes de gènes

importants liés aux fonctions des cellules NK (*i.e.* KIR et NKG2D) pourraient être associés au risque de développer un CCA (Chiossone *et al.*, 2018; Wadsworth *et al.*, 2019). Cela conforte l'idée que ces cellules jouent un rôle important dans la surveillance immunitaire contre le CCA. En outre, l'infiltration par les cellules NK a été associée à une meilleure OS des patients après résection (Fukuda *et al.*, 2020).

Les macrophages associés aux tumeurs (MAT) sont également des régulateurs importants de la réponse immunitaire tumorale. Dans le foie, les MAT dérivent de cellules de Kupffer ou de macrophages circulants dérivés de la moelle osseuse. Les MAT sont divisés en deux sous-types : le sous-type M1 qui favorise la réponse immunitaire antitumorale et le sous-type M2 qui exerce des effets immunosuppresseurs (Noy and Pollard, 2014). La présence de MAT, et plus particulièrement de MAT de type M2, a été associée à un pronostic plus défavorable pour les patients hospitalisés pour un CCA (Subimerb *et al.*, 2010; Thanee *et al.*, 2015). De plus, il a été démontré *in vitro* qu'il existe une interaction fonctionnelle entre les MAT et les cellules tumorales de CCA. D'un côté, les cellules de CCA exercent des actions sur les macrophages qui leur confèrent des capacités pro-tumorales (Noy and Pollard, 2014) (Zeng *et al.*, 2018). De l'autre côté, la présence importante de MAT est capable de stimuler directement la croissance des cellules tumorales de CCA *via* la voie Wnt/ β -caténine (Loilome *et al.*, 2014).

L'importance du système immunitaire dans l'initiation et la progression du CCAi a été mis en évidence dans une étude de Job *et al.* qui démontre l'existence de sous-type de CCAi caractérisés par une composition du MT et une activité immunitaire spécifique. Cette étude a notamment démontré la valeur pronostique de ces sous-classes basées sur le MT et suggère des cibles thérapeutiques potentielles. La sous-classe présentant l'activité immunitaire la plus importante (11 % des CCAi étudiés) est notamment associée à un meilleur pronostic. Elle est caractérisée par une infiltration importante de lymphocytes T et une activation des voies impliquant les points de contrôle immunitaires (Job *et al.*, 2020).

E- La matrice extracellulaire (MEC)

La présence abondante de FAC dans le MT est également responsable d'une production aberrante de MEC (Figure 8A). La progression et la dissémination du CCAi sont notamment soutenues par cette MEC dense (Fabris *et al.*, 2020). La MEC est formée par un large éventail de molécules multifonctionnelles, comprenant des collagènes et des glycoprotéines

non collagéniques, des protéoglycanes, des glycosaminoglycanes et des polysaccharides (Fabris *et al.*, 2020). Le remodelage de la MEC est étroitement associé à la régulation de voies de signalisation impliquées dans la progression tumorale du CCAi. Par exemple, le stroma particulièrement dense et fibreux associé au CCAi exerce des forces mécaniques anormales sur les cellules qu'il entoure. Il est de plus en plus démontré que ces forces mécaniques peuvent activer dans ces cellules des voies de signalisation intracellulaires participant à la progression tumorale. La *Yes-associated protein* (YAP) et le *Transcriptional co-activator with PDZ-binding motif* (TAZ) sont deux capteurs intracellulaires décrits comme des senseurs clés de ces phénomènes de mécanotransduction. Ils agissent notamment en adaptant la balance entre prolifération et apoptose en réponse aux contraintes physiques qu'ils détectent. YAP/TAZ déclenche également de nombreux processus pro-invasifs, tels que l'induction de la prolifération *via* la voie mTOR ou encore l'échappement à l'apoptose *via* la voie AKT (Fabris *et al.*, 2020).

La MEC est également un réservoir de facteurs solubles impliqués dans le dialogue entre les cellules tumorales et le stroma. Par conséquent, il est naturel de considérer la dégradation de ce stroma fibreux comme une stratégie thérapeutique pour améliorer la prise en charge des patients atteints de CCAi. Par exemple les thérapies actuelles employées pour le CCA pourraient être combinées avec un ciblage de la synthèse du collagène ou à celui de l'hyaluronane pour améliorer leur efficacité. La régulation des voies en amont de la synthèse de la MEC pourrait également constituer une option. Notamment, l'inhibition de la voie de signalisation TGF β afin de réduire la rigidité de la MEC pourrait être une stratégie pertinente. Cependant, comme mentionnée précédemment, l'abondance du stroma a été récemment associée à un meilleur pronostic dans le CCAi (Guedj *et al.*, 2021). Il semble donc crucial de prendre en considération non seulement la quantité de dépôt de MEC, mais également la composition et l'organisation de cette dernière.

II- La voie du « Transforming Growth Factor Beta » (TGF β) dans le CCAi

Le CCA est caractérisé par la présence d'un stroma desmoplastique important et par une forte sécrétion de TGF β . Cette cytokine est connue pour participer à la progression tumorale en favorisant la prolifération, la migration, l'invasion et la résistance à l'apoptose des cellules de CCA. Par conséquent, l'inhibition de la voie de signalisation du TGF β a été proposée

comme stratégie thérapeutique potentielle pour le CCA. Des études précliniques ont montré que les inhibiteurs de la voie du TGF β peuvent ralentir la croissance et l'invasion des cellules de CCA. Certains essais cliniques étudient actuellement la sécurité et l'efficacité ces inhibiteurs chez les patients atteints de CCAi. De plus, une présence importante de TGF β a été associée à un mauvais pronostic pour les patients avec un CCAi. Une meilleure connaissance des effecteurs de la voie de signalisation du TGF β impliqués dans la progression du CCAi pourrait donc permettre d'identifier des biomarqueurs innovants.

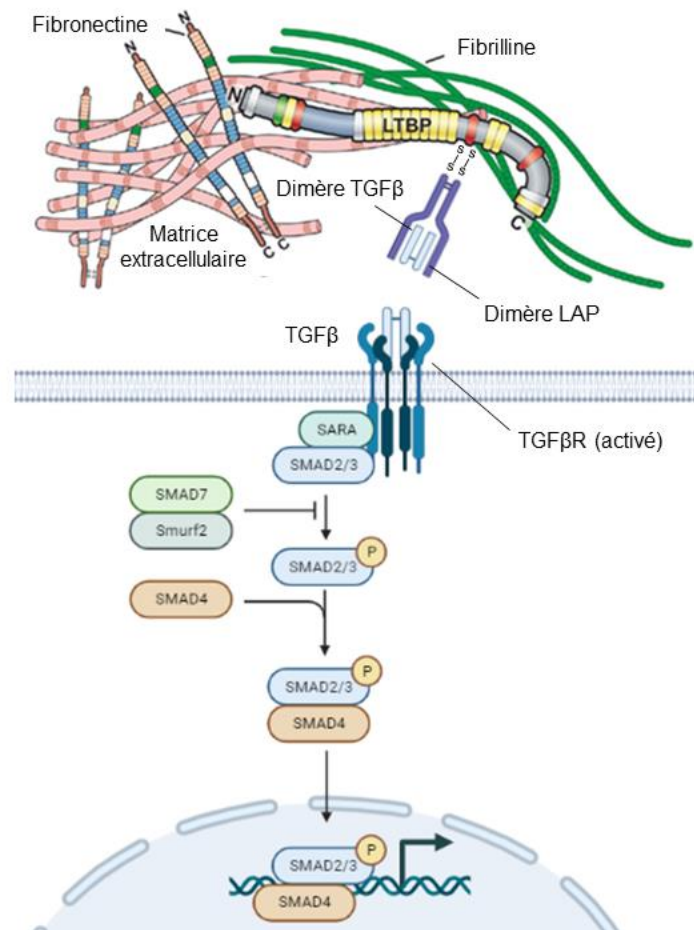
1. La voie du TGF β

Les molécules participant à la voie du TGF β sont des cytokines pléiotropiques qui jouent un rôle important dans l'homéostasie tissulaire, la différenciation cellulaire et le développement embryonnaire. Les ligands extracellulaires du TGF β se lient aux récepteurs TGF β de type I et de type II (respectivement TGF β RI et TGF β RII), initiant ainsi une cascade de signalisation qui régule l'expression de gènes cibles codant pour des protéines (David and Massague, 2018) et des ARNs non codants (Wang et al., 2016). L'activation de la voie du TGF β est amorcée par la liaison des ligands à TGF β RII, puis par la formation d'un complexe hétérotétramérique entre TGF β RII et TGF β RI et enfin par la phosphorylation de TGF β RI par TGF β RII (Figure 9).

La phosphorylation de TGF β RI déclenche son activité sérine/thréonine kinase, entraînant la phosphorylation de Smad2 et Smad3 qui forment alors un complexe avec le médiateur commun Smad4 et transfèrent ainsi le signal dans le noyau. Dans le noyau, ce complexe s'associe à divers co-facteurs pour réguler positivement ou négativement la transcription de gènes cibles (Figure 9). Outre l'activation de cette voie canonique, les récepteurs du TGF β peuvent également induire l'activation de voies non dépendante de Smad, telles que les voies MAPK et PI3K (Miyazono et al., 2018).

Avant de se fixer au récepteur, le TGF β est séquestré sous une forme biologiquement inactive (latente) au niveau de la MEC. Ces stocks de TGF β latents se présentent sous la forme d'un complexe trimérique composé du *latency-associated peptide* (LAP) et des protéines de liaison au TGF β latent (LTBP). Il existe quatre isoformes de LTBP dans le génome humain (LTBP-1, -2, -3 et -4), qui semblent toutes interagir avec la fibrilline (Jensen et al., 2009). Les LTBP sont majoritairement décrites pour leur rôle dans la structuration de la MEC et dans la régulation de l'activité extracellulaire du TGF β , tant par la modulation de sa biodisponibilité (libération du TGF β latent) que par son implication dans sa latence au

niveau de la MEC. Il a été démontré que l'isoforme LTBP-1 pourrait aussi participer directement à l'activation du TGF β *via* une association avec les intégrines, et moduler son activité *via* la régulation de protéases. La libération du TGF β de ce complexe est une étape critique dans la régulation de la signalisation du TGF β (Robertson et al., 2015).



2. Réponses physiologiques dans le foie

La voie de signalisation du TGF β occupe une place importante dans la régulation de programmes transcriptionnels et influencent ainsi de nombreux processus physiologiques

dans le foie, tels que l'arrêt du cycle cellulaire, l'apoptose, la transition épithélio-mésenchymateuse (TEM) ou encore la surveillance immunitaire. Toute altération de ces mécanismes finement régulés peut conduire à un contexte pathologique. Une activité excessive de cette voie peut initier le développement de maladies fibrotiques ou de tumeurs. Par exemple, les phénomènes de fibrose hépatique sont souvent initiés par une production et une accumulation anormales de composants de la MEC au niveau hépatique, notamment de collagène (Bataller and Brenner, 2005). Cette production excessive est la résultante de l'action combinée de réponses inflammatoires, de cellules immunitaires infiltrées et de plusieurs cytokines (e.g. TGF β) qui déclenchent l'activation des CSH et leur différenciation en myofibroblastes. Ce phénomène favorise un micro-environnement pro-fibrotique persistant, conduisant à des maladies fibrotiques chroniques sévères. Les signalisations dépendantes de Smad ou non peuvent médier les effets du TGF β dans les CSH, en induisant l'expression de *connective growth factor* (CTGF) (Caja et al., 2018).

Dans les cellules épithéliales, le TGF β bloque la prolifération cellulaire principalement par l'induction d'inhibiteurs de la kinase dépendante de la cycline (CDK), tels que p21, p15 et p27 ; et par la réduction de l'oncogène MYC (Zhang et al., 2017b).

Les effets proapoptotiques du TGF β sont bien décrits dans le foie normal ou dans des lignées de CHC. Par exemple, le TGF β possède une action proapoptotique *via* l'activation de caspases dans Hep3B, une lignée cellulaire de CHC. Dans le cas des cellules Huh7, des phénomènes d'apoptose sont également médiés par le TGF β qui participe l'activation de caspases et à la répression de protéines anti-apoptotiques, telles que Bcl-XL et XIAP (Schuster and Krieglstein, 2002). Dans d'autres modèles cellulaires, le rôle de la voie JNK (Yamashita et al., 2008), l'induction du suppresseur de tumeur *TGF β inducible early growth response protein 1* (TIEG1) (Bender et al., 2004) ou encore la *Death-Associated Protein Kinase 1* (DAPK1) (Jang et al., 2002) ont été rapportés comme médiateurs de l'apoptose en aval de la voie de signalisation TGF β .

Le TGF β joue également un rôle central dans la promotion de la TEM, un processus par lequel les cellules épithéliales perdent leurs caractéristiques morphologiques et leurs propriétés d'adhésion pour acquérir des traits mésenchymateux. Cette transformation les rend plus promptes à migrer et à quitter leur tissu d'origine (Tsubakihara and Moustakas, 2018). Le processus de TEM est un processus crucial de l'embryogenèse. Cependant, à des stades avancés de la progression tumorale, les carcinomes épithéliaux sont sujets à une

hyperactivation de ces voies de signalisation induisant la TEM, telles que les voies Wnt et TGF β . Cette suractivation conduit à l'acquisition de propriétés migratoires responsables des métastases. De nombreux rapports se rejoignent et attribuent une corrélation positive entre la TEM et les métastases. Néanmoins certains d'entre eux soutiennent que la TEM n'est pas un facteur déterminant pour les métastases pancréatiques (Zheng et al., 2015) ou pulmonaires (Fischer et al., 2015), mais qu'elle serait plutôt à associer à une résistance accrue aux chimiothérapies.

La TEM est également associée à la génération de cellules souches cancéreuses (CSC). Ces dernières sont définies comme des cellules cancéreuses indifférenciées aux propriétés souches qui contribuent à l'hétérogénéité tumorale et présentent une faible sensibilité aux chimiothérapies (Singh and Settleman, 2010). Le TGF β , qui est un régulateur positif de la TEM, est par conséquent capable de conférer un caractère souche aux cellules cancéreuses, notamment aux cellules de CHC (Malfettone et al., 2017). Au niveau moléculaire, le TGF β est capable d'initier la transition de cellules de phénotypes épithéliaux. Cette transition passe soit par une diminution de l'expression, soit par une redistribution des composants des jonctions serrées (e.g. E-cadhérine) loin de la membrane cellulaire, conduisant ainsi à leur désassemblage. De plus, le TGF β induit de manière simultanée l'expression de plusieurs facteurs de transcription impliqués dans la TEM (Snail, Slug, ZEB1, ZEB2, Twist, SOX4) et de différents marqueurs mésenchymateux (fibronectine, vimentine, collagènes) pour aboutir à une TEM complète (Moustakas and Heldin, 2016).

Les réponses du système immunitaire sont également contrôlées par la voie du TGF β , qui exerce généralement des effets immunosuppresseurs impliqués dans l'échappement immunitaire des cellules cancéreuses. Le TGF β contrarie notamment la capacité des cellules immunitaires à reconnaître et à éliminer les cellules cancéreuses, en bloquant la différenciation des cellules CD4⁺ naïves en cellules régulatrices T, limitant ainsi la production de cellules T effectrices. En outre, il empêche les NK de détruire les cellules tumorales. Un exemple typique du rôle du TGF β dans la réponse immunitaire est présenté dans une étude de 2018 (Thepmalee et al., 2018). Elle suggère que les perturbations de la voie TGF β provoquées par des anticorps neutralisants ciblant T β RII dans les cellules dendritiques (CD) conduisent à une activation des cellules T effectrices. Ainsi, la réponse immunitaire serait améliorée, entraînant un ciblage et une lyse plus efficaces des cellules de CCA présentes en coculture.

En outre, une inflammation chronique, causée par un traumatisme tissulaire ou par la présence de niveaux élevés de cytokines pro-inflammatoires au niveau du canal cholédoque, est souvent à l'origine du développement de CCA, (Landskron et al., 2014). Le TGF β joue également un rôle dans ces processus inflammatoires, notamment en induisant l'expression de l'IL-6 (Shimizu et al., 2006). Les MAT constituent un autre facteur important de la progression tumorale. Ces cellules immunitaires sont recrutées sur le site de la tumeur et sécrètent diverses cytokines, telles que le TGF β et les IL, créant ainsi un micro-environnement pro-inflammatoire qui favorise la progression tumorale. Une étude a notamment démontré que l'ajout de surnageant de culture de cellules de CCAi sur des MAT conduisait à une production importante de TGF β , d'IL-10 et de VEGF. Ceci souligne l'importance de l'interaction entre le TGF β et les MAT dans le MT du CCA (Hasita et al., 2010).

3. La dualité fonctionnelle du TGF β dans la cancérogenèse

Le TGF β représente un défi clinique pour le développement de thérapies ciblées en raison de sa dualité fonctionnelle (*i.e.* capacité à la fois à inhiber et à faciliter la progression tumorale). Au cours de l'initiation tumorale, le TGF β endosse plutôt le rôle de suppresseur des tumeurs en induisant l'arrêt de la prolifération et la mort cellulaire programmée. En revanche, dans les tumeurs malignes de stades avancés, le TGF β exerce préférentiellement des actions pro-tumorales en affectant le comportement des cellules cancéreuses elles-mêmes, ou en créant un micro-environnement favorable à la croissance tumorale (Huang and Blobel, 2016). La résistance de certains cancers avancés aux propriétés antitumorales du TGF β peut être la conséquence de mutations génétiques de gènes cyostatiques ou encore de mutations de certains acteurs majeurs de la voie de signalisation canonique, telles que T β R2 et SMAD4 (Massague, 2008). Il a par exemple été prouvé que les cellules de CCA opposent une résistance aux effets cyostatiques du TGF β , en raison de niveaux d'expression élevés de la cycline D1. Des cellules épithéliales biliaires normales exprimant des niveaux physiologiques de cycline D1, subissent au contraire un arrêt du cycle cellulaire en réponse à un traitement au TGF β (Zen et al., 2005).

Dans un contexte de carcinome hépatique, le TGF β induit des facteurs cyostatiques et pro-apoptotiques limitant la progression tumorale à des stades précoces. Néanmoins, il favorise la TEM à des stades plus avancés, et stimule la génération de FAC dans le stroma tumoral. Cette action combinée a pour conséquence le maintien de l'activité de la voie de signalisation

du TGF β , favorisant ainsi les métastases (Figure 10) (Fabregat and Caballero-Diaz, 2018; Roberts and Wakefield, 2003).

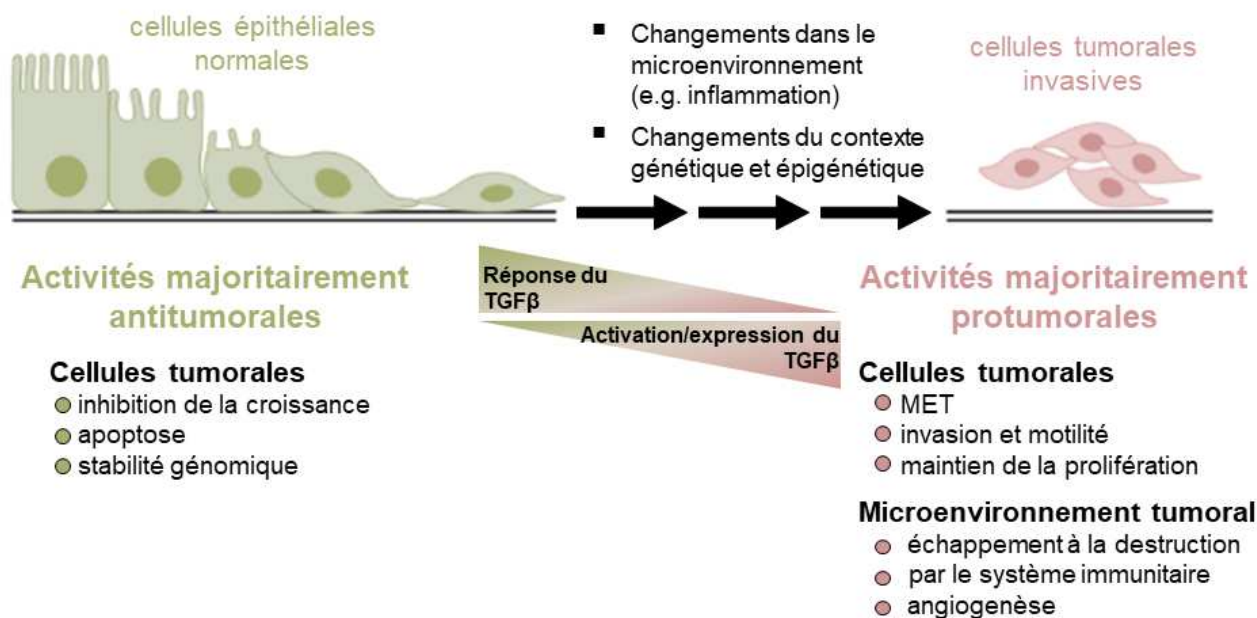


Figure 10 : Dualité fonctionnelle du TGF β dans la cancérogenèse

Le TGF β passe du statut de suppresseur de tumeur dans les stades précoces de la tumorigenèse à celui de pro-oncogène à des stades plus avancés, conduisant notamment à un phénotype métastatique. L'évolution du contexte pathologique s'accompagne généralement d'une diminution ou d'une altération de la réactivité du TGF β et d'une augmentation de l'expression ou de l'activation du ligand du TGF β . Ces perturbations, ainsi que d'autres modifications du contexte génétique ou épigénétique de la cellule tumorale et de son stroma, se combinent pour modifier le spectre des réponses biologiques au TGF β . Adapté de (Roberts and Wakefield, 2003)

4. Le TGF β participe à la progression tumorale du CCA

A- Altérations génomiques

Dans de nombreux cancers, dont le CCA, les gènes codant pour les membres de la voie du TGF β sont fréquemment sujets à des mutations génomiques. Ceci reflète l'importance de cette voie dans la modulation de la progression tumorale (Seoane and Gomis, 2017). En effet, des études impliquant un séquençage du génome entier ou de l'exome ont identifié les principales voies de signalisation touchées par des aberrations génomiques dans le CCA. Parmi elles, le TGF β occupe une place particulière. Ainsi, en s'appuyant sur une large cohorte de 103 patients atteints de CCAi, Zou *et al.* ont identifié des altérations de l'exome qui touchent les voies Ras/PI3K, p53 et TGF β . *SMAD4* a notamment été décrit comme l'un

des 25 gènes significativement mutés, avec un taux de mutation de 4 % (Zou et al., 2014). Dans une autre étude, *SMAD4* était muté chez 3,6 % des 55 patients avec un CCAi et chez 25 % des 20 patients avec un CCAe (Churi et al., 2014). L'expression de *SMAD4* a également été évaluée par immunohistochimie dans des tissus de foie normal et dans des CCAi. Cette expression a alors été associée à différentes caractéristiques cliniques, telles que le stade de différenciation et le niveau de métastases. Ainsi, les résultats suggèrent une corrélation négative de l'expression de *SMAD4* avec un stade tumoral avancé et un haut potentiel métastatique (Yan et al., 2013). Une perte complète de l'expression de *SMAD4* et des gènes codant pour les inhibiteurs du cycle cellulaire a été constatée par immunohistochimie chez 35,7 % des patients atteints d'un CCAi (n=42), (Kang et al., 2002).

Des mutations ponctuelles affectant *SMAD4* au niveau de leurs zones d'interaction (*linkers*) altèrent grandement leurs fonctions régulatrices. En effet, la phosphorylation de ses *linkers* est un mécanisme important pour l'activation de *SMAD4*. Elle permet la liaison de *SMAD4* à d'autres protéines et sa translocation vers le noyau où elle peut réguler l'expression de différents gènes. De telles mutations sont par exemple fréquemment retrouvées dans le cancer du pancréas ou le cancer colorectal, où elles sont responsables de l'inhibition de la voie canonique régulant les actions cytotostatiques du TGF β (Demagny and De Robertis, 2016).

B- La voie de signalisation du TGF β dans le CCA

Le TGF β est l'une des voies de signalisation qui favorisent la progression du CCA. De nombreuses études soutiennent que le TGF β joue un rôle central dans l'induction de la TEM dans des lignées cellulaires de CCA. La stimulation par un traitement TGF β sur les lignées CCKS-1 et TFK-1 a notamment conduit à une induction transcriptionnelle de *SNAIL*, *VIM* (*Vimentin*) et de *S100A4* et à une diminution de l'expression de *CDH1* et de la cytokératine 19 (CK19). Une telle régulation transcriptionnelle favorise ainsi la migration et l'invasion des cellules de CCA (Sato et al., 2010). En outre, des expériences *in vivo* utilisant des modèles de xénogreffes de souris ont montré une dissémination accrue et une croissance tumorale plus importante dans les souris injectées avec des cellules CCKS-1 traitées préalablement au TGF β comparé au groupe contrôle. Ces effets pro-tumorigènes du TGF β ont ensuite pu être abolis par l'administration d'une forme soluble de T β RII (TGF β sRII) qui entre en compétition avec le T β RII, diminuant ainsi l'activité de la voie TGF β (Sato *et al.*, 2010).

Le rôle du TGF β dans l'induction de la TEM a également été décrit dans KKK-M213 et HuCC-A1, deux lignées de CCAi. Un traitement TGF β sur ces cellules a notamment induit une augmentation des niveaux d'expression de *VIM* et de *Slug*, et une sécrétion plus importante de la métalloprotéinase 9 (MMP9). Cette activation s'accompagne d'une augmentation de la migration et de l'invasion cellulaire (Sritananuwat et al., 2017). De manière générale, l'activité des voies TGF β canonique et non canonique est illustrée respectivement par les niveaux de phosphorylation de Smad2/3 et ERK1/2. De manière intéressante, l'inhibition de l'activité kinase de MEK, en aval de ERK, diminue l'induction TGF β -dépendante de la TEM, tout en potentialisant les actions antiprolifératives du TGF β (Sritananuwat *et al.*, 2017). Cette démonstration suggère la possibilité d'un blocage sélectif de la signalisation non canonique du TGF β pour supprimer les effets pro-tumorigènes, tout en préservant les effets anti-tumorigènes dans le CCA.

De la même manière, l'inhibition de la voie du TGF β dans des cellules humaines primaires de CCA par un inhibiteur sélectif du TGF β RI LY2157299 (*galunisertib*), entraîne une diminution de la migration cellulaire. Néanmoins, les processus du cycle cellulaire ou d'apoptose ne sont pas impactés par cet inhibiteur, indiquant ici encore un blocage ciblé de la voie TGF β (Lustri et al., 2017).

Un lien intéressant entre la signalisation du TGF β , la TEM et la génération de cellules de CCA avec des propriétés de cellules souches a été soulevé par Shuang *et al.* (Shuang et al., 2014). Selon cette étude, l'exposition des cellules TFK-1 au TGF β a entraîné l'acquisition d'un phénotype mésenchymateux et une augmentation de la population de cellules positives pour le marqueur de cellules souches cancéreuses aldéhyde déshydrogénase1 (ALDH1). De plus, une diminution du taux de mort cellulaire des cellules de CCA TFK-1 a été observée en réponse à l'agent endommageant l'ADN 5-fluorouracile (5-FU) à la suite d'un traitement au TGF β . Ceci suggère que le TGF β pourrait conférer une chimiorésistance aux cellules de CCA (Shuang *et al.*, 2014).

Le ciblage de la voie TGF β semble ainsi pertinent pour le traitement du CCA. Néanmoins, la dualité fonctionnelle et la complexité de la voie de signalisation du TGF β rendent ce ciblage complexe. Ainsi, l'identification de nouveaux régulateurs et effecteurs de la voie du TGF β permettrait d'élargir l'arsenal thérapeutique déjà disponible pour le traitement du CCAi et potentiellement d'identifier des biomarqueurs diagnostiques et pronostiques innovants.

5. Les ARN non-codants : de nouveaux effecteurs de la voie du TGF β

Parmi les pistes explorées pour identifier de nouveaux effecteurs de la voie du TGF β , les ARN non codants (ARNnc) ont été envisagés dans de nombreuses études. En effet, les ARNnc jouent un rôle régulateur et structurel clés dans les cellules. La singularité de cette classe d'ARN repose sur le fait qu'ils ne soient pas traduits en protéines.

A- Les ARN longs non codants (ARNlnc)

Les ARNnc de plus de 200 nucléotides sont définis comme de ARNlnc. De nombreuses études ont évalué le rôle de ces ARN dans la voie de signalisation du TGF β , et notamment leur rôle potentiel dans la progression tumorale.

Par exemple dans le CHC, l'ARNlnc-activé par le TGF β (ARNlnc-ATB) est un gène cible de la voie de signalisation TGF β qui contribue à l'apparition de métastases en épongeant miR-200. La séquestration de ce miRNA participe ainsi à stabiliser les facteurs de transcription ZEB1 et ZEB2 impliqués dans la TEM et à augmenter les niveaux d'IL-11, une cytokine pro-tumorale (Yuan et al., 2014).

B- Les micro-ARN (miARN)

Les autres ARNnc sont classiquement désignés comme des ARNnc courts. Cette deuxième catégorie comprend notamment les miRNA, dont le rôle fonctionnel est de cibler les ARN messagers (ARNm) pour les dégrader ou inhiber leur traduction, contribuant ainsi à la régulation de l'expression de nombreux gènes (Bunch, 2018). L'implication des miARN dans la voie de signalisation du TGF β , et son rôle dans la modulation de la TEM, ont été évalués dans des échantillons provenant de patients atteints de CCA. Une mesure des niveaux d'expression de marqueurs épithéliaux et mésenchymateux dans les tissus tumoraux et adjacents non néoplasiques de 20 patients a ainsi été effectuée. Ces résultats révèlent une faible expression des marqueurs épithéliaux (*e.g.* CDH1) et du miRNA miR-200b accompagnée d'une expression élevée des marqueurs mésenchymateux tels que la fibronectine et l' α -smooth muscle actin (α -SMA) dans les tissus tumoraux (Zhang et al., 2017a). En outre, des niveaux élevés de TGF β ont été détectés dans les tissus tumoraux comparés aux tissus normaux adjacents. De plus, des expériences *in vitro* utilisant les lignées cellulaires humaines HCCC et RBE ont confirmé la régulation négative de miR-200b par le TGF β , conduisant ainsi à l'établissement d'un programme transcriptionnel favorisant la TEM. Les ARNm codant pour les protéines AP2 α et MAPK7 ont notamment été identifiés

comme des cibles directes de miR-200b. En outre, la surexpression de miR-200b a conduit à une diminution de la formation de tumeurs et de la TEM dans un modèle de souris. Cette expérience souligne ainsi son rôle de suppresseur de tumeurs et l'importance de sa répression par le TGF β pour réguler la TEM (Zhang *et al.*, 2017a).

Dans un modèle de rat développant naturellement des CCAi, une augmentation de la taille de la tumeur et du nombre de métastases intrahépatiques a été enregistrée en conséquence d'une surexpression de TGF β (Huang *et al.*, 2016). Les auteurs ont également démontré que l'inhibition de la voie TGF β , grâce à un ARN interférent spécifique ou en abolissant l'activité kinase de TGF β RI *via* deux inhibiteurs chimiques (LY2157299 et SB431542), affectait négativement la croissance cellulaire et la migration de lignées cellulaires de CCA (RBE et SSP25). Les efforts visant à identifier les mécanismes moléculaires à l'origine de ces changements phénotypiques ont révélé miR-34a comme un effecteur central de la voie de signalisation du TGF β . En effet, il a été mis en évidence que l'expression de miR-34a est réduite en réponse au TGF β , ce qui conduit à une augmentation des niveaux d'expression des cibles directes de miR-34a, (*i.e.* CDK6, cycline D1 et c-Met). La stabilisation des niveaux de CDK6 et de cycline D1 contribue ainsi à induire la prolifération cellulaire, et l'élévation des niveaux de c-Met favorise la migration cellulaire (Huang *et al.*, 2016).

Une autre étude décrit également le rôle anti-migratoire du miR-34a dans le CCA, mais propose ici un mécanisme alternatif suggérant une intervention de ce miRNA dans la régulation de la TEM. Le miR-34a est en effet décrit pour cibler *SMAD4* et ainsi supprimer la TEM induite par le TGF β . Cet axe de régulation est particulièrement pertinent, puisque les niveaux de miR-34a et de Smad4 sont inversement corrélés. Ainsi, des niveaux d'expression de Smad4 particulièrement élevés ont été associés à des faibles niveaux de détection de miR-34a dans des tissus de CCAe humains (Qiao *et al.*, 2015).

Toutes ces données indiquent que les miARN sont étroitement impliqués dans la régulation de la voie TGF β . Avec l'avènement des techniques de séquençage à haut débit, des études sont parvenues à identifier de manière non supervisée des signatures de miARN régulées par le TGF β dans le CCAi. Ces études permettent d'avoir une vision plus globale et donc une meilleure compréhension du réseau d'ARN régulé par le TGF β , offrant ainsi de nouvelles perspectives pour cibler cette voie sur le plan clinique. De cette manière, Ota *et al.* ont identifié 73 miARN significativement dérégulés par le TGF β dans des lignées de CCAi (HuCC-T1). Cette signature a notamment permis d'identifier le miARN antitumoral miR-30e.

L'expression induite de miR-30e a conduit à une diminution de l'expression de SNAIL et de plusieurs marqueurs de la TEM dans ces cellules. De plus, le miR-30e est présent dans des VE secrétées par les cellules tumorales et agit de façon paracrine sur les cellules tumorales environnantes, dans lesquelles il exerce ses fonctions anti-TEM. Un traitement au TGF β a permis de réduire l'abondance de ce miARN dans les vésicules extracellulaires (VE) secrétées par les cellules de CCA, élargissant encore un peu plus les fonctions régies par le TGF β dans la progression tumorale (Ota et al., 2018).

III- Les ARN circulaires

D'autres effecteurs de la voie TGF β pourraient encore complexifier ce réseau d'ARN régulateurs déjà très intriqué. Les ARNcirc pourraient notamment représenter de nouveaux protagonistes dans ce labyrinthe d'ARN. En effet, les nombreuses fonctions régulatrices et les caractéristiques prometteuses des ARNcirc ont suscité beaucoup d'intérêt au cours des dernières années.

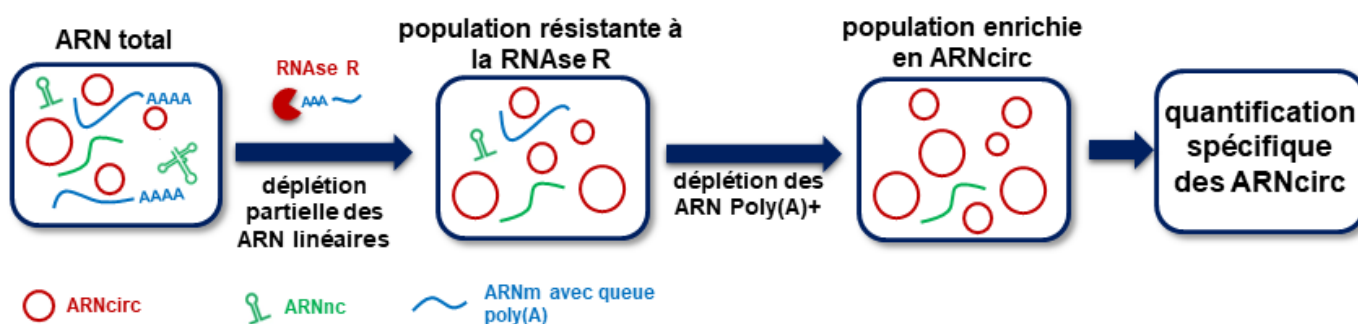
1. Découverte des ARNcirc

Les ARNcirc ont été identifiés pour la première fois en 1976 sur des particules virales (Sanger et al., 1976). Trois ans plus tard, la microscopie électronique a permis d'observer ces mêmes structures circulaires intrigantes dans le cytoplasme de cellules eucaryotes, notamment dans les cellules humaines HeLa (Perry and San Clemente, 1979). Ces entités étaient alors encore considérées comme des artefacts et ne soulevaient que peu d'intérêt. Il a fallu attendre 1993 avant que l'existence d'un ARNcirc, l'ARNcirc Sry (circSry dérivé de la région Y déterminant le sexe), soit démontrée chez la souris par des approches de biologie moléculaire. Pour prouver sa forme circulaire, la méthode suivante a été utilisée : l'ARN total a d'abord été hybridé avec des oligonucléotides spécifiques, puis digéré par un traitement RNase H qui dégrade les régions double brin des hybrides ARN-ADN. Ainsi, si le transcrit Sry existe sous forme de molécule linéaire, ce traitement entraîne la détection de deux bandes sur un Northern blot avec une sonde ciblant ces oligonucléotides spécifiques. En revanche, si le transcrit est une molécule circulaire, l'hybridation avec un oligonucléotide spécifique ne donnerait qu'une bande après traitement avec RNase H (Capel et al., 1993).

Par la suite, de nouveaux ARNcirc ont été identifiés, tels que l'ARNcirc provenant du gène 2C24 du cytochrome P450 du rat (Zaphiropoulos, 1996). Ils étaient alors décrits comme

des "scrambled exon RNA". Toutefois, l'intérêt pour les ARNcirc restait limité à cette époque, puisqu'aucune fonction biologique spécifique ne leur avait encore été attribuée.

L'avènement des technologies de séquençage à haut débit et le début des études à l'échelle du génome ont marqué un tournant pour la recherche sur les ARNcirc, en permettant l'identification et la caractérisation à grande échelle de cette nouvelle classe d'ARN. Les pipelines de séquençage d'ARN traditionnels éliminaient jusqu'alors les lectures non conventionnelles qui ne correspondent à aucune référence connue du génome. Par exemple, les lectures issues de la fusion de gènes ou celles provenant d'événements d'épissage alternatif étaient éliminées. Néanmoins, de nouveaux algorithmes bio-informatiques de cartographie du génome ont incrémenté une nouvelle fonction permettant la reconnaissance de ces jonctions chevauchantes issues d'épissages alternatifs, et donc la détection d'ARNcirc *de novo* et de leurs homologues linéaires (*i.e.* DCC, Acfs, CIRCexplorer3-CLEAR) (Cheng et al., 2016; You and Conrad, 2016). En outre, des protocoles spécifiques pour la préparation de bibliothèques de *Next Generation Sequencing* (NGS) ont été établis afin d'enrichir la fraction d'ARN en ARNcirc. Ce protocole comprend notamment un appauvrissement des ARN ribosomiques et poly(A)⁺ et un traitement RNase R qui conduit à une dégradation des ARN linéaires (Figure 11) (Szabo and Salzman, 2016).



La démocratisation de ces approches a conduit à la création de plusieurs bases de données répertoriant les ARNcirc identifiés. Parmi elles, circBase s'est appuyée sur des études pionnières dans le domaine (Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2013) pour centraliser et annoter la plupart des ARNcirc identifiés chez l'homme à cette époque. L'objectif de cette base de données était d'offrir un outil simple d'utilisation visant à homogénéiser et développer la recherche autour des ARNcirc. Circbase est régulièrement incrémentée par de nouvelles bases de données (Glazar et al., 2014). Récemment, une méthode utilisant l'amplification « *rolling circle* », suivie d'un séquençage nanopore à lecture longue (*long read sequencing*), a permis de décrypter la séquence complète d'ARNcirc, apportant ainsi des informations précieuses sur l'existence de nombreuses isoformes d'ARNcirc (Xin et al., 2021). Il est même maintenant admis que les isoformes d'ARNcirc sont abondantes et proviendraient de milliers de gènes humains (Salzman et al., 2012) et que leur expression a été conservée à travers le règne des Eucaryotes (Wang et al., 2014).

2. Biogenèse des ARNcirc

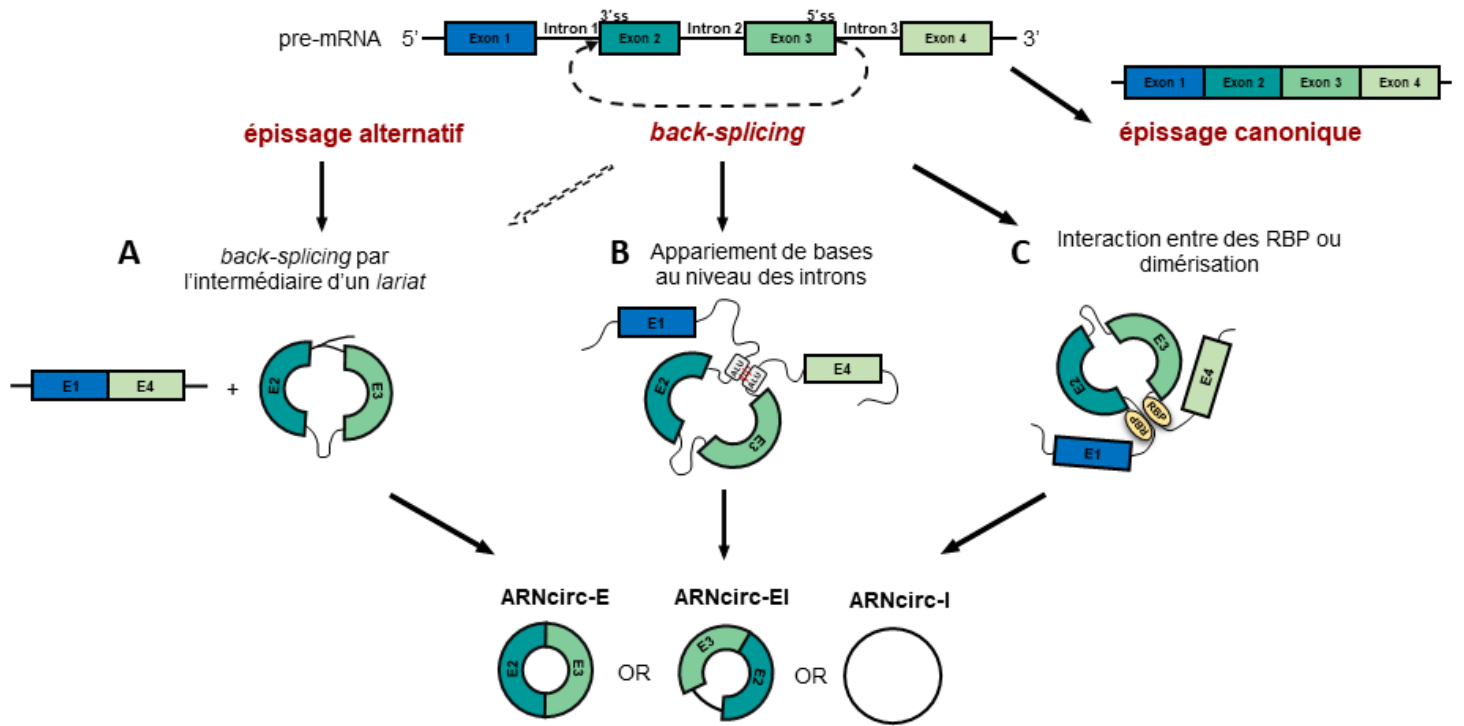
Contrairement à leur homologue linéaire, les ARNcirc sont générés à la suite d'un épissage non canonique appelée rétroépissage (*back-splicing*). Un site donneur d'épissage 5' en aval de l'ARN pré-messager réagit avec un site accepteur d'épissage 3' en amont, ce qui entraîne la jonction de l'extrémité 3' d'un exon en aval avec l'extrémité 5' d'un exon en amont. Ainsi, une jonction de circularisation est formée par deux exons mal ordonnés au regard de leur emplacement génomique. Le *back-splicing* des ARNcirc est un processus actif qui peut être favorisé par différents mécanismes (Figure 12).

Par exemple, de nombreuses études ont démontré que des séquences complémentaires localisées dans les introns flanquant la jonction d'exons circularisés, pouvaient favoriser la circularisation d'un ARN (Liang and Wilusz, 2014; Zhang et al., 2014). En s'hybridant les unes aux autres, ces séquences complémentaires forment une boucle qui permet de rapprocher des sites d'épissage distants, ce qui favorise les événements de *back-splicing* (Figure 12B).

D'autres études ont rapporté que la biogenèse des ARNcirc pouvait également être favorisée par la fixation de protéines de liaison à l'ARN (RBP) en amont et en aval des exons circularisés, favorisant le *back-splicing* par interaction directe ou par dimérisation (Figure 12C). En effet, une combinaison d'éléments agissant en *cis* et de facteurs agissant en *trans* pourrait rapprocher un site d'épissage donneur en 5' d'un site accepteur d'épissage

en 3' en amont, régulant ainsi finement les événements de *back-splicing*. Par exemple, en se liant à des motifs introniques spécifiques, la RBP Quaking (QKI) est responsable de la biogenèse d'ARNcirc spécifiques de la TEM. De manière plus spécifique, l'expression de QKI5 est régulée par le TGF β , suggérant que la biogenèse des ARNcirc pourrait être activement régulée par ce facteur de croissance (Conn et al., 2015). Il a également été démontré que l'insertion artificielle de sites de liaison pour des motifs QKI introniques était suffisante pour induire un *back-splicing*.

Un autre mécanisme nommé "épissage en lariat" a également été décrit pour favoriser la formation d'ARNcirc. Lors de cet épissage alternatif, les exons exclus sont incorporés dans un lariat circulaire intermédiaire contenant une structure intron-exon qui pourrait être épissée une seconde fois et générer ainsi un ARNcirc (Figure 12A) (Jeck *et al.*, 2013; Zaphiropoulos, 1996). Alors que la majorité des ARNcirc est composée de séquences exoniques (ARNcirc-E), une partie non négligeable des ARNcirc comprend également des séquences introniques. De tels ARNcirc sont appelés ARNcirc Exonic-Intronic (ARNcirc-EI). Cette catégorie d'ARNcirc serait plutôt localisée dans le noyau où ils interagissent avec snRNP U1, régulant ainsi la transcription de leur propre gène parental (Li et al., 2017). De même, certains ARNcirc ne sont constitués que de séquences introniques (ARNcirc-I ; ARNcirc dérivés de lariat), et seraient présents dans la même proportion que les ARNcirc-E (Figure 12) (Stoll et al., 2020).



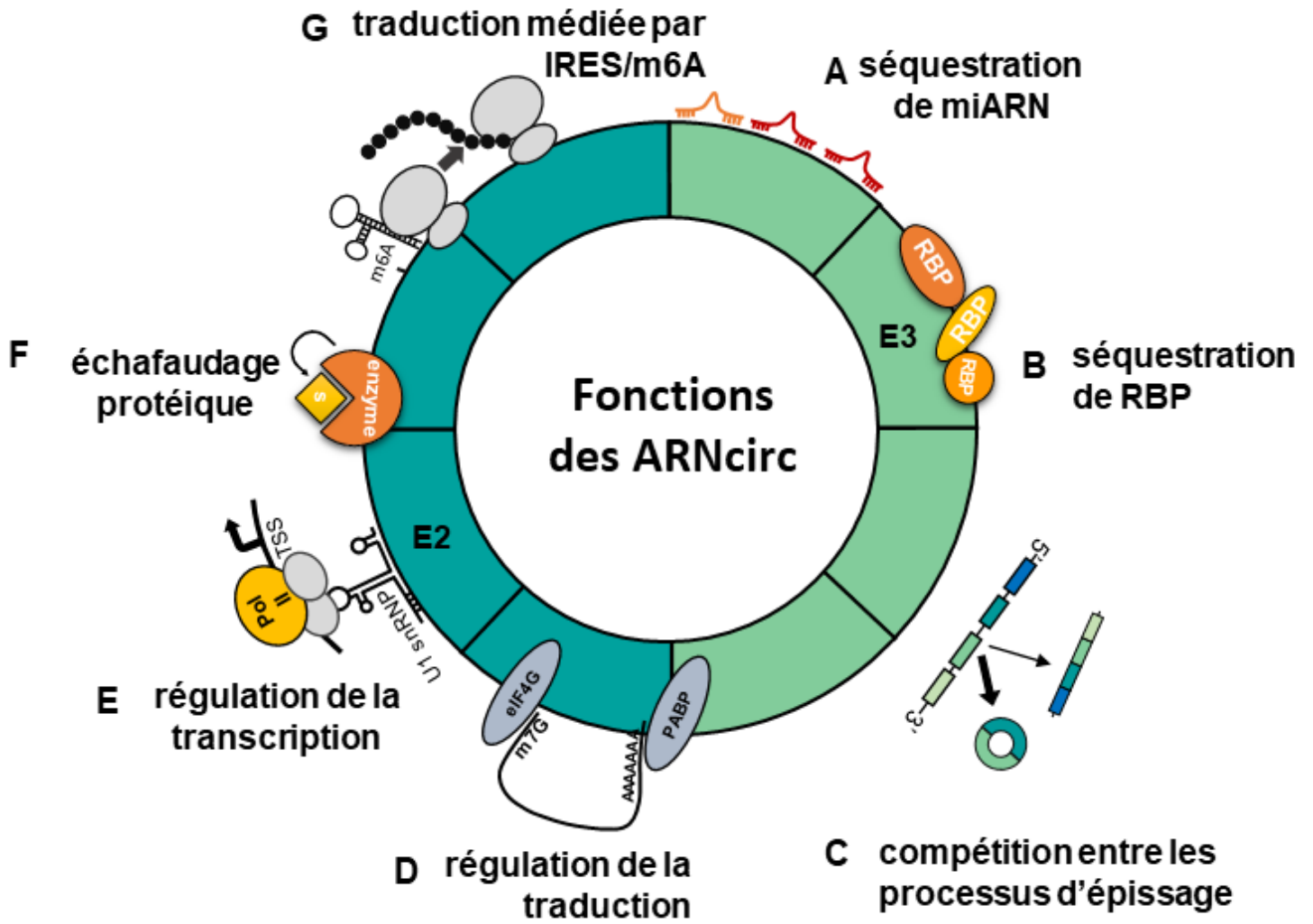
3. Fonctions des ARNcirc

Bien que les ARNcirc aient été découverts au cours du siècle dernier, établir une liste exhaustive des fonctions qu'ils assurent reste un défi majeur. En effet, on rencontre plusieurs difficultés lorsque l'on s'intéresse aux ARNcirc. Leur faible abondance et les similarités de séquences avec les ARN linéaires les rendent notamment difficiles à détecter spécifiquement. À ce jour, la fonction des ARNcirc la plus largement décrite est sans aucun doute leur capacité à éponger les miRNA (Hansen et al., 2013). En se liant aux régions 3' non traduites des ARN messagers (ARNm), les miARN peuvent réprimer la traduction des ARNm et/ou induire leur dégradation (Bartel, 2009). En conséquence, l'abondance et l'activité des miRNA sont étroitement régulées. Les miARN peuvent notamment être séquestrés par des éponges à ARN, dont les ARNcirc (Tay et al., 2014). À titre d'exemple, l'ARN circulaire antisens CDR1 humain (circCDR1as) contient plus de 70 sites de liaison pour miR-7. Ainsi, grâce à sa capacité d'éponge à miRNA, circCDR1as agit comme un ARN compétitif empêchant le suppresseur de tumeur miR-7 de se lier à ses ARNm cibles et ainsi de les réprimer (Figure 13A) (Memczak *et al.*, 2013). En outre, des outils reposant sur des algorithmes de prédiction bio-informatique et des données expérimentales de CLIP-seq ont été développés afin d'identifier le réseau ARNcirc/miRNA (Dudekula et al., 2016; Li et al., 2014). Ces bases de données regroupent également des données concernant les interactions RBP-ARNcirc. En effet, plusieurs démonstrations suggèrent que les ARNcirc sont capables d'éponger les RBP, avec des conséquences fonctionnelles sur leur activité et leur localisation subcellulaire (Figure 13B) (Kristensen et al., 2019). Par exemple, il a été récemment démontré que circFOXK2 interagit avec YBX1 et hnRNPK pour promouvoir l'expression des oncogènes NUF2 et PDXK dans le cancer du pancréas (Wong et al., 2020).

Le *back-splicing* et l'épissage canonique utilisent la même machinerie. La balance entre ces deux épissages peut ainsi représenter un des modes d'action des ARNcirc dont la biogenèse rentre en compétition avec celle de leur ARNm parental (Figure 13C). Une étude rapporte notamment que l'ARN de la lésion musculaire (MBL) contient plusieurs motifs introniques pour la protéine MBL. Lorsqu'elle est surexprimée, la protéine MBL se lie à des motifs introniques spécifiques de l'ARN, favorisant ainsi un événement d'épissage alternatif de l'exon 2 et réduisant indirectement l'expression de sa propre protéine par un mécanisme de rétroaction (Ashwal-Fluss et al., 2014).

Une autre étude décrit un réseau de régulation impliquant l'ARNcirc YAP (circYAP) et son ARNm linéaire associé. Dans des lignées cellulaires de cancer du sein, il a été démontré que l'ARN circYAP interagit avec plusieurs partenaires, dont l'ARNm YAP, PABP et la protéine d'initiation de la traduction eIF4G. Cette interaction inhibe l'assemblage du complexe d'initiation de la traduction sur l'ARNm YAP, ce qui entraîne une inhibition de sa traduction (Figure 13D) (Wu et al., 2019). De manière intéressante, les ARNcirc peuvent aussi promouvoir la transcription de leur gène parental en interagissant avec U1snRNP et le complexe ARN polymérase II (Figure 13E) (Li et al., 2015b). Certains ARNcirc seraient également responsables du recrutement de protéines, facilitant leur activité enzymatique (Figure 13 F).

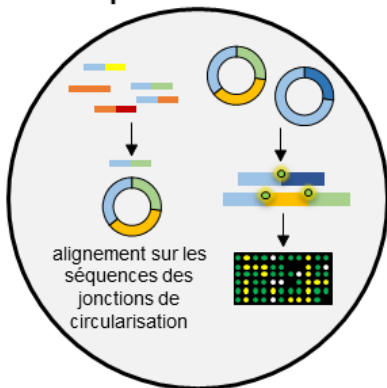
Il est également important de souligner que, bien que considérés pendant plusieurs années comme des ARNnc, certains ARNcirc sont traduits en protéines. Cela a notamment été démontré chez la drosophile en utilisant une approche de séquençage de l'empreinte ribosomique axé spécifiquement sur les lectures chevauchant les jonctions de circularisation des ARNcirc (Pamudurti et al., 2017). La traduction des ARNcirc peut ainsi être initiée *via* des sites d'entrée internes des ribosomes (IRES) (Legnini et al., 2017). En outre, des données émergentes suggèrent que la traduction des ARNcirc pourrait être médiée par des modifications épi transcriptomiques, notamment par la N6-méthyladénosine (m6A) (Figure 13G) (Chen et al., 2019c). Bien que le rôle de ces ARNcirc transcrits n'ait pas encore été complètement élucidé, de nombreuses fonctions régulatrices leur ont déjà été attribuées. Par exemple, la β -caténine-370aa est une isoforme de β -caténine qui comporte 370 acides aminés (aa). Elle est encodée par le gène circ β -caténine (hsa-circ-0004194) et partage 361 aa à l'extrémité N et 9 aa à l'extrémité C-terminale avec la protéine β -caténine complète. En tant que composant crucial de la voie Wnt, la β -caténine cytoplasmique est associée à un complexe de dégradation impliquant la *glycogen synthase kinase* (GSK3 β). Lorsque des ligands se fixent aux récepteurs activant la voie Wnt, la β -caténine est libérée de ce complexe, puis elle est transloquée dans le noyau où elle active la transcription de ses gènes cibles. La β -caténin-370aa cytoplasmique issue de cet ARNcirc agit comme un leurre qui se lie de manière préférentielle à GSK3 β , protégeant ainsi la β -caténine complète de la dégradation dirigée par GSK3 β . Ainsi, une surexpression de cet ARNcirc contribue à une libération excessive de β -caténine et stimule la voie Wnt/ β -caténine favorisant ainsi la progression du cancer du foie (Liang et al., 2019).



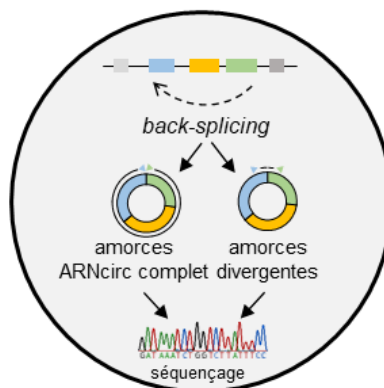
4. Développement d'outils spécifiques

L'intérêt croissant de la communauté scientifique pour les ARNcirc a naturellement conduit au développement d'outils dédiés. Outre les approches de séquençage abordées précédemment, des puces à ADN conçues avec des sondes spécifiques chevauchant la jonction de circularisation d'ARNcirc connus ont permis de multiplier le nombre d'études à grande échelle (Figure 14A). Les approches supervisées permettant de quantifier un ARNcirc donné se sont également démocratisées. Des sets d'amorces spécifiques ont notamment été développés, permettant d'amplifier soit la séquence de la jonction de circularisation (amorces divergentes), soit la séquence totale de l'ARNcirc. Il est ainsi possible de quantifier spécifiquement un ARNcirc en réadaptant des techniques déjà communément utilisées en laboratoire (*i.e.* RT-q-PCR) (Figure 14B). Au vu des fonctions régies par les ARNcirc, il paraît primordial de caractériser les molécules qu'ils séquestrent. C'est pourquoi des approches de *pulldown* utilisant des ASO biotinylés chevauchant les jonctions de circularisation ont été développées. Il est ainsi possible d'identifier tous les miARN et toutes les RBP séquestrés par les ARNcirc portant cette jonction, en réalisant respectivement un séquençage des miARN et une spectrométrie de masse à partir de la fraction récupérée de l'expérience de *pulldown* (Figure 14D). La localisation des ARNcirc étant intimement liée à la fonction qu'ils exercent, il paraît également crucial de déterminer leur position dans la cellule. Pour cela, des méthodes de *fluorescent in situ hybridization* (FISH) ont été réadaptées pour les ARNcirc, avec des sondes conçues pour chevaucher la jonction de circularisation (Figure 14E). De plus, afin de mieux comprendre le rôle régulateur des ARNcirc, des expériences de gain et de perte de fonction utilisant des outils spécifiques peuvent être réalisées. Il est notamment possible d'induire leur expression grâce à des plasmides contenant la séquence d'un ARNcirc d'intérêt. Ces plasmides ont pour spécificité des séquences qui favorisent la circularisation. Pour les expériences de perte de fonction, des siARN ou des GapmeRs complémentaires de la séquence de circularisation se sont montrés efficaces (Bejugam *et al.*, 2020) (Figure 14F).

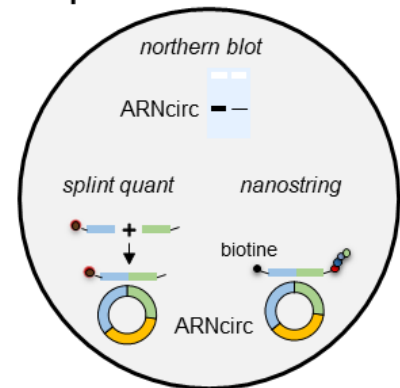
A Séquençage d'ARN et puces à ADN



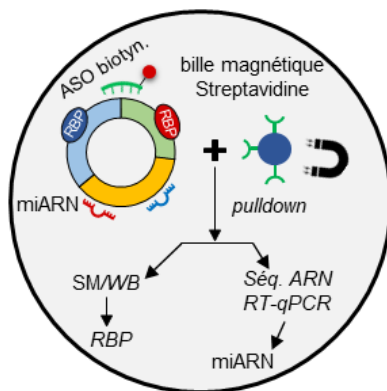
B RT-PCR



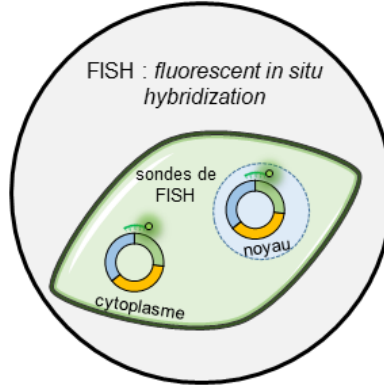
C Alternatives pour quantifier les ARNcirc



D Pulldown d'ARNcirc



E Imagerie



F Gain et perte de fonction

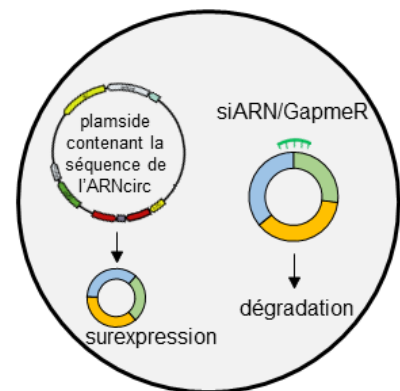


Figure 14 : Approches méthodologiques pour l'étude des ARNcirc

(A) Le séquençage d'ARN et les puces à ADN sont les deux approches privilégiées pour identifier et quantifier les ARNcirc de façon non supervisée. (B) La RT-PCR utilisant des amorces divergentes conçues pour chevaucher la jonction de circularisation ou des amorces conçues pour couvrir toute la séquence d'un ARNcirc permet de quantifier un ARNcirc spécifique. (C) Des méthodes alternatives (northern blot, split quant et nanostring) sont moins fréquemment utilisées, mais permettent également la quantification d'ARNcirc. (D) Il est également possible d'identifier les miARN et les protéines de liaison à l'ARN (RBP) séquestrés par un ARNcirc via des approches de pull-down suivies respectivement par un séquençage de miARN ou une spectrométrie de masse (SM). (E) Des sondes fluorescentes conçues pour chevaucher la jonction de circularisation permettent la localisation et la quantification des ARNcirc. (F) Les expériences de gain et de perte de fonction utilisent des outils spécifiquement conçus pour les ARNcirc. Il est possible d'induire l'expression d'un ARNcirc grâce à un plasmide dédié contenant sa séquence. Pour la perte de fonction, des siARN et des GapmeRs ciblant spécifiquement la jonction de circularisation permettent de dégrader efficacement un ARNcirc donné. Adapté de (Bejugam et al., 2020).

5. Fonctions des ARNcirc dans la cancérogenèse

Bien que les fonctions des ARNcirc n'aient pas encore toutes été explorées à ce jour, les données regroupées jusqu'à présent suggèrent un rôle clé des ARNcirc en tant que régulateurs de l'expression de gènes. Il est donc pertinent de se questionner sur l'implication de ces ARNcirc dans la progression tumorale.

En tant que processus pathologique complexe, le développement du cancer présente des caractéristiques bien connues, autrement appelées *hallmarks*. En effet, la progression tumorale profite systématiquement d'une autosuffisance en signaux de croissance, d'une insensibilité aux signaux inhibiteurs de la croissance, d'une capacité à éviter l'apoptose, d'une capacité de se répliquer indéfiniment, d'une induction de l'angiogenèse et de la capacité à former des métastases. Plusieurs études corroborent l'idée que les ARNcirc seraient impliqués dans chacun de ces différents *hallmarks* du cancer (Li et al., 2022b). Il existe également de plus en plus de preuves démontrant que les ARNcirc sont différentiellement exprimés dans les cancers, soit comme oncogènes, soit comme suppresseurs de tumeur. Ils permettent notamment de distinguer le tissu tumoral du tissu normal adjacent (Chen, 2020). De plus, certains ARNcirc ont été spécifiquement associés à un type de cancer. Certaines études soulèvent même la corrélation entre leur niveau d'expression et certaines caractéristiques cliniques, telles que le stade tumoral (TNM), la différenciation ou encore la survie globale (Tang and Hann, 2020).

De plus, il est important de noter que le niveau d'expression d'un ARNcirc n'est pas obligatoirement corrélé à celui de l'ARNm linéaire associé. Cela signifie par exemple que, si l'expression d'un ARNcirc est induite, le niveau d'expression de l'ARN linéaire associé peut rester inchangé, voire être anticorrélé. Cette observation suggère que la régulation du *back-splicing* est un processus actif et donc potentiellement impliqué dans le cancer. En 2019, une expérience de perte de fonction utilisant un shRNA dirigé spécifiquement contre des ARNcirc hautement exprimés a révélé que 11,3 % des ARNcirc sont nécessaires à la prolifération cellulaire d'une lignée de cancer de la prostate. En revanche, parmi les transcrits linéaires apparentés, 91,8 % n'étaient pas essentiels au maintien de cette fonction (Chen et al., 2019b). Ces résultats mettent en évidence la participation des ARNcirc dans l'activité oncogène ou antitumorale, suggérant ainsi qu'ils pourraient jouer un rôle dans l'initiation et la progression des tumeurs.

Comme mentionné précédemment, la fonction majoritairement attribuée aux ARNcirc est leur capacité à éponger les miRNA. Ils favorisent ou empêchent ainsi la progression tumorale, en fonction de la nature des cibles qui sont davantage préservées ou détruites (Han et al., 2017; Yu et al., 2018). L'ARN circHIPK3 est un des ARN le mieux décrits pour son rôle dans la carcinogenèse de plusieurs cancers. Il s'illustre notamment par son grand nombre de sites de liaison pour des miRNA suppresseurs de tumeurs (18 sites de liaison) impliqués dans la croissance cellulaire de plusieurs cancers (*e.g.* cancer du sein, cancer colorectal et CHC) (Zheng et al., 2016). Le développement d'algorithmes permettant d'identifier des réseaux ARNcirc/miARN/ARNm pertinents est donc devenu d'un intérêt majeur. Ils sont utilisés pour proposer des hypothèses quant aux mécanismes d'action d'un ARNcirc donné. Ils permettraient aussi de déceler les ARNcirc ayant un potentiel thérapeutique (Xiong et al., 2018).

Un projet majeur visant à approfondir nos connaissances sur l'expression des ARNcirc dans le cancer a utilisé des approches de séquençage par capture d'exome sur 2000 échantillons tumoraux. Ainsi, cette étude a permis d'identifier des signatures d'ARNcirc spécifiquement exprimés dans des lignées cellulaires, dans des tissus non pathologiques et dans divers types de tumeurs. Les données récoltées ont été compilées dans MiOncoCirc, le premier recueil international d'ARNcirc associés au cancer (Vo et al., 2019).

6. Rôle des ARN circulaires dans les processus pathologiques du CCAi

À ce jour, la participation des ARNcirc dans les *hallmarks* du cancer du foie a largement été démontrée. Nous avons reporté certains d'entre eux dans une revue parue récemment (Louis et al., 2022). Ainsi, bien que de nombreux ARNcirc aient été identifiés dans le CHC, seules quelques études ont rapporté leur rôle dans le CCAi (Figure 15).

Les ARNcirc décrits jusqu'alors dans le CCAi sont principalement impliqués dans le maintien de la prolifération cellulaire, dans la résistance à la mort cellulaire et dans l'acquisition de propriétés invasives. Par exemple, l'expression altérée de l'ARN antitumoral circ-0001649 et l'ARN oncogénique circ-0005230 a été associée à la régulation de la prolifération, de la migration et de l'invasion de cellules de CCA (Xu et al., 2019; Xu et al., 2018). Une autre étude s'est intéressée à circSMARCA5, un ARNcirc suppresseur de tumeur déjà largement décrit pour son rôle dans la progression de plusieurs cancers, dont le CHC. Ainsi, de faibles niveaux de circSMARCA5 ont été mesurés dans des tissus de CHC et son expression a été négativement corrélée à un stade tumoral avancé et une mauvaise OS. En outre, la

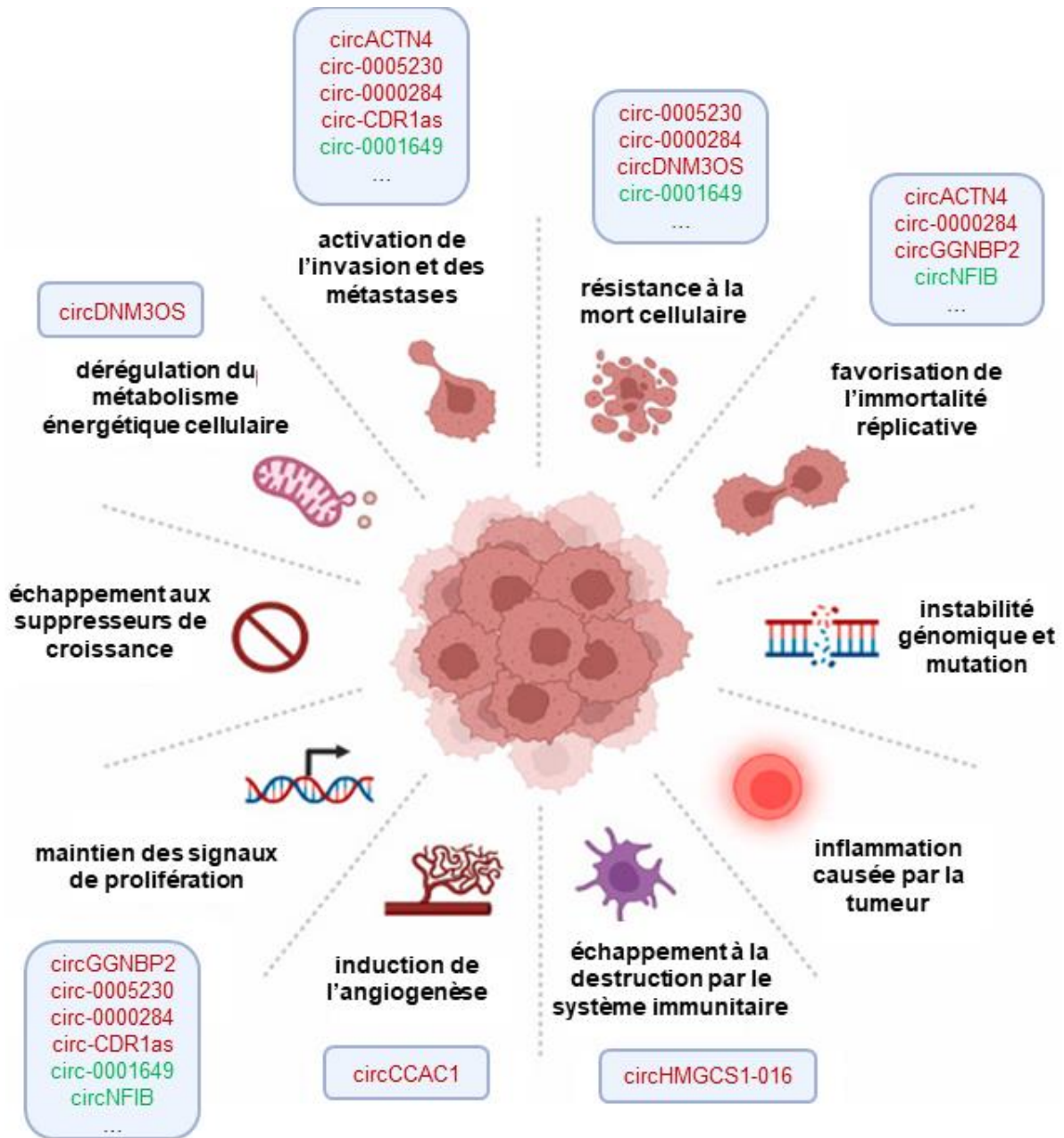
surexpression de circSMARCA5 a été associée à une diminution de la prolifération des cellules de CCA (Lu and Fang, 2020). Très récemment, des travaux ont démontré que la perte d'expression de l'ARN circNFIB était associée à des caractéristiques agressives et prédisait un pronostic défavorable chez les patients atteints de CCAi. De plus, des études fonctionnelles ont révélé que l'ARN circNFIB inhibait la prolifération et la migration des cellules de CCA *in vitro*, et participait à la croissance tumorale et au développement de métastases *in vivo* (Li et al., 2022a). Li *et al.* ont également identifié un ARNcirc pro-tumoral impliqué dans la progression du CCAi. En utilisant des approches de séquençage, ils sont parvenus à identifier une signature d'ARNcirc régulés par l'IL-6 dans le CCAi. Ils se sont plus particulièrement intéressés à l'ARN circGGNBP2, dont la biogenèse est induite par l'IL-6. Cet ARNcirc est traduit en une protéine tronquée qui induit la prolifération et la migration des cellules de CCAi *in vitro* et *in vivo* (Li et al., 2022a).

L'ARN circHMGCS1-016 fortement exprimé dans des échantillons de CCAi a également été décrit pour sa contribution au développement de CCAi. Il participe notamment à la tolérance immunitaire de la tumeur en épongeant le miR-1236-3p, régulant ainsi l'expression de CD73 et GAL-8. Les auteurs de cette étude ont également rapporté que des patients ayant un niveau d'expression important de circHMGCS1-016 montraient une résistance accrue à un traitement anti-PD1 comparé aux patients présentant des niveaux d'expression faibles pour cet ARNcirc (Figure 15) (Xu et al., 2021b).

La croissance du CCAi est également favorisée par la formation de nouveaux vaisseaux alimentant la tumeur et facilitant les métastases. Ainsi, l'identification d'ARNcirc régulant ce processus pourrait être pertinente. Les travaux de Xu *et al.* soulignent notamment le rôle de circCCAC1 dans l'induction de ces phénomènes d'angiogenèse. Cet ARNcirc associé à un mauvais pronostic a été identifié dans des tissus et circulant dans la bile de patients atteints de CCA. CircCCAC1 agit notamment *via* la séquestration de miR-514a-5p qui ne peut plus cibler l'ARN Yin Yang 1 (YY1). YY1 active alors la transcription du *calcium-modulating ligand* (CML) conduisant à une désorganisation de la barrière endothéliale et la formation de nouveaux vaisseaux sanguins et favorisant ainsi l'invasion cellulaire et les métastases (Figure 15) (Xu et al., 2021a).

Certains ARNcirc ont également été décrit pour leur rôle dans la régulation du métabolisme énergétique cellulaire. L'ARNcirc DN3OS a notamment été décrit pour ses capacités à

réguler le métabolisme de la glutamine des cellules de CCA *via* la séquestration du miR-145-5p. (Su et al., 2021).



7. Le rôle des ARNcirc dans les voies de signalisation associées au CCAi

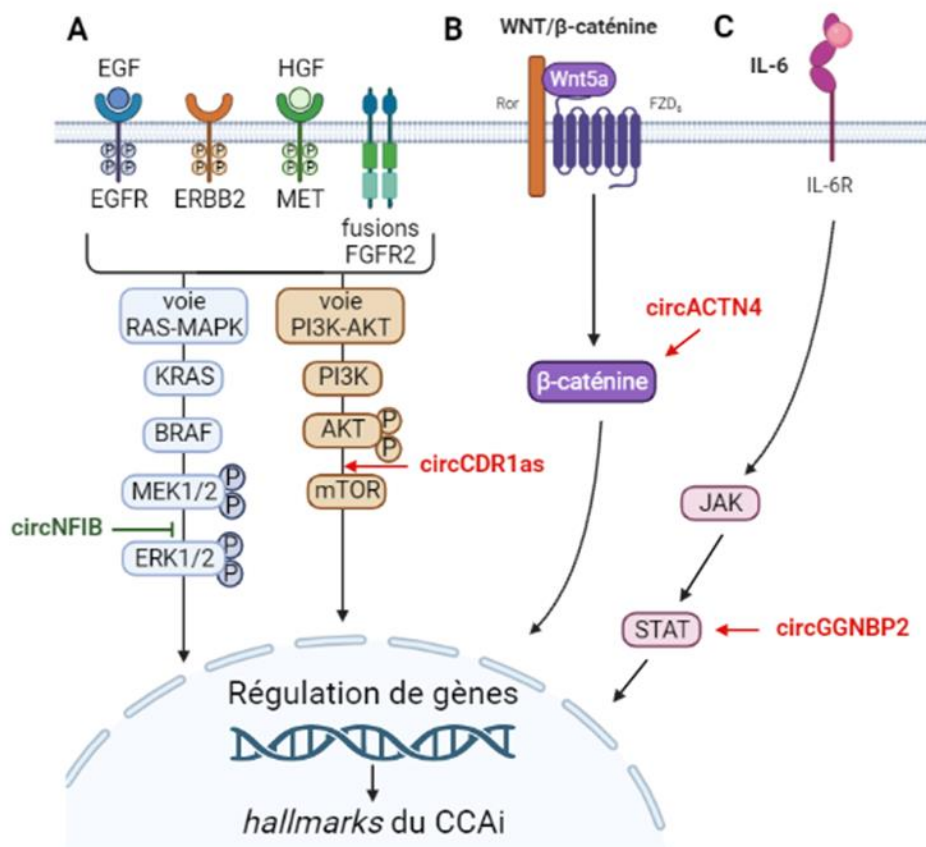
L'expression aberrante des ARNcirc est impliquée dans l'activation de plusieurs voies de signalisation oncogènes, notamment de voies décrites précédemment dans ce travail comme étant impliquées dans la progression du CCAi.

De multiples ARNcirc jouent par exemple un rôle dans la progression tumorale *via* l'activation de la voie de signalisation PI3K/AKT. Li *et al.* ont notamment découvert que l'expression particulièrement élevée de circCCR1as dans le CCAi contribuait à induire la prolifération cellulaire, la migration et l'invasion en activant la voie de signalisation AKT3/mTOR (Li *et al.*, 2020a) (Figure 16 A).

Les protéines ERK1/2, sont une classe de protéines kinases sérine/thréonine qui jouent un rôle essentiel dans la voie MAPK et régulent ainsi divers processus biologiques, tels que la prolifération, l'invasion et la migration (Roberts and Der, 2007). Il a été montré que des altérations de plusieurs voies impliquant des RTK conduisent à une expression accrue de ERK1/2, participant ainsi à la progression du CCAi (Schmitz *et al.*, 2007). Une étude récente a rapporté le rôle de l'ARN antitumoral circNFIB dans la régulation de la voie MEK1/ERK. Ainsi, la diminution de son expression a été associée à une croissance tumorale plus importante et au développement de métastases. Cet ARNcirc est associé à un meilleur pronostic chez les patients atteints d'un CCAi (Du *et al.*, 2022) (Figure 16 A).

Les voies de signalisation du développement ne font pas exception et sont également régulées par des ARNcirc. Par exemple, des travaux récents ont mis en évidence l'ARN circACTN4 qui est induit dans le CCAi et qui favorise la croissance des cellules tumorales et les métastases en régulant les voies de signalisation Hippo et Wnt (Figure 16 B). Il est intéressant de noter que les actions pro-oncogéniques de circACTN4 passent par deux mécanismes distincts : i) en tant qu'éponge pour le miR-424-5p qui dégraderait ainsi moins l'oncogène YAP dans le cytoplasme et ii) *via* le recrutement de YBX1 initiant la transcription de *FZD7* dans le noyau. Cette étude décrit ainsi circACTN4 comme un acteur central de la signalisation, permettant l'activation coordonnée des voies Hippo et Wnt dans le CCAi (Chen *et al.*, 2022). Toutefois, la régulation dynamique du passage des ARNcirc du noyau au cytoplasme est un point important qui reste à élucider. En effet, en fonction de leur localisation cellulaire, les ARNcirc possèdent des fonctions différentes. Une régulation active de ces processus pourrait donc offrir des leviers intéressants pour moduler l'activité des ARNcirc.

L'inflammation chronique associée au CCAi conduit à l'induction de plusieurs médiateurs inflammatoires qui activent des voies impliquées dans la progression tumorale. Ces médiateurs, tels que l'IL-6 et le TGF β , pourraient également être responsables de l'expression aberrante de certains ARNcirc impliqués dans le CCAi. Par exemple, la protéine traduite à partir de l'ARN circGGNBP2 induit par l'IL-6 interagit directement avec STAT3 et exerce un rétrocontrôle positif qui maintient l'activité de la voie IL-6/STAT3 (Figure 16 C) (Li *et al.*, 2022a). Au sein de l'équipe, nous avons également identifié, plusieurs ARNcirc dérivés de *CASC15*, un ARNlnc induit par le TGF β et dont l'expression a été associée à un micro-environnement inflammatoire dans les CCAi (Merdrignac *et al.*, 2018).



8. Potentiel thérapeutique des ARNcirc dans le CCAi

A- Les ARNcirc sont-ils des biomarqueurs idéaux ?

Les ARNcirc possèdent des caractéristiques qui leur sont propres et qui offrent des opportunités pour améliorer la prise en charge des patients atteints de CCAi. En effet, même si la plupart des ARNcirc sont faiblement exprimés (moins de 10 % du niveau d'expression de leur homologue linéaire) (Liang et al., 2017), leur structure circulaire leur confère une plus grande résistance à l'activité des exonucléases et donc une demi-vie plus longue. De plus, les méthodes de détection des ARNcirc sont beaucoup plus spécifiques et sensibles que les approches couramment utilisées pour détecter les biomarqueurs protéiques (Nimse et al., 2016). En effet, en tant qu'acides nucléiques, les ARNcirc peuvent être facilement et spécifiquement amplifiés et détectés par RT-q-PCR, une technique sensible et quantitative, en utilisant des amorces dédiées qui chevauchent la jonction de *back-splicing* (Figure 14B) (Mi et al., 2022).

B- Valeur diagnostique des ARNcirc

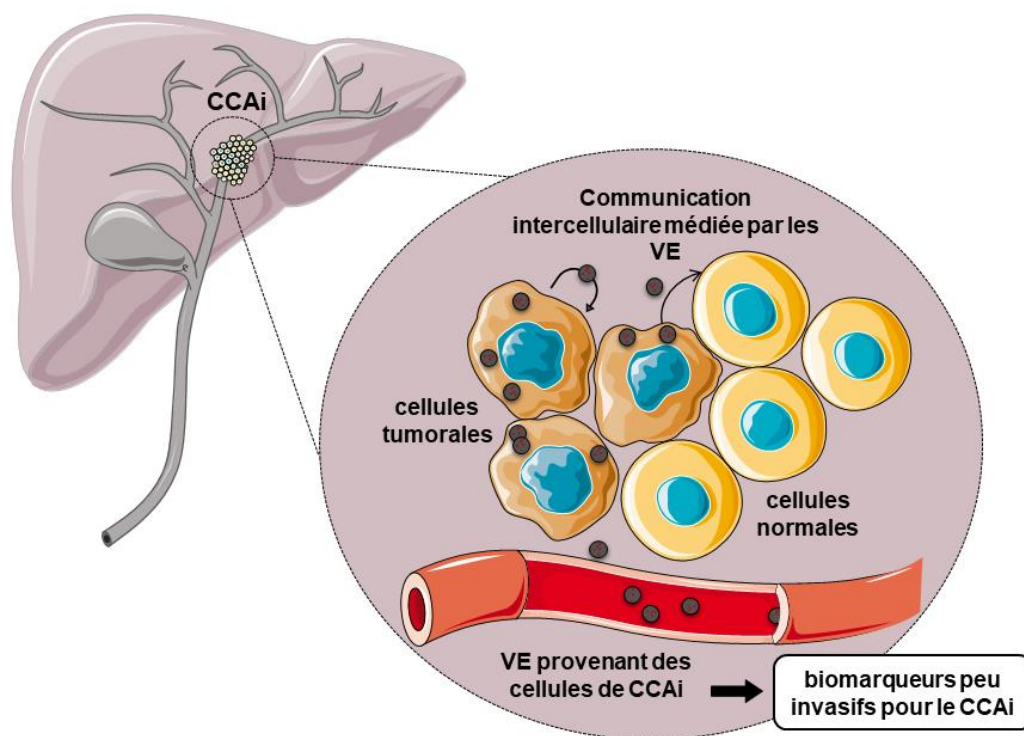
En outre, il a été démontré que certains ARNcirc sont exprimés de façon spécifique dans des tissus et des cellules (Xia et al., 2017). Cette caractéristique pourrait notamment être utilisée pour appuyer le diagnostic, comme celui des CCAi qui présente de nombreux défis (*e.g.* différencier les CCAi des CHC). Ces caractéristiques physiques intrinsèques confèrent aux ARNcirc un futur prometteur en tant que biomarqueurs diagnostiques. (Meng et al., 2017).

De plus, compte tenu de l'absence de symptômes associés à la survenue du CCAi et du degré d'agressivité de ce type de tumeur, des outils permettant un diagnostic précoce sont d'une importance cruciale (Razumilava and Gores, 2014). Dans ce but, quelques études se sont concentrées sur l'évaluation de l'abondance des ARNcirc dans les tissus et dans les biopsies liquides (sang, urine, salive, bile) et sur leur potentiel en tant que biomarqueurs diagnostiques dans le CCA (Liu et al., 2019a; Vo et al., 2019).

Les ARNcirc qui circulent dans les fluides corporels sont présents soit sous forme libre, soit encapsulés dans des VE (Louis et al., 2019). Il a notamment été suggéré qu'ils seraient davantage enrichis et stables dans les VE que dans les cellules sécrétrices d'origine (Li et al., 2015a). Ces ARN vésiculaires pourraient ainsi être des témoins de ce qu'il se passe localement au niveau de la tumeur (Figure 17). Ils représentent par conséquent des candidats sérieux en tant que biomarqueurs innovants et peu invasifs. Ainsi, plusieurs essais

cliniques en cours explorent le potentiel des ARNcirc en tant que biomarqueurs pour le cancer, y compris pour les tumeurs des voies biliaires (e.g. NCT03334708, NCT04584996, NCT04792437).

La plupart des travaux s'intéressant aux ARNcirc dans le CCA se sont jusqu'à présent principalement concentrés sur l'expression des ARNcirc au niveau tissulaire. Néanmoins, quelques études ont investigué l'action de ces ARNcirc circulants dans le CCA. L'ARN circ-0000284 a notamment été identifié dans des VE sécrétées par des lignées cellulaires de CCA. Les VE enrichies en ARN circ-0000284 produites par les cellules de CCA ont ainsi été capables d'augmenter la migration, la prolifération et l'invasion des cellules normales environnantes à la fois *in vitro* et *in vivo* (Wang et al., 2019).



C- Valeur pronostique des ARNcirc

L'avènement des techniques de séquençage à haut débit dédiées aux ARNcirc a permis l'identification de plusieurs signatures d'ARNcirc exprimés de façon aberrante dans le CCA. La plupart des ARNcirc induits dans les tissus de CCA se sont avérés impliqués dans la progression tumorale et le développement de métastases. Parmi les études ayant identifié ces signatures, le premier critère pour sélectionner des ARNcirc cliniquement pertinents repose systématiquement sur leur valeur pronostique (OS et RFS). Ainsi, l'expression particulièrement importante des ARN circACTN4, circGGNBP2, circHMGCS1-016 et circCCAC1 dans le CCA est associée à une mauvaise OS (Chen *et al.*, 2022; Li *et al.*, 2022a; Xu *et al.*, 2021a; Xu *et al.*, 2021b).

D- Valeur prédictive des ARNcirc

Plusieurs thérapies sont en cours d'évaluation clinique dans le cancer du foie. Dans ce contexte, le développement d'outils diagnostiques robustes pour sélectionner une thérapie personnalisée la plus adaptée pourrait être précieux.

En particulier, il a été démontré que de faibles niveaux d'ARN circSMARCA5 dans le CCAi sont corrélés à une mauvaise OS des patients. De manière intéressante, la surexpression de circSMARCA5 dans des lignées cellulaires de CCAi améliore la chimio sensibilité à la *gemcitabine* et au *cisplatine* (Lu and Fang, 2020). De même, une répression de l'expression de l'ARN circ-0003418 induit une chimiorésistance au *cisplatine* dans les cellules de CHC, *via* l'activation de la voie Wnt/ β -caténine (Chen *et al.*, 2019a). Dernièrement, l'ARN circ-SORE a également été décrit pour son rôle dans la résistance au *sorafenib*, un inhibiteur multikinase utilisé pour le traitement du CHC. L'ARN circ-SORE agit en piégeant YBX1, empêchant ainsi la dégradation d'oncogènes par le système ubiquitine E3. Plus intéressant encore, il a été démontré que la chimiorésistance induite par l'ARN circ-SORE pouvait être transmise aux cellules tumorales environnantes *via* les processus de transport vésiculaire (Xu *et al.*, 2020a).

De même, lorsque des cellules de CCA ont été traitées conjointement par chimiothérapie (*trametinib*, inhibiteur de la kinase MEK) et avec un vecteur contenant l'ARN circNFIB, une meilleure réponse au traitement a été observée *in vitro* et dans des modèles de souris. Ces observations suggèrent l'idée que les ARNcirc pourraient être utilisés en tant que biomarqueurs de réponse aux traitements et ainsi aiguiller le choix de thérapie pour les patients.

Ainsi, les ARNcirc apparaissent comme des molécules prometteuses pour la prise en charge des patients atteints de cancer. Tout d'abord, ils offrent de nouveaux outils pour le diagnostic et le pronostic. De plus, ils présentent de nombreux atouts attendus d'un biomarqueur : ils sont résistants, spécifiques, sensibles et nécessitent une intervention peu invasive pour être détectés dans les fluides corporels. Le potentiel thérapeutique des ARNcirc dans le CCA est un domaine de recherche encore inexploité qui offre de nombreuses opportunités.

E- Perspectives pour les ARNcirc

Le potentiel clinique des ARNcirc en tant que biomarqueurs est maintenant relativement bien décrit. Néanmoins, d'autres utilisations cliniques des ARNcirc sont étudiées. Comme mentionné précédemment, de plus en plus de preuves montrent que les ARNcirc sont dérégulés dans le cancer et que ces ARN sont impliqués dans les différents *hallmarks* du cancer (Figure 15). Ainsi, on peut imaginer que cibler directement ces ARNcirc peut représenter une option thérapeutique valable. La multitude de fonctions régulatrices attribuée aux ARNcirc offre de nombreuses possibilités pour moduler leur activité au cours de la progression tumorale. Par exemple, de nombreux ARNcirc sont décrits pour leur activité pro-oncogénique qui repose sur la séquestration de miRNA suppresseurs de tumeurs (Han *et al.*, 2017; Yu *et al.*, 2018). Ainsi, plusieurs stratégies pourraient être développées pour atténuer l'activité pro-oncogénique de ces ARNcirc. Il faut néanmoins rester prudent avec l'utilisation de ce genre d'approches, puisqu'il est difficile de cibler spécifiquement un miARN sans en affecter d'autres qui peuvent avoir des fonctions similaires ou complémentaires dans les cellules. Ce genre de difficulté a été rencontré avec l'utilisation des antagomirs qui ont également été développés pour cibler et limiter l'activité des miARN. De même, une des limites des antagomirs repose sur les effets indésirables secondaires qu'ils peuvent causer en ciblant involontairement des miARN qui ont une fonction dans les cellules saines. Les premiers résultats d'essais cliniques sur les antagomirs ont été encourageants, mais sont encore à un stade préliminaire et il est nécessaire de poursuivre les recherches pour évaluer leur efficacité et leur sécurité à long terme (Rupaimoole and Slack, 2017).

Une première stratégie consisterait à limiter l'activité des ARNcirc. Dans ce cas, il s'agirait d'empêcher la séquestration des miARN anti tumoraux par les ARNcirc, en bloquant leurs sites de liaison spécifiques. Pour cela, plusieurs outils sont déjà utilisés, tels que les *Target Site Blockers* (TSB) qui sont des oligonucléotides antisens (ASO) initialement développés pour empêcher les miARN de se lier à leurs ARNm cibles. Ils seraient ici utilisés pour

restaurer la disponibilité des miARN. Bien que cette stratégie n'ait pas encore été appliquée aux ARNcirc, elle s'est montrée d'une grande efficacité *in vitro* et *in vivo* lorsqu'elle a été appliquée aux ARNinc qui ont également pour fonction de séquestrer des miARN (Gilot et al., 2017). Les thérapies utilisant des acides nucléiques représentent un domaine prometteur. En effet, plusieurs traitements basés sur des ASO ont déjà été approuvés par la *Food and Drug Administration* (FDA), et beaucoup sont en cours d'évaluation clinique (Kaczmarek et al., 2017).

Une deuxième stratégie serait de diminuer directement l'abondance des ARNcirc en les dégradant. Ici encore, des outils permettant de cibler spécifiquement et de détruire des acides nucléiques existent déjà. L'utilisation d'oligonucléotides antisens, autrement appelés *gapmeRs*, s'avère très efficace pour ce genre de tâche. Ces courtes séquences d'ADN sont conçues pour s'hybrider par complémentarité de séquences à un ARNm cible, formant ainsi un complexe qui sera dégradé par la RNase H. Il serait donc pertinent de concevoir des *gapmeRs* ciblant spécifiquement la jonction de *back-splicing* d'un ARNcirc pro tumoral afin de réduire son abondance, sans pour autant affecter le niveau d'expression de l'ARNm linéaire associé. Plusieurs études ont déjà démontré l'efficacité de cette stratégie non seulement en utilisant des *gapmeRs*, mais aussi *via* de siRNA et des *small hairpin RNA* (shRNA) ciblant également la jonction de *back-splicing*. Il a d'ailleurs été montré que l'utilisation d'un shRNA ciblant l'ARN circ β -caténine contribuait à limiter son action oncogénique dans le CHC. L'amenuisement des niveaux de circ β -caténine se traduit notamment par une baisse de la prolifération, de la migration et de l'invasion cellulaires *in vitro*, et par une diminution de la croissance tumorale et du nombre de métastases *in vivo* (Liang et al., 2019). De la même manière, les approches utilisant le système CRISPR/Cas13 pourraient être utiles pour diminuer spécifiquement le niveau d'expression d'un ARNcirc (Abudayyeh et al., 2017).

Une troisième stratégie consisterait à moduler l'abondance d'ARNcirc pro-oncogéniques en régulant la machinerie d'épissage pour contrôler l'occurrence des événements de *back-splicing*. En effet, comme mentionné précédemment, la biogenèse des ARNcirc au cours de la TEM est étroitement régulée par la RBP QKI5 (Conn et al., 2015). Ainsi, on peut émettre l'hypothèse que la modulation du niveau d'expression de RBP pourrait contrôler l'expression des ARNcirc. Il existe également des preuves solides que la biogenèse des ARNcirc est médiée par l'hybridation de régions introniques complémentaires flanquant les séquences des ARNcirc (Liang and Wilusz, 2014; Zhang et al., 2014). Ainsi, on peut supposer que des

ASO conçus pour cibler ces séquences complémentaires sur l'ARN prémessager seraient en mesure de bloquer la biogenèse des ARNcirc en empêchant l'interaction des régions impliquées dans le back-splicing.

F- ARNcirc synthétiques

Certains ARNcirc sont également connus pour leurs propriétés antitumorales, notamment *via* la séquestration de miARN oncogènes. Une autre approche thérapeutique consisterait donc à synthétiser en grande quantité ces ARNcirc qui agiraient ainsi comme un bouclier contre ces miARN. Cette idée est d'autant plus envisageable que les ARNcirc sont des molécules plus stables que leurs homologues linéaires et que les miARN. Plusieurs méthodes ont notamment été développées pour produire des E-ARNcirc synthétiques (Petkovic and Muller, 2015). L'avantage de cette approche est la possibilité de concevoir spécifiquement des ARNcirc qui possèdent un grand nombre de sites de liaison pour un miARN d'intérêt. Par exemple, la conception d'un ARNcirc présentant 5 sites de liaison pour miR-21 (un miARN oncogène bien connu), a ainsi permis de limiter l'activité de miR-21 en induisant l'apoptose de lignées cellulaires de cancer gastrique (Liu et al., 2018).

Il est également possible de concevoir des ARNcirc synthétiques pour capturer des protéines pro-oncogéniques. En effet, une équipe allemande a déjà créé un ARNcirc artificiel spécifique qui est capable de capturer et désactiver la protéine hnRNP (Schreiner et al., 2020). Étant donné que les RBP jouent un rôle dans la progression du cancer, notamment en perturbant l'épissage, cette preuve de concept ouvre la voie à des thérapies innovantes utilisant des ARNcirc (Schreiner et al., 2020).

Une idée encore plus innovante consisterait à utiliser le fait que les ARNcirc exogènes peuvent produire efficacement des protéines *in vitro* (Wesselhoeft et al., 2018). Ainsi, les ARNcirc portant des *IRES* pourraient être spécifiquement synthétisés pour exprimer des protéines anti tumorales.

9. ARNcirc régulés par le TGF β

Ces stratégies pourraient être envisagées afin de développer des solutions thérapeutiques innovantes pour le CCAi. L'implication de la voie TGF β dans la carcinogenèse du CCAi laisse penser que des ARNcirc régulés par cette cytokine pourraient contribuer à la progression tumorale. Cette hypothèse est renforcée par une étude montrant que l'ARNcirc PWWP2A induit par le TGF β favorise la fibrose hépatique en activant les CSH (Liu et al., 2019b).

Cependant, aucune recherche n'a encore identifié de signature d'ARNcirc régulés par le TGF β dont l'expression serait altérée dans le CCAi ou dans le cancer en général. La découverte de tels ARN circulaires pourrait cependant ouvrir la voie à l'identification de biomarqueurs diagnostiques et pronostiques innovants pour le CCAi. Ils pourraient également être utilisés comme biomarqueurs compagnons pour orienter le choix des thérapies ciblées. Cela pourrait par exemple permettre de repérer les patients les plus réceptifs aux traitements basés sur l'inhibition de la voie TGF β (par exemple *Bintrafusp Alfa*) en cours de développement pour traiter les tumeurs des voies biliaires (Yoo *et al.*, 2020).

Chapitre 2 : Objectifs

Les cholangiocarcinomes (CCA) comprennent un groupe hétérogène de tumeurs malignes émergeant à tous les niveaux de l'arbre biliaire. Ils sont classifiés en fonction de leur localisation anatomique en trois grandes catégories : intrahépatique (CCAI), périhilaire (CCAp) et distal (CCAd). Au cours de ma thèse, mes efforts se sont essentiellement concentrés sur la forme intrahépatique, laquelle représente 10 à 20 % des cholangiocarcinomes. Les CCAi représentent la seconde tumeur maligne du foie la plus fréquente après le carcinome hépatocellulaire (CHC). En raison de leur nature asymptomatique, les CCA sont généralement diagnostiqués trop tardivement, réduisant les options thérapeutiques. La meilleure alternative, à ce jour, passe par une intervention chirurgicale et la résection de la tumeur. Néanmoins, les tumeurs de taille trop imposante, les métastases et l'invasion ganglionnaire associée aux CCA limitent le nombre de patients éligibles à la chirurgie. Le manque de connaissances sur la pathogenèse agressive des CCA pourrait expliquer cette histoire clinique défavorable. L'amélioration de la prise en charge des patients pourrait de ce fait reposer sur une meilleure compréhension des mécanismes d'initiation et de progression tumorale, le développement de thérapies ciblées associées, et l'identification de biomarqueurs diagnostiques et pronostiques cliniquement pertinents.

Dans cette optique, l'étude du micro-environnement est particulièrement pertinente puisque l'une des caractéristiques histologiques du CCAi est la présence d'un stroma desmoplastique abondant, connu pour contribuer au déclenchement et à la progression de la tumeur. C'est dans ce contexte que l'équipe s'est intéressée à caractériser les altérations transcriptomiques présentes dans le micro-environnement tumoral du CCAi en démontrant que le TGF β fortement présent dans le stroma est associé à un mauvais pronostic (Sulpice et al., 2013). Des travaux réalisés plus récemment au sein de l'équipe ont également identifié plusieurs ARNcirc dérivés de *CASC15*, un ARNInc induit par le TGF β et dont l'expression a été associée à un micro-environnement inflammatoire dans les CCAi (Merdrignac *et al.*, 2018). Ces données suggèrent un rôle important de la voie du TGF β dans la progression du CCAi et sont pleines de promesses quant à son potentiel thérapeutique puisqu'il existe des inhibiteurs efficaces de cette voie déjà testés en clinique (*e.g. Galunisertib, Bintrafusp Alfa*). Le TGF β est une cytokine pléiotropique impliquée dans de nombreux processus biologiques régissant le destin cellulaire, tels que l'apoptose, la prolifération ou encore la différenciation cellulaire. Le TGF β représente un défi de taille en tant que cible thérapeutique dans le cancer du fait de sa dualité fonctionnelle. En effet, le TGF β est à la fois capable d'endiguer et de faciliter la progression tumorale. A un stade précoce de la tumorigenèse, le TGF β permet

l'activation transcriptionnelle de gènes qui auront un effet cytotatique comme *CDKN1A* (P21) ou *CDKN2B* (P15). *A contrario*, à un stade avancé, le TGFβ promeut la progression tumorale *via* son action pro-métastatique en activant *SNAIL1/2*, *TWIST* ou encore des métalloprotéases. Ces derniers permettront la transition épithélio-mésenchymateuse (TEM) impliquée dans la dissémination métastatique.

Par conséquent, l'ensemble des travaux de notre équipe visent à identifier des régulateurs et effecteurs innovants de la voie du TGFβ, ainsi que des biomarqueurs pertinents et spécifiques de tumeurs dans lesquelles le bras pro-métastatique du TGFβ est activé, facilitant ainsi le développement de thérapies ciblées.

Mon projet de thèse vise plus précisément à évaluer le potentiel d'une nouvelle classe d'ARN de structure circulaire (ARNcirc) régulés par le TGFβ en tant que biomarqueurs pronostiques innovants dans le CCAi. Nous nous sommes également escrimés à mieux comprendre le rôle de ces ARNcirc dans la progression du CCAi. Les résultats obtenus sont présentés dans l'article ci-dessous. Cet article est en préparation pour soumission et il s'agit ici d'une version réadaptée et rallongée pour les besoins de ce manuscrit de thèse.

Chapitre 3 : Résultats

TGFβ-induced circLTBP2 predicts a poor prognosis in intrahepatic cholangiocarcinoma and mediates gemcitabine resistance by sponging miR-338-3p

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Abstract

Background & Aim: Intrahepatic cholangiocarcinoma (iCCA) is a deadly cancer worldwide with an increasing incidence and limited therapeutic options. Therefore, there is an urgent need to open the field to new concepts for identifying clinically relevant therapeutic targets and biomarkers. Here, we explored the role and the clinical relevance of circular RNA circLTBP2 in iCCA.

Methods: TGFβ-regulated circRNA were identified by dedicated microarrays in human HuCC-T1 iCCA cell line and their clinical relevance was evaluated in independent cohorts of patients. Gain and loss of function of circLTBP2 combined with functional tests was performed *in vitro* and *in vivo* in mice. RNA pulldown, miRNA-seq, RNA-IP were performed to explore the sponging activity of circLTBP2.

Results: CircLTBP2 (hsa-circ-0032603) was identified as a novel TGFβ-induced circRNA in several CCA cell lines. CircLTBP2 promotes tumor cell proliferation, migration and resistance to gemcitabine-induced apoptosis *in vitro* and tumor growth *in vivo*. Mechanistically, circLTBP2 acts as a competitive RNA regulating notably the activity of the tumor suppressor microRNA miR-338-3p, leading to the overexpression of its pro-metastatic targets including SOX4. The restoration of miR-338-3p levels in iCCA cells reversed the pro-tumorigenic

effects driven by circLTBP2, including the resistance to gemcitabine-induced apoptosis. In addition, circLTBP2 expression predicted a reduced survival, as detected in tumor tissues but also in serum exosomes isolated from patients with iCCA.

Conclusions: CircLTBP2 is a novel effector of the pro-tumorigenic arm of TGF β and a potential clinically relevant therapeutic target and biomarker for iCCA easily detected from liquid biopsies.

Introduction

Cholangiocarcinoma (CCA) is a deadly cancer that affects people worldwide. It is a highly heterogeneous form of tumor that can occur anywhere in the biliary tree. The incidence of CCA varies depending on the region, but it is estimated to account for 15% of all primary liver cancers worldwide (Rumgay et al., 2022). The silent presentation of CCA, combined with its aggressive nature and resistance to chemotherapy, contributes to its high mortality rate, which represents 2% of all cancer-related deaths worldwide each year.

The current methods for diagnosing CCA are not accurate enough, and the high heterogeneity of CCA severely limits the effectiveness of therapies. Therefore, there is a pressing need to identify new biomarkers and therapeutic targets to improve the survival of patients with CCA. Despite recent advances in our understanding of CCA diagnosis and treatment, patient prognosis has not improved significantly in the past decade, with 5-year survival rates (7-20%) and tumor recurrence rates after surgery still being poor. Therefore, a more detailed study of this type of cancer is urgently needed to improve patient outcomes. Given the high heterogeneity of CCA, a personalized approach to studying the genomic, epigenetic, and molecular characteristics of these tumors are essential to understand their pathogenesis and develop new therapeutic options and personalized medicine. This was recently reviewed in a consensus statement endorsed by the European Network for the Study of Cholangiocarcinoma (ENSCCA) (Banales *et al.*, 2020).

The development and progression of cancer is not just caused by genetic changes in tumor cells, but also by changes in their microenvironment. Therefore, targeting the microenvironment, including the cytokines that mediate the interactions between tumor cells and their surroundings, has been proposed as a promising therapeutic strategy (Clara et al., 2020). CCA provides an ideal model to study the tumor microenvironment, given that the presence of a prominent desmoplastic stroma is a histological characteristic of intrahepatic CCA (iCCA). While previous studies have primarily focused on molecular changes in tumor cells, we have characterized genomic changes that occur in the iCCA microenvironment (Sulpice *et al.*, 2013). Through laser microdissection of the stroma and gene expression profiling, we provided the first evidence of clinically relevant gene signatures in the stroma of iCCA that can distinguish between patients with good and poor prognosis. Importantly, we demonstrated that the expression of Transforming Growth Factor beta (TGF β) in the stroma

of iCCA predicts a poor prognosis, suggesting that TGF β may be a potential therapeutic target in iCCA, as we have recently reviewed (Papoutsoglou et al., 2019).

Furthermore, recent studies have shown that non-coding RNAs (ncRNA), such as microRNA (miRNA) and long non-coding RNAs (lncRNA) play a role in regulating the dual functions of TGF β in cancer (Yuan et al., Cancer Cell 2014), particularly by interfering with key elements of the TGF β pathway, such as CDKN1A/P21 involved in cell growth regulation, or transcriptional activators of the process called Epithelial-mesenchymal transition (EMT) ZEB1/2. For instance, an autocrine TGF β /ZEB1/miR-200 signaling network has been found to regulate the transition of cells between epithelial and mesenchymal states, with miR-200 acting as an inhibitor of ZEB1 (Gregory et al., 2011). Notably, TGF β -induced lncRNA have also been found to be part of this network, specifically by acting as "sponges" for miR-200 (Yuan *et al.*, 2014). The sequestration of miR-200 by lncRNA releases the inhibition of ZEB1 and thus promotes EMT. More specifically in iCCA, we previously identified a TGF β -induced lncRNA called T-LINC, which is involved in promoting an inflammatory microenvironment. These findings further strengthen the idea that ncRNA can play significant roles in the progression of iCCA (Merdrignac *et al.*, 2018).

More recently, circular RNA (circRNA) have emerged as another class of RNA that may play crucial roles in cancer biology (Goodall and Wickramasinghe, 2021). CircRNA are a large group of novel and widespread endogenous RNA that form a covalently closed continuous loop without a 5' or 3' end, as opposed to linear RNA. A large number of circRNA are generated from back-spliced exons during the splicing of coding or non-coding RNAs (Li et al., 2020b). Increasing evidence suggests that circRNA play important roles in tumor growth and invasion in various types of cancers and may serve as potential stable diagnostic and predictive biomarkers (Jahani et al., 2020). Due to their circular structure, circRNA are not sensitive to the activity of exonucleases and are therefore much more stable than linear RNA. Recent studies have demonstrated that circRNA are altered in cancer, including liver cancers. However, there are currently very few reports of circRNA changes in iCCA, and these studies are mainly descriptive (Liao *et al.*, 2023). We have recently reviewed the several of them showing the role of circRNA in the hallmarks of liver cancer (Louis *et al.*, 2022). At the functional level, there is evidence that circRNA may function as miRNA sponges, which regulate fundamental cellular processes such as gene transcription and translation (Lv et al., 2020). Thus, circRNA may represent innovative key regulators of signaling pathways involved in carcinogenesis, including TGF β . Supporting this hypothesis, circRNA_010567 has been

recently shown to act as a sponge for miR-141, a TGF β -associated microRNA involved in fibrosis (Zhou and Yu, 2017). More recently, a descriptive screening identified EMT-related circRNA and miRNA in prostate cancer (Yan et al., 2020). Taken together, the data reported in the literature fully support the hypothesis of a key role of circRNA in TGF β -associated cancer biology. However, a deep understanding of the molecular mechanisms involved and the clinical relevance of TGF β -regulated circRNA is currently lacking in iCCA. With the exception of our initial identification of TGF β -induced lncRNA TLINC and its derivative circRNA (Merdrignac *et al.*, 2018), there is no report of TGF β -modulated circRNA in iCCA.

In summary, this project aims to investigate and understand the role of TGF β -regulated circRNA in iCCA. Specifically, the project focuses on the identification and study of novel circRNA that act as miRNA sponges and are involved in propagating the effects of TGF β by regulating TGF β -modulated gene regulatory networks in iCCA tumors. The hypothesis is that understanding these circRNA and analyzing their expression in human iCCA will reveal new mechanisms that modulate TGF β actions in cancer and identify clinically relevant biomarkers that can be used to target TGF β in tumors using clinically available inhibitors. Additionally, we will also investigate the potential of circulating vesicular-circRNA as innovative biomarkers in patients with iCCA.

Materials and Methods

Patients and biological samples

Freshly frozen human CCA were obtained from surgical specimens after hepatectomy, which were provided by the Biological Resource Centre (BB-0033-00056). Three independent cohorts of patients were analyzed: 1) a training set of 20 iCCA patients from a French national cohort, 2) a validating set of 31 iCCA patients from another French national cohort, 3) an external validating set of 39 patients from a Salamanca cohort, which included 12 iCCA, 5 dCCA, 2 pCCA and 20 HCC. Serum samples were also obtained from patients with advanced metastatic iCCA (n= 104) from the CLCC Eugène Marquis (Rennes, France). Among these samples, 17 sera were paired with tissues from the first cohort. The research protocol was in compliance with national laws and regulations and was approved by the local ethics committee and the institutional review board of Inserm (IRB00003888).

Cell culture and reagents

HuCC-T1 (RCB-1960) and HuH-28 (RCB-1943) CCA cell lines were obtained from the RIKEN BioResource Center in Japan. SG231, Mz-ChA-1, TFK-1 and SK-ChA-1 were kindly provided by Dr. Laura Fouassier from the Centre de Recherche Saint-Antoine (Inserm U938, Paris, France). Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 (HuCC-T1, TFK-1), minimal essential medium (MEM) (HuH-28) or Dulbecco's Modified Eagle Medium (DMEM) (SG231, Mz-ChA-1, SK-ChA-1) culture media supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. The cells were kept at 37°C in an atmosphere containing 5% CO₂ and were serum-starved overnight before each treatment. Unless otherwise specified, the cells were treated with 1 ng/mL of recombinant human TGFβ (R&D Systems, Minneapolis, MN) and 10 µM of LY2157299 (TGFβR1 inhibitor) (Sigma-Aldrich, St. Louis, MO).

RNA isolation and q-RT-PCR from mRNA

Quantitative reverse-transcription polymerase chain reaction (q-RT-PCR) was performed as previously described by (Bevant et al., 2022) was used. The reaction was carried out using a SYBR Green master mix (Applied Biosystems, Carlsbad, CA). The RT reaction was primed with a mixture of deoxythymidine oligomers (250 ng) and random hexamers (100 ng). For

each RT reaction, 500 ng of total RNA was used (Superscript III RT; Invitrogen). The list of DNA primers used in this study is provided in Supporting Table S1.

CircRNA microarray analysis

Total RNA, including circRNA, was purified with an miRNeasy kit (Qiagen, 217004). Arraystar Human circRNA Microarray v2.0 (Arraystar, Rockville, MD, USA) were used to screen for novel TGF β -regulated circRNA in human CCA cell lines. Each microarray contains probes for over 13,617 circRNA isoforms and is designed to detect both exonic and intronic circRNA. RNA samples were extracted from the CCA cell lines and labeled with fluorescent dye using the Arraystar circRNA Microarray Labeling Kit (Arraystar). The labeled samples were then hybridized to the microarray slides and scanned using the Arraystar Microarray Scanner (Arraystar). The data was analyzed using BRB Array Tools software (available for download at <http://linus.nci.nih.gov/BRB-ArrayTools.html>). Differentially expressed genes were identified by a 2-sample univariate t test and a random variance model as previously described (Merdrignac *et al.*, 2018). Differentially regulated genes between the different experimental conditions were selected based on stringent criteria ($P < 0.01$; $FC > 2$). The results were further validated by q-RT-PCR

CircRNA quantification

In order to enrich for circRNA, total RNA was first treated with RNase R (1 $\mu\text{g/mL}$) at 37 $^{\circ}\text{C}$ for 30 minutes. RNase R selectively degrades linear RNA. Next, Poly(A)⁺ RNA were removed by using only random hexamers (100 ng) before performing the reverse transcription (RT) reaction. Quantitative Real-time PCR (q-RT-PCR) was carried out using sets of primers that specifically target circRNA, known as divergent primers. These primers were designed to overlap the circularization junction, allowing only the detection of circular transcripts. A list of DNA primers used to detect circRNA is provided in Supporting Table S1.

Immunoblotting

Protein analysis by immunoblotting was done as previously described by (Bevant *et al.*, 2022). Cells were washed with phosphate-buffered saline (PBS) and harvested in RIPA lysis buffer containing protease inhibitors (ThermoFisher, Waltham, MA, USA). The lysates were sonicated and centrifuged. The protein content was then quantified by BCA assay. Twenty micrograms of total protein were separated on NuPAGE Bis-Tris 4-12% gels and transferred

to nitrocellulose membranes (Invitrogen). The membranes were incubated with primary antibodies (Ab) diluted as recommended by the manufacturer overnight at 4°C. Subsequently, the membranes were incubated for one hour with an appropriate secondary HRP-coupled diluted 1:2000, followed by ECL detection using the ImageQuant LAS imaging system (General Electric, Boston, MA, USA). For immunofluorescence experiments, cells were fixed with 4% PFA in PBS for 15 minutes at room temperature. A list of antibodies used in this study is provided in Supporting Table S1.

circLTBP2 gain and loss of function

HuCC-T1 cells were infected with a specific circLTBP2 overexpressing vector (pLV-circLTBP2) generated by Creative Biogene (NY, USA). A clonal stable cell line overexpressing circLTBP2 was generated after a 2 weeks puromycin selection (5 µg/mL) and subsequent limited dilution in 96-well plates. Overexpression was validated by q-RT-PCR. Antisense LNA GapmeRs that target the circularization junction of circLTBP2 (5'-GACGTTCCCTCCTGTGC-GG-3') (#339511, Qiagen) were used for loss of function experiments. HuCC-T1 cells were transfected at 70% confluence with the Antisense LNA GapmeRs (50nM) using RNAiMAX lipofectamine reagent and Opti-MEM medium according to the manufacturer's instructions (Invitrogen).

Cell proliferation test

Three genetically engineered HuCC-T1 clonal cell lines overexpressing circLTPB2 were seeded in 96-well plates (37°C and 10% FBS), at a density of 2,500 cells per well. After 12 hours, the FBS was reduced to 1%. Cell proliferation was monitored in real-time using an Incucyte S3 device (Sartorius, USA). Pictures were taken every 4 hours for 132 hours. The Incucyte S3 software was used to analyze the images and calculate the rate of cell proliferation by calculating the ratio between the area occupied at T0 and that of each other time point (relative cell proliferation expressed as percentage).

Cell migration assay

To evaluate the effect of circLTBP2 on cell migration, a wound healing assay was performed using the Incucyte S3 device. HuCC-T1 cells (3 independent clones as above) were seeded in 96-well plates (37°C and 10% FBS) at a density of 30,000 cells per well. Once the cells reached confluency, a wound was created using an Incucyte 96-Well Woundmaker Tool

provided by Sartorius. The plate was then placed in the Incucyte S3 and an initial image of the wound area was taken. Images of the wound area were taken every 4 hours until the wound was fully closed. The Incucyte S3 software was used to analyze the images and calculate the rate of cell migration and the percentage of wound closure (relative wound density expressed as percentage).

Cell apoptosis detection

HuCC-T1 cells (3 independent clones as above) were seeded in 96-well plates (37°C and 10% FBS), at a density of 5,000 cells per well. After 12 hours, the cells were treated with Gemcitabine (10 ng/mL, G6423-10mg-Sigma-Aldrich) and Annexin V Red Dye (Sartorius). The quantitative analysis was performed using the Incucyte S3 (Sartorius). The fluorescence intensity collected for each condition was normalized to the untreated condition. Photos were taken every 8 hours until 140 hours. HuCC-T1 cells resistance to gemcitabine-induced apoptosis (10 ng/mL) was evaluated every 4 hours using a red fluorescent Annexin V labelling, in both the circLTBP2 overexpressing and control groups.

Tumor xenograft models

HuCC-T1 cells were modified to overexpress either an empty vector (pLV-circRNA-GFP) or circLTBP2 (pLV-circLTBP2-GFP) and were infected with GL261-Luc (CMV-Firefly luciferase lentivirus (Neo), PLV-10064-50, Cellomics Technology, USA). 4,000,000 cells (100µL in PBS + 100µL matrigel) were implanted on the flanks of 8-week-old female NSG mice (Charles River, USA) that were immune-deficient. All animal procedures followed the European Community Directive guidelines (Agreement B35- 639 238-40, Biosit Rennes, France; DIR #7163) and were approved by the local ethics committee. Mice were monitored daily to ensure their well-being according to the law and the 3R rule (Reduce-Refine-Replace). Tumor growth was evaluated by measuring the size of the tumor with caliper. 90 days after implantation, the mice were sacrificed and the tumors, liver, and lung tissues were isolated and analyzed at the molecular level. The presence of metastases in the liver and in the lungs was evaluated by bioluminescence.

The method for isolating and extracting RNA from xenograft tumors involves cutting the tumors into smaller pieces, homogenizing them using a tissue homogenizer and ceramic beads, and then extracting the RNA using the RNeasy Lipid Tissue Mini Kit. The homogenization was done at a high speed of 5,500 rpm for 3 cycles of 20s each with a pause

of 20s between cycles. The extracted RNA was then used for further analysis and downstream experiments.

circRNA pulldown assay

A circLTBP2 pulldown assay was carried out by Creative Biogene (NY, USA). Briefly, a labeled circRNA expression vector was generated and co-transfected into HuCC-T1 cells with a capture protein expression vector. Afterwards, a pulldown assay was done by using a labeled antibody that binds to the induced capture protein, which allows for the pulldown of circLTBP2-RNA and circLTBP2-RBP complexes. The detection of eluted miRNA was done by miRNA-sequencing.

Data mining

The GSEA software version 2.0.13 was utilized to perform Gene Set Enrichment Analysis. The GSE102109 signature was used to identify the enrichment of miRNA targets in the TGF β signature of HuCC-T1 cells. The results were displayed as a normalized enrichment score (NES) and presented in an enrichment map format.

The Starbase software, which combines data from sources such as miRNA-seq, mRNA-seq, and ChIP-seq, was utilized to identify the binding sites of miRNA on circRNA and to infer the connections between circRNA, miRNA, and mRNA (Dudekula *et al.*, 2016; Li *et al.*, 2014).

miRNA immunoprecipitation (IP)

miRNA IP was carried out according to the manufacturer's instructions (miRNA Target IP kit #25500, Active Motif, Inc). Briefly, miR-338-3p mimics (miRCURY LNA miRNA Mimic, Qiagen, 5nM) were transfected into HuCC-T1 cells for 24 hours. The IP was performed using a pan-Ago antibody that recognizes Ago1, Ago2, and Ago3. This antibody pull-down miRNA/mRNA and miRNA/circRNA complexes that have been associated as part of a RISC complex. IP was also performed with a Negative Control IgG. Additionally, the IP experiment was also performed on cells that have been transfected with a non-targeting control miRNA to be certain the effect seen with the miRNA of interest is not due to non-specific binding. First, cells were lysed in a Complete Lysis Buffer. Then, antibodies were incubated with Protein G Magnetic Beads at room temperature for 30 minutes. The lysate was mixed with antibody-beads complexes and incubated under rotation overnight at 4°C. After a proteinase

K digestion, RNA complexes were extracted using a phenol: chloroform: isoamyl alcohol RNA extraction method. The abundance of RNA bound to miR-338-3p was evaluated by q-RT-PCR analysis.

EV isolation and characterization

Extracellular vesicles (EV) were isolated by ultracentrifugation (UC). Supernatant from cell culture was collected and centrifuged at 1000xg for 10 minutes to remove cells and debris. The resulting supernatant was then centrifuged at 10,000xg for 1h30 to pellet EV. The EV pellet was washed with PBS, and centrifuged again at 10,000xg for 1h30. The final EV pellet was resuspended in PBS and stored at -80°C until further analysis. The size and concentration of EV were assessed using a tunable resistive sensing method (qNano, Izon Science technology). To confirm the presence of EV markers and EV morphology, scanning electron microscopy (SEM) and immunoblotting were used. The relative levels of circLTBP2 in EV and HuCC-T1 cells were determined by q-RT-PCR. Samples volume used to extract EV ranged from 1mL (for sera) to 10 mL for HuCC-T1 cells supernatant.

Statistical analyses

All statistical analyses were conducted using Prism 8 software (from GraphPad Software) and the results were presented as mean values \pm standard deviation or median values. Comparison between variables were made using either Student's t-test or Mann-Whitney U test. For multiple sets of multivariate comparisons, two-way analysis of variance was applied. Survival curves were analyzed through Kaplan-Meier method and log-rank test. A *P* value < 0.05 was considered statistically significant.

Results

HuCC-T1 is a TGF β -responsive iCCA cell line

CCA is a rare cancer with limited characterized *in vitro* models. Therefore, we first characterized 3 iCCA (HuCC-T1, HuH-28, SG231) and 3 eCCA (Mz-ChA-1, TFK-1, SK-ChA-1) cell lines. Mz-ChA1 and TFK-1 displayed an epithelial phenotype (high expression of E-Cadherin, CDH1 and no expression of Vimentin, VIM) while HuH-28 displayed a mesenchymal phenotype (high VIM / no CDH1). SK-ChA-1, SG231 and HuCC-T1 exhibited an intermediate phenotype with expression of both CDH1 and VIM (**Figure S1A-C**). Next, we evaluated their response to TGF β by measuring the level of phosphorylated SMAD3 (**Figure 1A, Figure S1D**) and the expression of known target genes (*SERPINE1*, *SMAD7*, *BIRC3*). HuCC-T1 and HuH-28 cell lines were fully responsive to TGF β , with a significant SMAD3 phosphorylation, up-regulation of *SERPINE1*, *SMAD7* and down-regulation of *BIRC3*. In addition, these effects were reversed in presence of galunisertib (LY2157299), a selective inhibitor of TGFBR1 (**Figure 1B, Figure S1E**). Subsequent experiments were performed in HuCC-T1 cells, which exhibited better cell culture conditions and transfection efficiency.

CircLTBP2 is a TGF β -induced circRNA predictive of poor prognosis in iCCA

Gene expression profiling using Arraystar microarrays identified 119 circRNA significantly ($P < 0.001$ and $FC > 2$) deregulated by TGF β (1 ng/mL, 16 hours) in HuCC-T1, including 85 up- and 34 down-regulated circRNA isoforms (**Figure 1C, Table S2**). Interestingly, more than 50% of circRNA and their linear counterparts were co-regulated in response to TGF β (**Figure S2**). Of the circRNA and linear RNA pairs, 48% were both induced and 4% were both repressed by TGF β . Thus, 48% of circRNA were regulated independently of their linear counterpart, being either induced or repressed while the linear counterpart remained unchanged (**Figure S2**). Out of the 119 TGF β -regulated circRNA, Starbase software (Dudekula *et al.*, 2016; Li *et al.*, 2014) predicted that 42 may act as miRNA sponges (**Figure S2**). A gene ontology (GO) analysis revealed that these circRNA were associated to biological processes regulated by TGF β , such as cell migration and apoptosis (**Figure 1D**). The expression of 5 randomly selected TGF β -induced circRNA was validated by q-RT-PCR in cells upon TGF β stimulation (**Figure 1E**). Next, we evaluated their clinical relevance in resected iCCA tumors (cohort 1, $n=20$). Interestingly, among the 5 candidates, circLTBP2 was associated with a poor prognosis since its expression was predictive of a significant reduced overall (OS) and relapse-free (RFS) survival ($P < 0.01$) (**Figure 1F-G, Figure S3A**).

The association of circLTBP2 expression and OS was further validated in two independent cohorts of resected iCCA (**Figure S3B-C, Figure 1I**). Interestingly, the expression of circLTBP2 was very low in HCC and not associated with OS (**Figure 1H-I**).

CircLTBP2 is a large exonic circRNA (2165 bases) composed of exons 2 to 16 of *LTBP2* gene and its circular structure was confirmed by Sanger sequencing (**Figure 2A**). We developed sets of convergent and divergent primers to specifically detect the circLTBP2 isoform hsa-circ-0032603 and its linear LTBP2 counterpart (**Supplementary Methods, Figure S4, Figure 2A-C**). CircLTBP2 was detected and induced by TGF β in both intrahepatic and extrahepatic CCA cell lines, with the exception of SG231 (**Figure 2D**). In HuCC-T1 cells, circLTBP2 induction by TGF β was the highest (**Figure 2D**) and abolished in presence of galunisertib (LY2157299), an inhibitor of TGFBR1, or SMAD3 inhibitor SIS3 (**Figure 2E-F**).

CircLTBP2 exerts pro-oncogenic actions in iCCA

First, we performed gain-of-function (GOF) assays by infecting HuCC-T1 cells with a vector that contained the sequence of circLTBP2 (pLV-circLTBP2) (**Figure 3A**). Overexpression was validated in several HuCC-T1 clones and 3 of them with similar levels of circLTBP2 expression as seen after TGF β treatment were selected for functional analysis. Importantly, the expression of linear LTBP2 was not affected by circLTBP2 overexpression (**Figure 3B**). TGF β contributes to CCA progression by stimulating cell proliferation, migration and by inhibiting apoptosis (Papoutsoglou *et al.*, 2019). Thus, we investigated the impact of circLTBP2 on these processes using an Incucyte S3 device. HuCC-T1 cells stably overexpressing circLTBP2 exhibited significantly higher proliferation and migration abilities when compared to cells infected with an empty vector (**Figure 3C-D**). In addition, circLTBP2 overexpression was shown to promote resistance to gemcitabine-induced apoptosis (**Figure 3E**). The pro-tumorigenic potential of circLTBP2 was further evaluated *in vivo* by injecting genetically engineered HuCC-T1 cells into NSG mice (**Figure 3F**). Tumors developed in mice injected with cells overexpressing circLTBP2 were significantly bigger in size (856 ± 336 vs 1478 ± 292.07 mm³, $P < 0.001$) and weight (0.6 ± 0.21 vs 1.12 ± 0.41 g, $P < 0.05$) (**Figure 3G-I**). CircLTBP2 overexpression in the resected tumors was confirmed by q-RT-PCR (**Figure S5A**). However, no difference was observed in the number or size of lung metastases in mice injected with cells overexpressing circLTBP2 vs control vector (**Figure S5B**).

Next, loss-of-function (LOF) assays using LNA GapmeRs specifically targeting circLTBP2 circularization junction were performed (**Figure S5C-G**). Importantly, these GapmeRs were

shown to specifically decrease the expression of circLTBP2 without impacting the expression of its linear counterpart (**Figure S5D**). Validation our previous observation, circLTBP2 LOF in HuCC-T1 cells resulted in a decrease of cell proliferation and migration, and an improved sensitivity to gemcitabine (**Figure S5E-G**).

CircLTBP2 acts as a sponge for miR-338-3p, a tumor suppressor miRNA

To gain insight into the molecular mechanisms driving the effects circLTBP2, we performed a circLTBP2 pull-down assay followed by miRNA sequencing. A decision-making pattern was established to select the most promising miRNA candidates (**Figure 4A**). We identified 94 miRNA physically interacting with circLTBP2 ($FC > 1.5$ between circLTBP2 IP and the input control), among them 69 were recorded in databases. In addition, the selection of miRNA relied on a strong dependency to circLTBP2, as determined by the presence of 2 or more predicted binding sites, and an enrichment of their predicted targets in HuCC-T1 cells stimulated by TGF β . In total, 8 miRNA met the selection criteria (miR-338-3p, miR-3064-5p, miR1270, miR-1294, miR-34c-5p, miR324-3p, miR-1914-3p, miR-34a-5p) (**Figure 4B**). Among them, miR-338-3p had the highest number of predicted binding sites ($n=7$) on circLTBP2 (**Figure 4C**), and the highest enrichment score ($NES=1.86$, $P < 0.01$) of its predicted targets in the gene expression profiles of HuCC-T1 cells stimulated by TGF β , as determined by GSEA (**Figure 4D**, **Figure S6**). Furthermore, miRNA sequencing demonstrated that miR-338-3p and miR-1294 were among the least abundant miRNA in HuCC-T1, suggesting a tightly regulated expression of these two miRNA. Ranking these miRNA based on the above criteria highlighted miR-338-3p as the most promising candidate (**Figure 4C**). Among 16 miR-338-3p targets involved in biological processes regulated by circLTBP2, the expression of 10 was significantly increased by TGF β (**Figure 4E**). Four of these targets (SOX4, ANHAK2, FURIN, NET1) also exhibited a small but significant decrease in expression in cells transfected with miR-338-3p mimics (**Figure 4F**). To confirm the direct interaction between miR-338-3p and SOX4, a known regulator of EMT, we performed a miRNA pulldown assay. Our results demonstrated that miR-338-3p not only binds to circLTBP2 and its linear counterpart LTBP2, but also SOX4 (**Figure 4G**). We used the key EMT genes ZEB1 and ZEB2 as negative controls. In addition, circLTBP2 overexpression induced SOX4. In order to determine if the pro-tumorigenic effects driven by circLTBP2 resulted from the sequestration of miR-338-3p, we conducted rescue experiments using miRNA mimics. Importantly, circLTBP2 expression does not affect the expression of miR-338-3p (**Figure 5A**). While rescuing miR-338-3p levels was not able to decrease circLTBP2-

induced cell proliferation, it restored normal levels of cell migration and resistance to gemcitabine-induced apoptosis (**Figure 5B-D**), demonstrating that circLTBP2 contributes to gemcitabine resistance by sponging miR-338-3p.

Circulating circLTBP2 is associated with poor OS in iCCA patients

Finally, we aimed at evaluating the clinical relevance of circLTBP2 as a prognostic biomarker for iCCA, easily detectable by non-invasive methods. Thus, we first determined whether circLTBP2 could be embedded into EVs and secreted. EVs (mainly exosomes) were isolated by ultracentrifugation and qualified by qNano, transmission electron microscopy and expression of specific surface markers (**Figure 6A-C**). Interestingly, circLTBP2 was expressed in EVs secreted by HuCC-T1 cells and its expression in EVs was greater in cells pre-treated by TGF β (**Figure 6D**). In addition, circLTBP2 was more stable than its linear counterpart with a half-life greater than 24 hours (**Figure 6E**). More interestingly, the expression level of circLTBP2 was significantly increased in EVs isolated from the serum of patients with advanced, metastatic iCCA (n=103) vs healthy individuals (**Figure 6F**). A significant correlation between the levels of circLTBP2 in the tumor tissues and in the serum was also highlighted (**Figure 6G**). From patients with available survival data (n=39) we generated two groups based on the median expression of circLTBP2 (**Figure 6H**). Statistical analysis demonstrated that a high expression of circulating circLTBP2 was significantly associated with a reduced OS (**Figure 6I**).

Figure 1

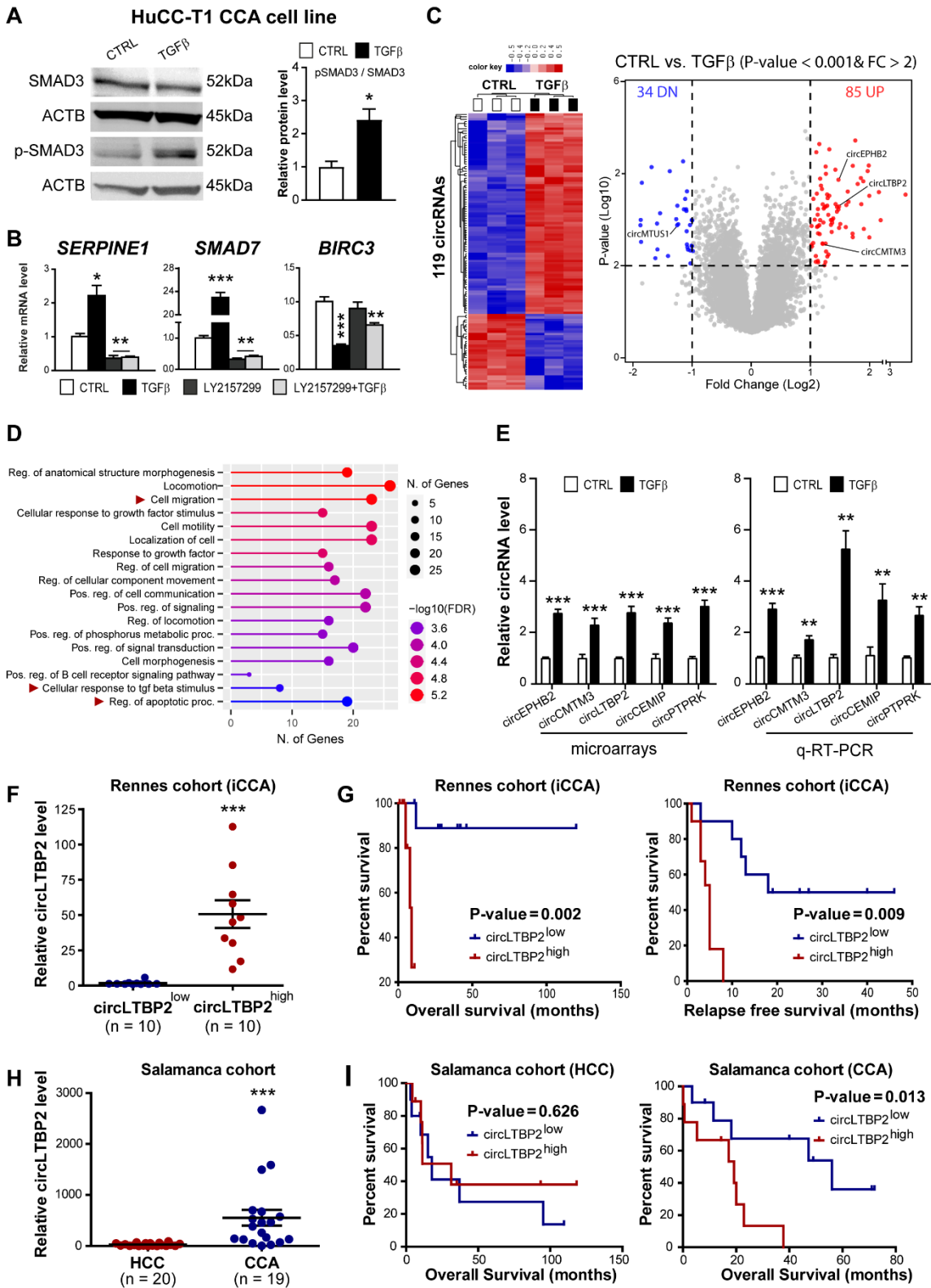


Figure 1. CircLTBP2, a TGF β -induced circular RNA, predicts a poor prognosis for iCCA patients

(A) Western blot analysis of p-SMAD3 and SMAD3 expression in HuCC-T1 iCCA cell lines in response to a TGF β treatment (1 ng/mL TGF β , 16 hours) (B) Expression of three well-known TGF β target genes in HuCC-T1: SERPINE1, SMAD7 and BIRC3 alone or in response to a TGF β , LY2157299 or a combined treatment (1 ng/mL TGF β ; 10 μ M LY2157299; 16 hours) (C) Hierarchical clustering and volcano plot of circRNA isoforms differentially expressed by TGF β in HuCC-T1 (1 ng/mL TGF β , 16 hours). HuCC-T1 cells treated with TGF- β display a specific signature of 135 probes (85 circRNA isoforms) up-regulated and 50 probes (34 circRNA isoforms) down regulated. Horizontal dashed lines on the volcano plot: P -value < 0.01. Vertical dashed lines: $FC > 2$. (D) Gene ontology (GO) analysis based on circRNA microarray signature showed that dysregulated genes upon TGF β treatment are involved in the regulation of cell migration and response to apoptosis. FDR, false discovery rate. P -values are calculated by permutation test (E) TGF β -induced circRNAs empirically selected and their relative expression, as determined by microarray and q-RT-PCR. (F) CircLTBP2 (has-circ-0032603) relative expression in 20 freshly frozen iCCA tumors from a French national cohort (separated in two groups: circLTBP2^{low} and circLTBP2^{high}, according to their circLTBP2 median expression). Stable expression of circWRNIP1 was observed among patients and served as a reference circRNA for normalization. (G) Kaplan-Meier plots and log-rank statistics analysis revealed a significant decreased overall survival (OS) and relapse free survival (RFS) for iCCA patients with a high expression of circLTBP2. (H) circLTBP2 relative expression in Salamanca cohorts including hepatocellular (HCC) and CCA tumors unraveled circLTBP2 specificity for CCA. Stable expression of circWRNIP1 was observed among patients and served as a reference circRNA for normalization. (I) Kaplan-Meier plots and log-rank statistics analysis endorsed the association of circLTBP2 expression with OS in the Salamanca independent CCA cohort while no correlation was found in HCC cohort. Statistical analyses for (A), (B) and (C) were performed by a Mann–Whitney test. Data are presented as means \pm SD. (* P -value < 0.05 ** P -value < 0.01, *** P -value < 0.001, $n \geq 3$). Abbreviations: iCCA (intrahepatic cholangiocarcinoma).

Figure 2

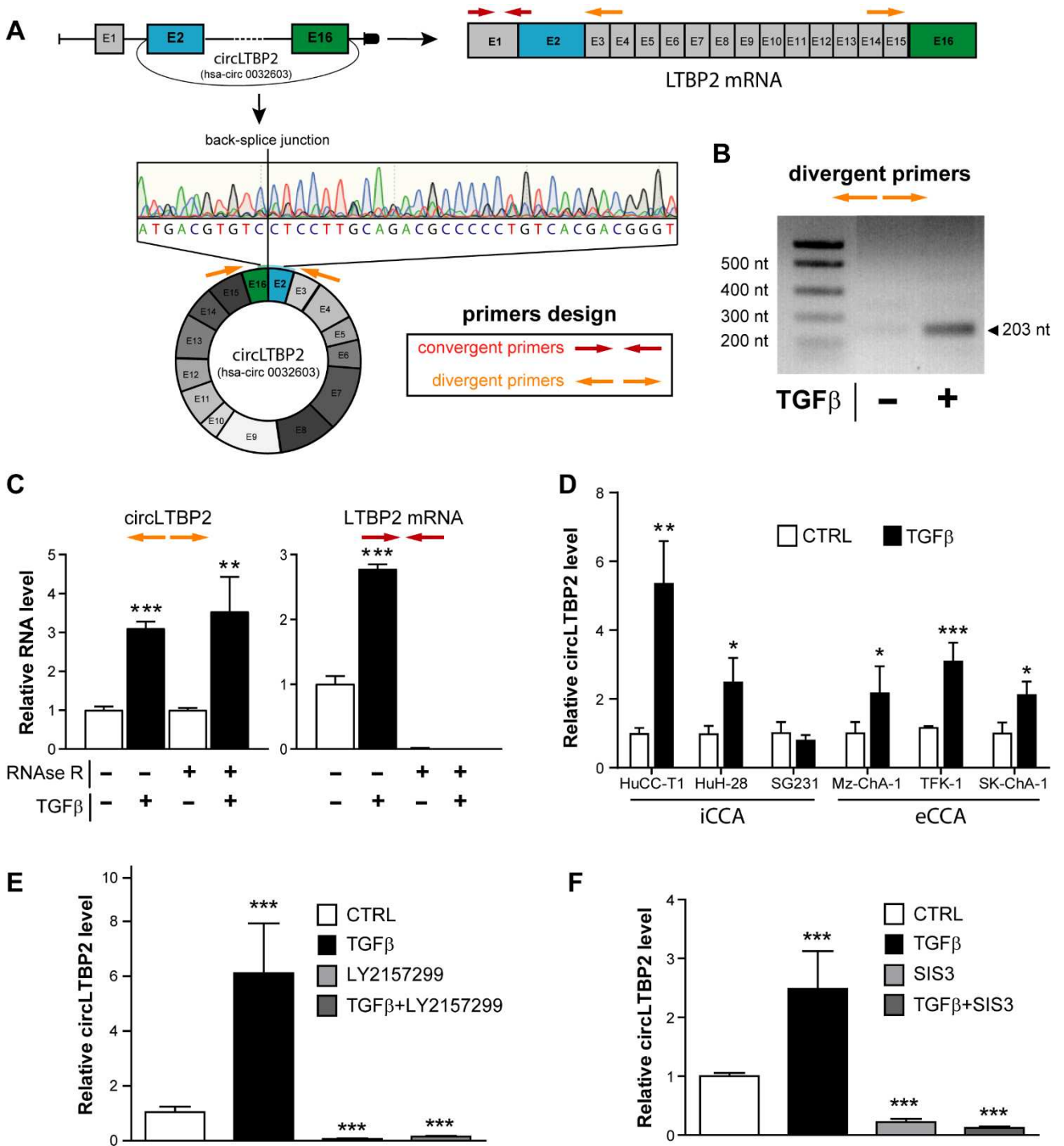


Figure 2. CircLTBP2 (hsa-circ-0032603) characterization in CCA cell lines

(A) Schematic illustration of circLTBP2 production via the circularization from exon 2 to exon 16 in LTBP2 gene. The back-splice junction sequences were confirmed by Sanger sequencing (B) q-RT-PCR showing the existence of circLTBP2 isoform (hsa-circ-0032603) after a RNase R treatment (C) Relative expression of circLTBP2 and its cognate mRNA LTBP2 in HuCC-T1 after a TGF β treatment and with or without RNase R pretreatment. A pair of divergent and convergent primers were respectively used to evaluate their expression (D) Relative expression of circLTBP2 in 6 CCA cell lines (including iCCA and eCCA cell lines). (E,F) Inhibition of circLTBP2 Smad-dependent expression in HuCC-T1 cell lines using the LY2157299 and SIS3 inhibitor. Statistical analyses for (C), (D) (E) and (F) were performed by a Mann–Whitney test (* P-value < 0.05 ** P-value < 0.01, *** P-value < 0.001, $n \geq 3$). Abbreviations: eCCA (extrahepatic cholangiocarcinoma), iCCA (intrahepatic cholangiocarcinoma).

Figure 3

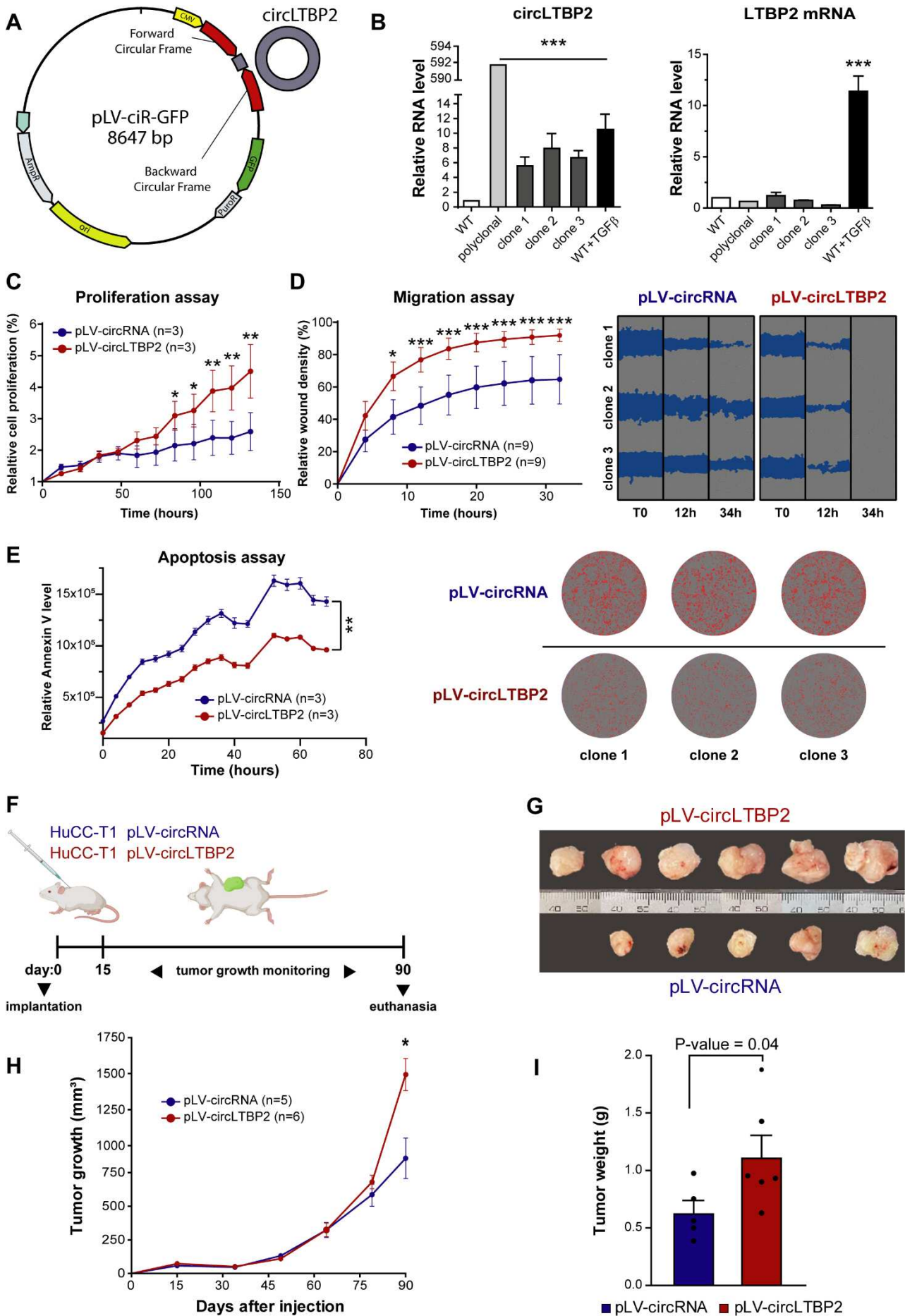


Figure 3. The overexpression of circLTBP2 has been shown to contribute to the progression of intrahepatic cholangiocarcinoma (iCCA) by promoting cell proliferation, migration, and resistance to gemcitabine-induced apoptosis in vitro, as well as promoting tumor growth in xenograft models.

(A) Schematic representation of circLTBP2 overexpression vector **(B)** Relative expression of circLTBP2 and its cognate mRNA in HuCC-T1 clones infected with circLTBP2 overexpression vector **(C)** Relative confluency of cultured HuCC-T1 cells was evaluated for 72 hours ($n=3$ per group). HuCC-T1 cells overexpressing circLTBP2 showed an induced proliferation **(D)** Cell migration was evaluated by wound healing assay performed on HuCC-T1 cells overexpressing circLTBP2 compared to the empty vector control group ($n=9$ per group). Relative wound density was assessed every 4 hours as a percentage of the initial wound surface, represented in blue on the right panel. HuCC-T1 cells were cultured in low FBS concentration medium (1% FBS) and with Actinomycin D ($10 \mu\text{g. mL}^{-1}$) to inhibit cell proliferation. **(E)** HuCC-T1 cells resistance to gemcitabine-induced apoptosis (10 ng/mL) was evaluated every 4 hours using a red fluorescent Annexin V labelling, in both the circLTBP2 overexpressing and control groups **(F)** Mice subcutaneous tumor models were used to evaluate the impact of circLTBP2 overexpression on tumor growth **(G, H)** and volume **(I)**. 5 mice were included in the control group and 6 mice were included in the circLTBP2 overexpression group. Statistical analyses for **(B)** and **(I)** were performed by a Mann–Whitney test. Statistical analyses for **(C)**, **(D)**, **(E)** and **(H)** were performed by a 2-way-Anova test (* P -value < 0.05 ** P -value < 0.01 , *** P -value < 0.001 , $n \geq 3$). Abbreviations: pLV-circLTBP2 (circLTBP2 overexpression vector), pLV-circRNA (empty vector), TGF β (Transforming-Growth Factor β).

Figure 4

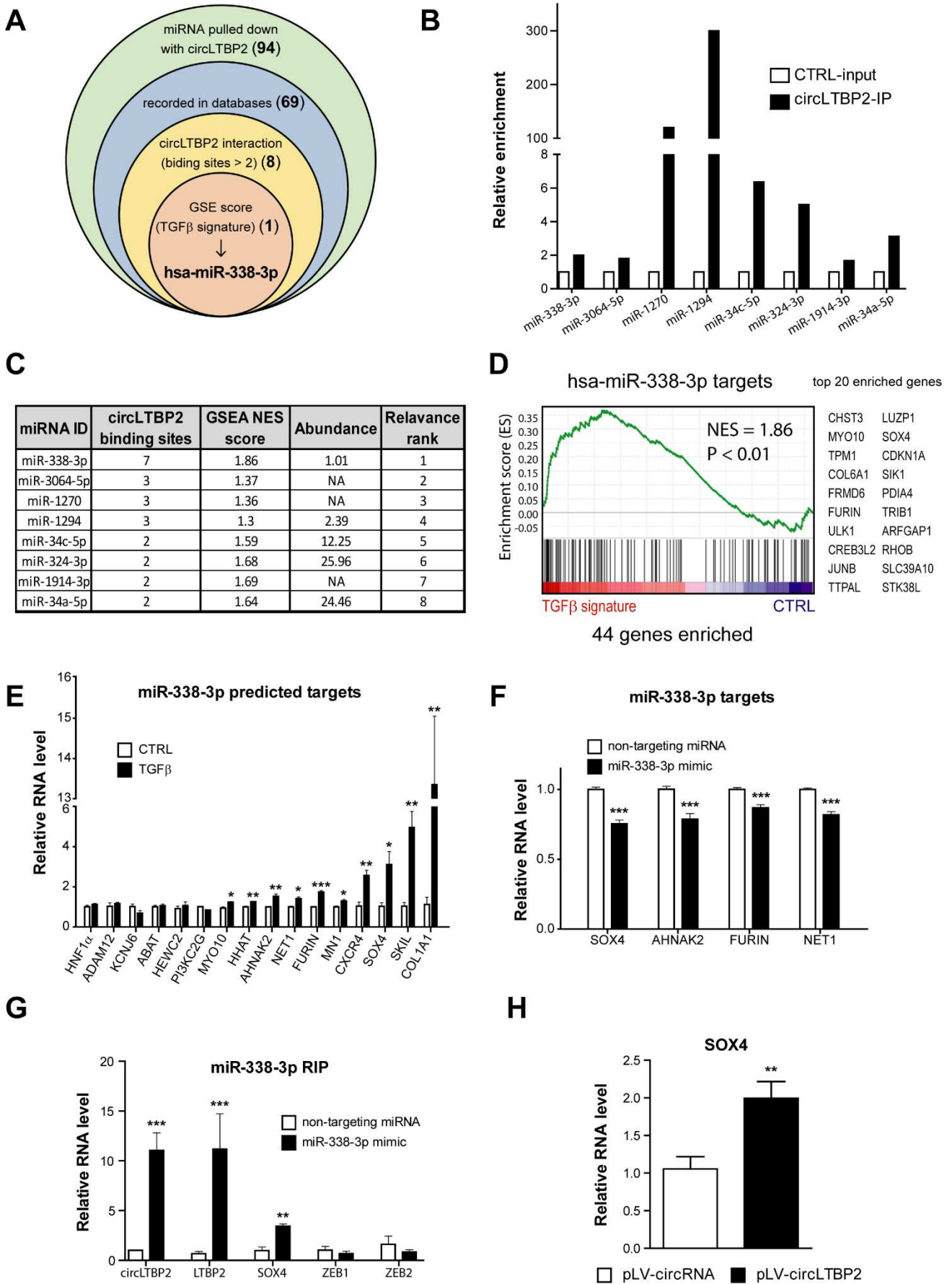


Figure 4. CircLTBP2 acts as a sponge for miR-338-3p, a known tumor suppressor, targeting SOX4

(A) Schematic representation of the strategy used to select a miRNA showing strong interactions with circLTBP2, based on the data obtained by pulldown assay followed by a miRNA-seq. (B) Relative enrichment of miRNA sponged by circLTBP2 obtained by circLTBP2 pulldown assay compared to CTRL-input. The top 10 enriched miRNA are represented here (FC > 1.5). (C) Relevance rank of circLTBP2 binding miRNA based on the predicted number of binding sites, the GSEA enrichment score of their mRNA targets in HuCC-T1 TGF β signature (GSE102109) and their relative abundance. (D) GSEA enrichment score of miR-338-3p mRNA targets in HuCC-T1 TGF β signature (GSE102109). We listed the top 20 enriched genes beside. (E) TGF β induction of miR-338-3p targets evaluated by q-RT-PCR. (F) miR-338-3p TGF β -induced targets relative mRNA level in HuCC-T1 cells transfected with miR-338-3p mimics (5nM). (G) RNA immunoprecipitation of AGO2 in HuCC-T1 cells transfected with miR-338-3p mimics and quantification by q-RT-PCR of putative miR-338-3p binding RNA (i.e., circLTBP2, LTBP2 and SOX4). Key EMT genes ZEB1 and ZEB2 were used as negative controls. (H) SOX4 expression was significantly induced in HuCC-T1 cells overexpressing circLTBP2. Statistical analyses for (E), (F), (G) and (H) were performed by a Mann–Whitney test. (* P-value < 0.05 ** P-value < 0.01, *** P-value < 0.001, n \geq 3). Abbreviations: GSE (Gene Set Enrichment), NES (Normal Enrichment Score), TGF β (Transforming-Growth Factor β).

Figure 5

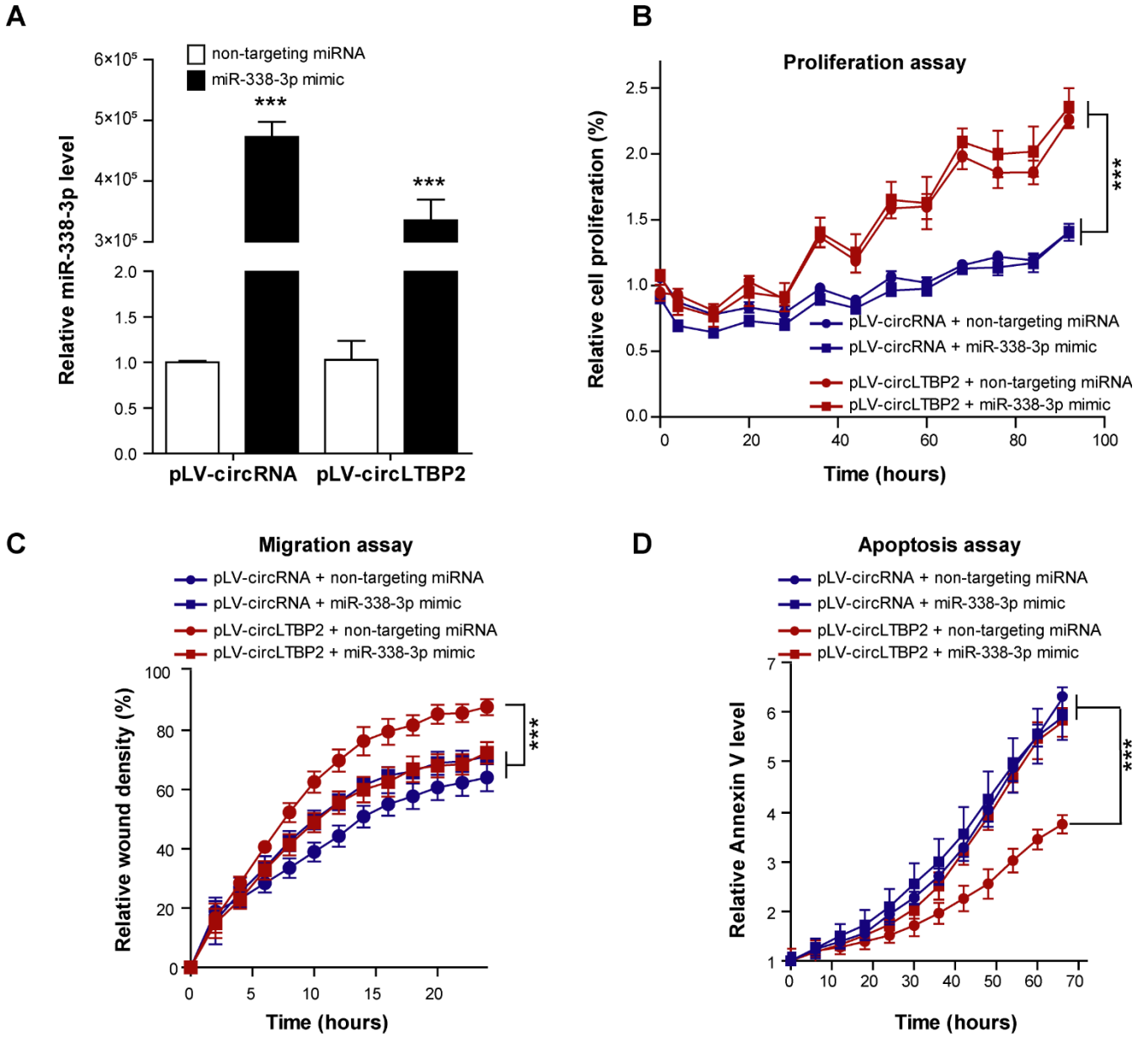


Figure 5. The restoration of miR-338-3p levels in iCCA cells reversed the pro-tumorigenic effects driven by circLTBP2

(A) Relative levels of miR-338-3p in HuCC-T1 were assessed by q-RT-PCR in cells overexpressing circLTBP2 vs. in cells transfected with the empty vector. In addition, miR-338-3p mimics (5nM) were added with the aim of rescuing the excessive sponging mediated by circLTBP2. **(B)** Relative confluency of cultured HuCC-T1 cells was evaluated for 96 hours in HuCC-T1 cells overexpressing circLTBP2 compared to the empty vector control group (n=3 per group). In addition, miR-338-3p mimics were added aiming at restoring the phenotype. **(C)** Cell migration was evaluated by wound healing assay performed on HuCC-T1 cells overexpressing circLTBP2 compared to the empty vector control group (n=3 per group). In addition, miR-338-3p mimics (5nM) were added aiming at restoring the phenotype. **(D)** HuCC-T1 cells resistance to gemcitabine-induced apoptosis (10 ng/mL) was evaluated every 4 hours using a red fluorescent Annexin V labelling, in both the circLTBP2 overexpressing and control groups. In addition, miR-338-3p mimics were added aiming at restoring the phenotype (5nM). Statistical analyses for (A) were performed by a Mann–Whitney test. Statistical analyses for (B), (C) and (D) were performed by a 2-way-Anova test (* P-value < 0.05 ** P-value < 0.01, *** P-value < 0.001, n ≥ 3). Abbreviations: pLV-circLTBP2 (circLTBP2 overexpression vector), pLV-circRNA (empty vector).

Figure 6

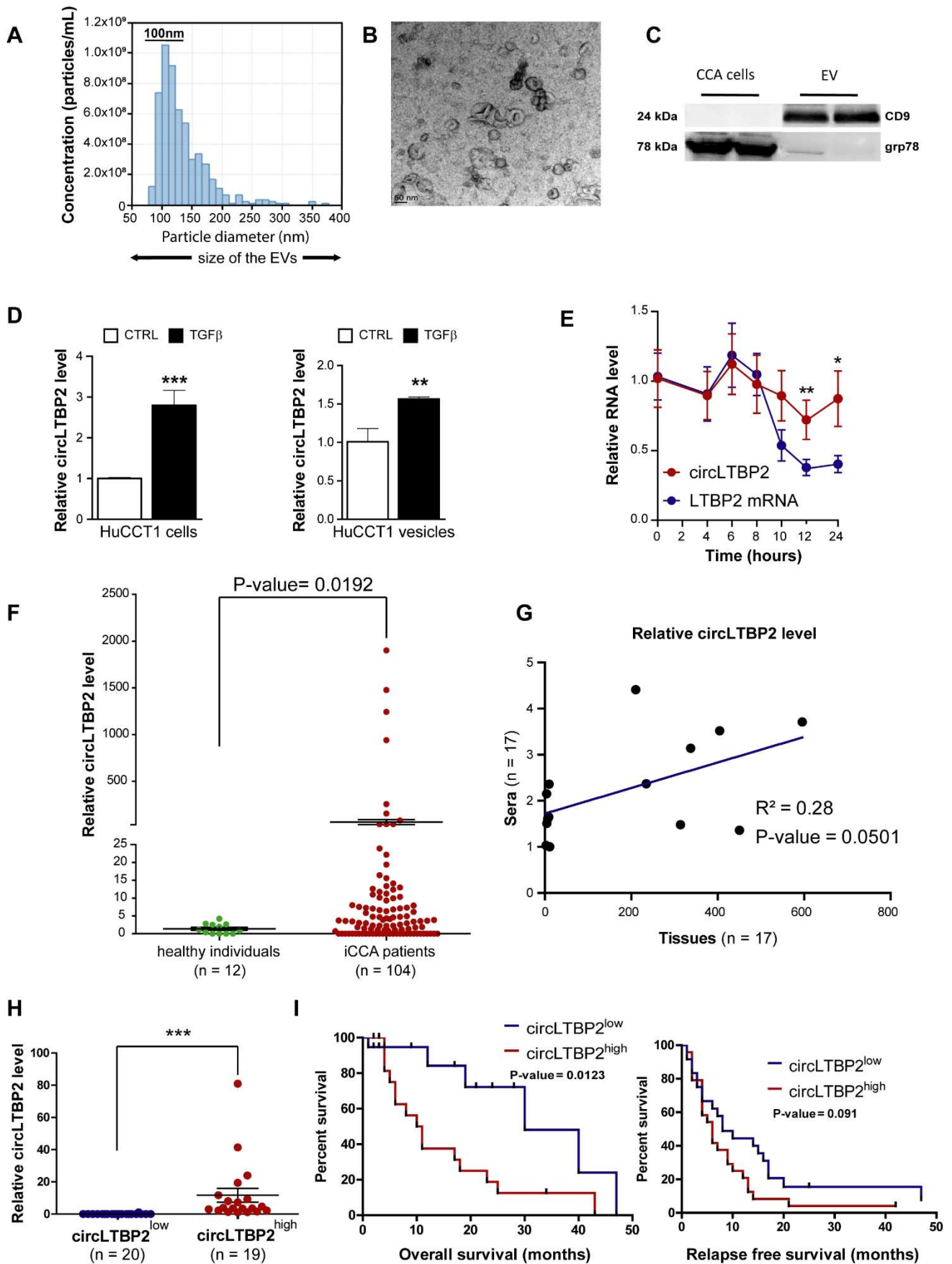


Figure 6. Circulating vesicles containing circLTBP2 in serum are associated with poor OS for iCCA patients.

(A) The size and the concentration of extracellular vesicles (EV) collected from HuCC-T1 supernatant by ultracentrifugation (UC) was assessed using a tunable resistive sensing method (qNano, Izon Science technology). EV with a size ranging from 100 to 200 nm were characterized. (B) Scanning electron microscopy was used to image EV collected after UC. The morphology and size observed match those of EV. (C) Immunoblotting of EV markers CD9 in HuCC-T1 cells and EV isolated from their supernatant. The protein GRP78 was used as a cell specific negative control. (D) Relative levels of circLTBP2 in HuCC-T1 cells and EV isolated from their supernatant by UC. HuCC-T1 cells were first treated with TGF β (1 ng/mL TGF β , 16 hours). (E) Half-life of circLTBP2 in HuCC-T1 cells treated with Actinomycin D (2 μ g/mL) for 24 hours. (F) Quantification by q-RT-PCR of EV-embed circLTBP2 in sera from metastatic advanced iCCA patients (Rennes cohort, n=104). Sera from healthy individuals were used as control (Établissement français du sang n=12). Stable expression of circWRNIP1 was observed among patients and served as a reference circRNA for normalization. (G) Positive correlation of circLTBP2 expression in matched tissues and serum samples (patients for which both samples are available, n=17) (H) EV-embed circLTBP2 relative expression in sera from iCCA patients whose survival data were available (separated in two groups: circLTBP2^{low} and circLTBP2^{high}, according to their circLTBP2 median expression). (I) Kaplan-Meier plots and log-rank statistics analysis revealed a significant decreased overall survival (OS) for iCCA patients with a high expression of vesicles-embed circLTBP2. Levels of circLTBP2 were not associated with the relapse-free survival (RFS). Statistical analyses for (A) were performed by a Mann–Whitney test. Statistical analyses for (B), (C) and (D) were performed by a 2-way-Anova test (* P-value < 0.05 ** P-value < 0.01, *** P-value < 0.001, n \geq 3). Abbreviations: pLV-circLTBP2 (circLTBP2 overexpression vector), pLV-circRNA (empty vector).

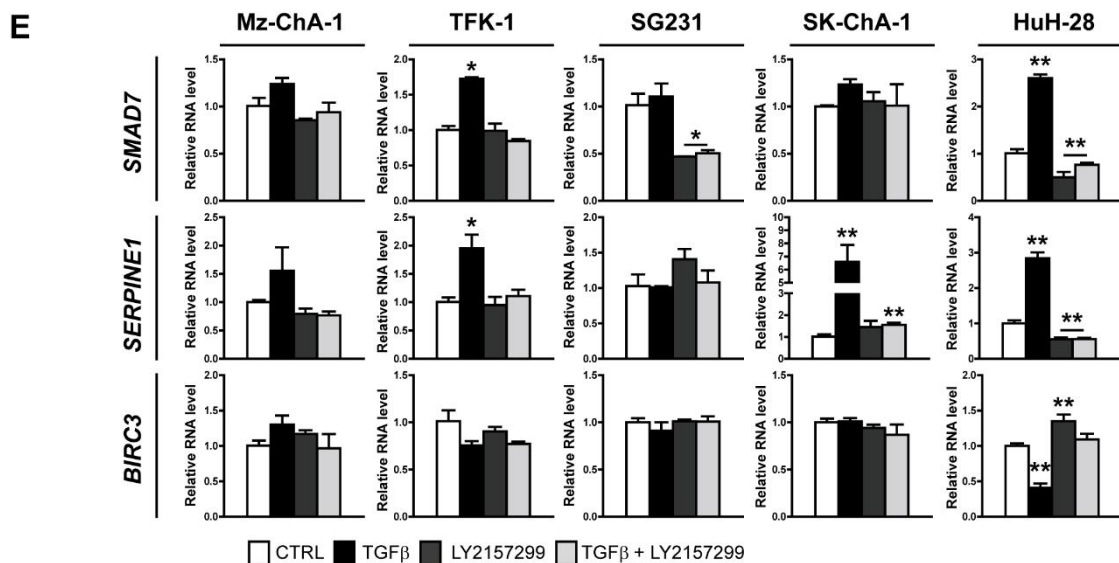
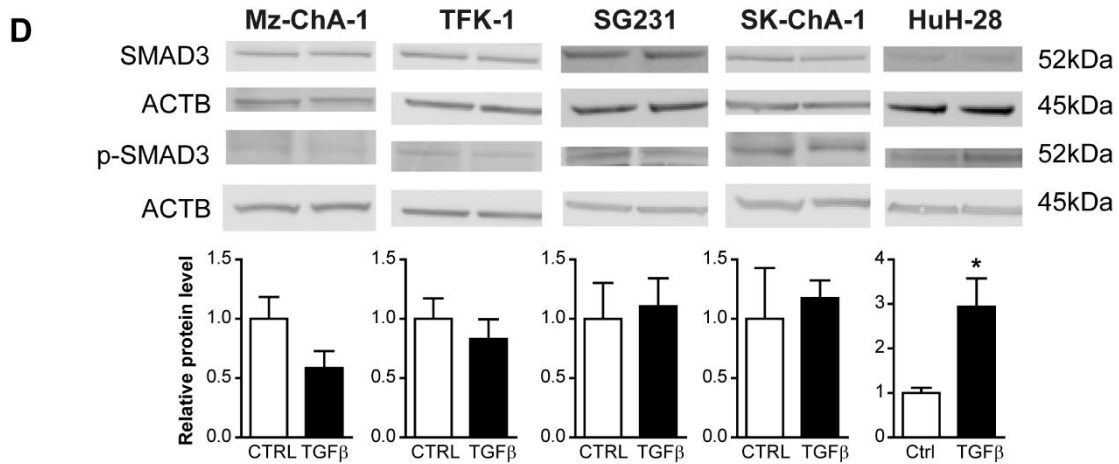
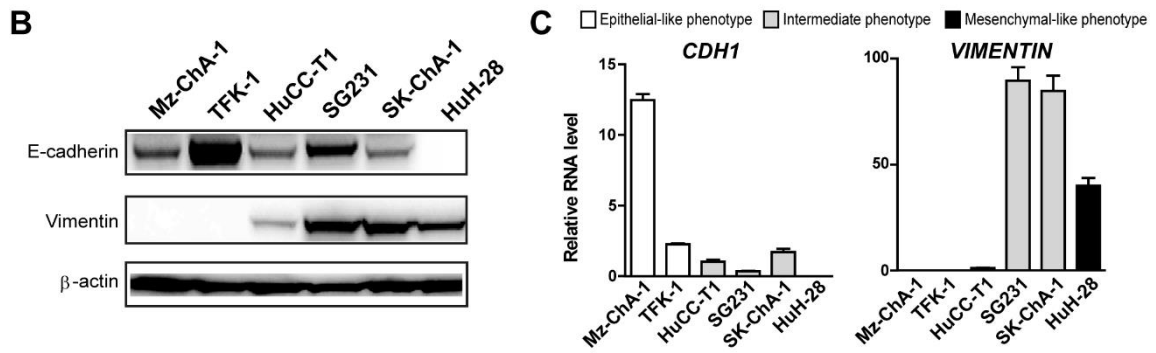
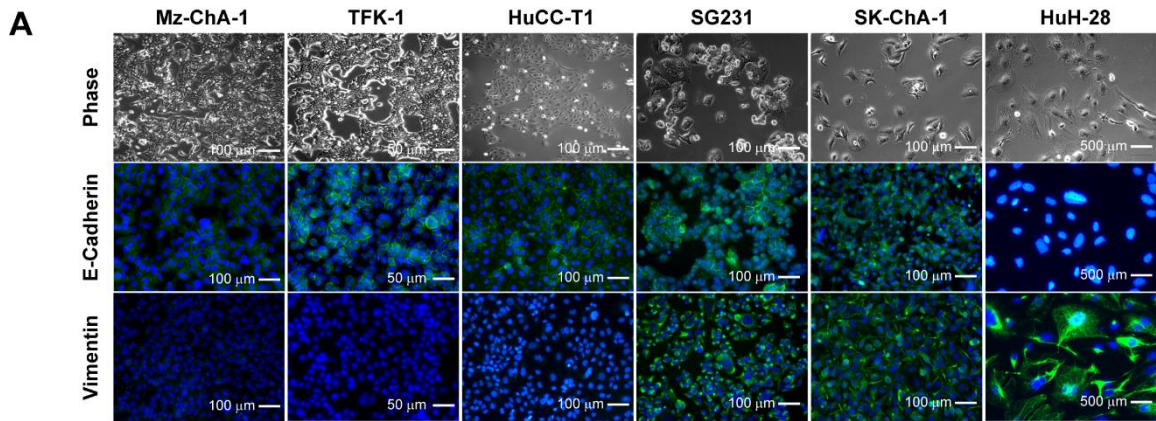


Figure S1

Figure S1. HuCC-T1 is the most TGF β -responsive CCA cell line

(A, B) Protein levels of epithelial markers (*E-Cadherin*) and mesenchymal markers (*Vimentin*) in three iCCA cell lines (*HuCC-T1*, *HuH-28* and *SG231*) and three eCCA cell lines (*Mz-ChA-1*, *TFK-1* and *SK-ChA-1*) assessed by immunofluorescence and western blot. **(C)** *CDH1* and *Vimentin* expression were validated at the RNA level by q-RT-PCR in the 6 CCA cell lines. **(D)** Western blot analysis of p-SMAD3 and SMAD3 expression in CCA cell lines in response to a TGF β treatment (1 ng/mL TGF β , 16 hours) **(E)** Expression of three well-known TGF β target genes (*SERPINE1*, *SMAD7* and *BIRC3*) in CCA cell lines alone or in response to a TGF β , LY2157299 or a combined treatment (1 ng/mL TGF β ; 10 μ M LY2157299; 16 hours).

Figure S2

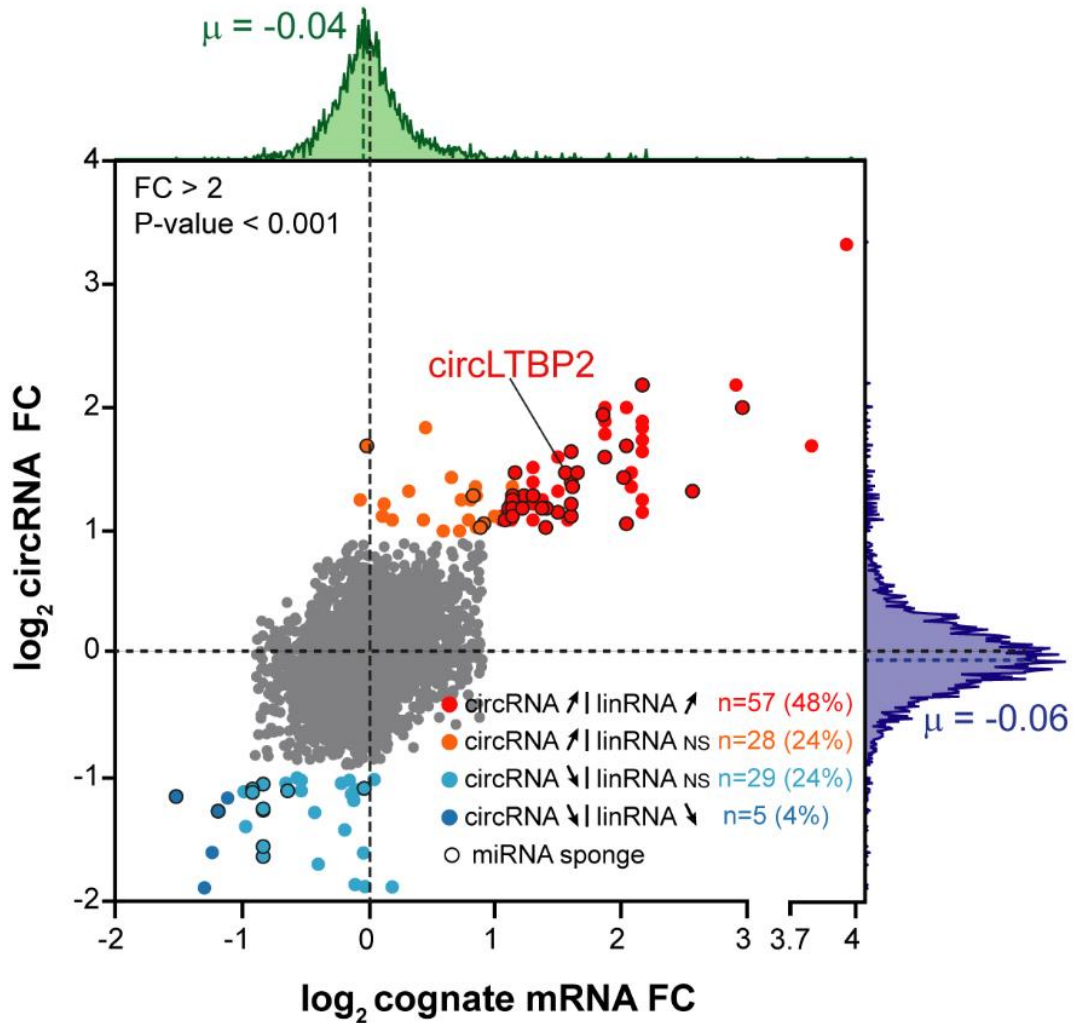


Figure S2. Correlation of circRNA and their cognate mRNA fold expression

Gene expression profiling shows $TGF\beta$ regulation of 119 circRNA, with 85 induced and 34 repressed. In the majority of cases, if a circRNA is induced, its corresponding linear RNA is also induced ($n=57$). However, decreased expression of a circRNA does not always indicate decreased expression of its linear RNA ($n=5$). In approximately half of the cases, circRNA is regulated while the expression of its corresponding linear RNA remains unchanged. CircRNA predicted to sponge miRNA are indicated with a black circle.

Figure S3

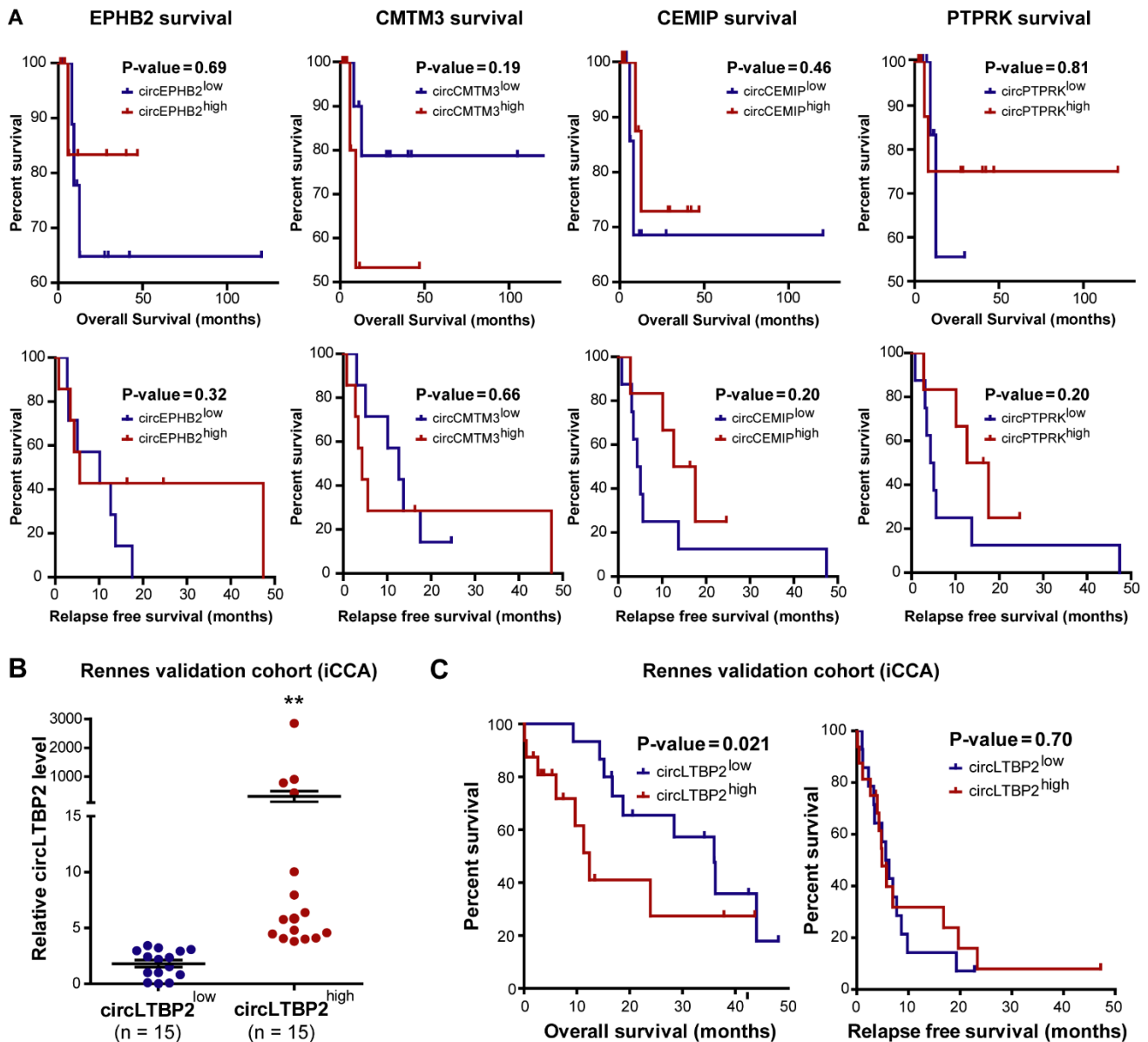


Figure S3. Prognostic value of circRNA regulated by TGF β

(A) TGF β -induced circRNA (*circEPHB2*, *circCMTM3*, *circCEMIP*, *circPTPRK*) relative expression in 20 freshly frozen iCCA tumors from a French national cohort (separated in two groups: *circRNA*^{low} and *circRNA*^{high}, according to their median expression) (B) Validation of *circLTBP2* relative expression in 31 freshly frozen iCCA tumors from a French national cohort (separated in two groups: *circLTBP2*^{low} and *circLTBP2*^{high}, according to their *circLTBP2* median expression) (C) Kaplan-Meier plots and log-rank statistics analysis revealed a significant decreased overall survival (OS) and relapse free survival (RFS) for iCCA patients with a high expression of *circLTBP2*.

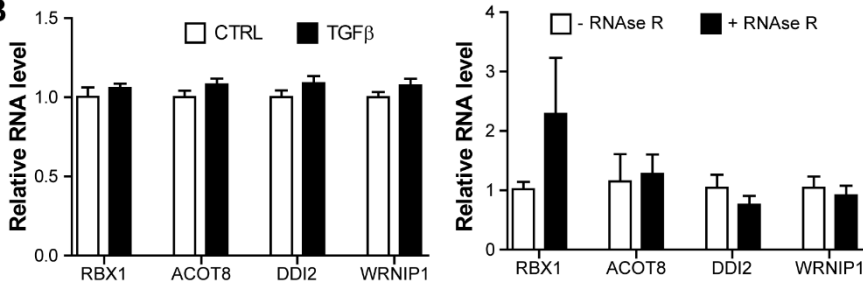
Figure S4

A

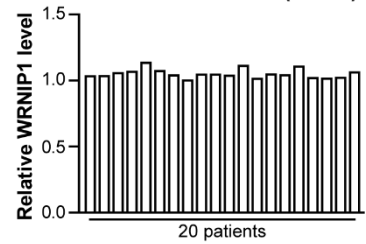
circRNA ID	GENE	HuCC-T1 CTRL	HuCC-T1 TGFβ	VAR	Rank
hsa_circRNA_101654	CHD2	6.28	6.26	0.00036	1
hsa_circRNA_005437	MED29	9.17	9.20	0.00037	2
hsa_circRNA_007302	RLF	7.12	7.17	0.00095	3
hsa_circRNA_007584	TULP3	7.15	7.16	0.00108	4
hsa_circRNA_102145	APPBP2	8.97	8.92	0.00110	5
...
hsa_circRNA_104046	WRNIP1	5.82	5.68	0.01068	510
...

ID	GENE	ISOFORMs
hsa_circRNA_103235	RBX1	1
hsa_circRNA_103071	ACOT8	3
hsa_circRNA_010048	DDI2	3
hsa_circRNA_104046	WRNIP1	3

B



C Rennes cohort (iCCA)



D

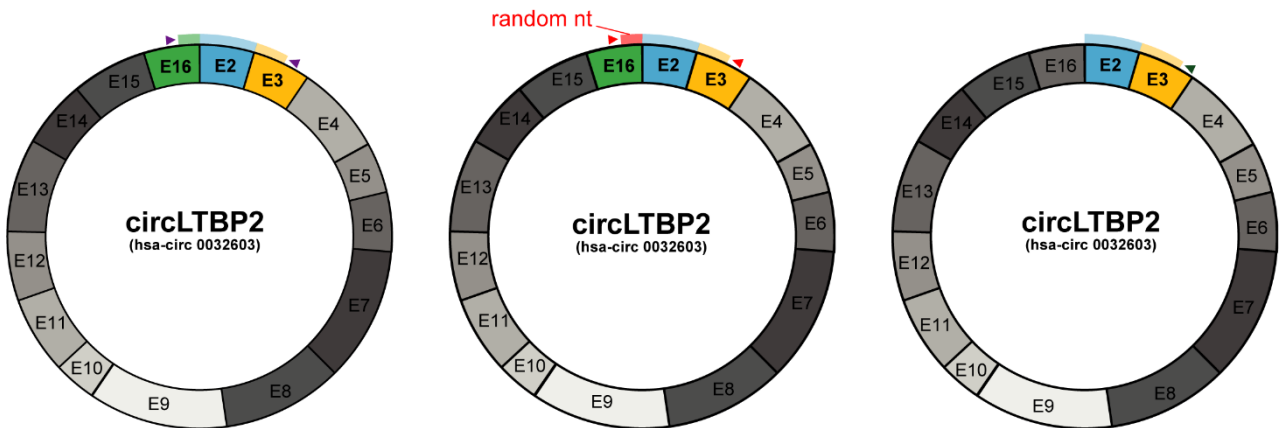
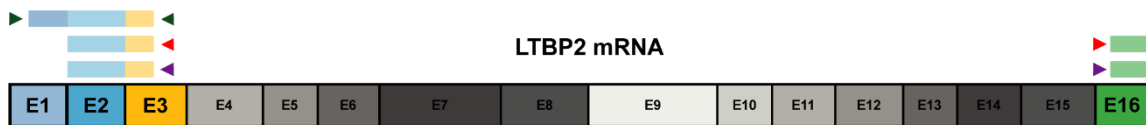
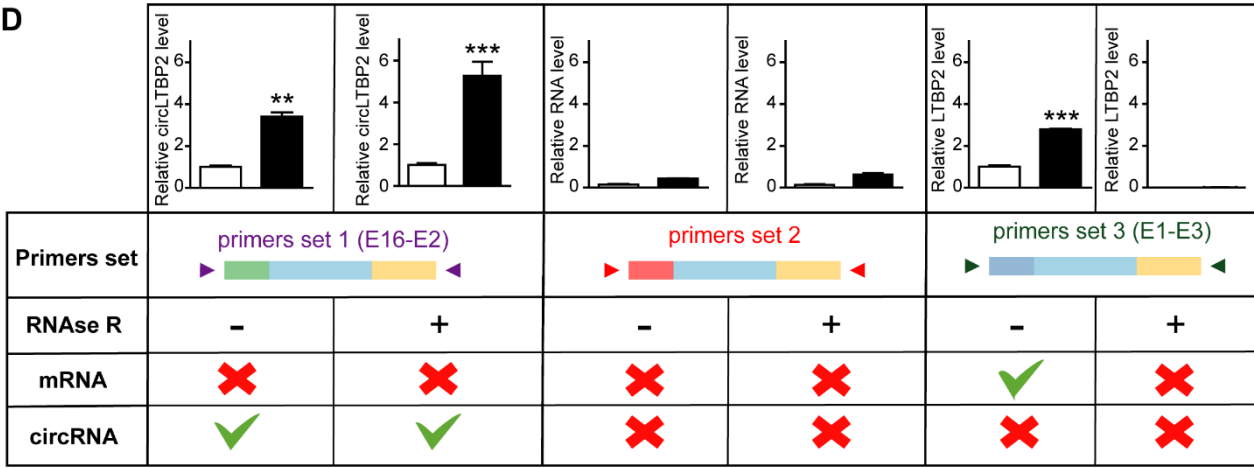


Figure S4. Strategy for circLTBP2 quantification

(A, B) Selection of circWRNIP1 as a housekeeping circRNA based on its stability after treatment with TGF β or RNase R. (C) CircWRNIP1 was also stably expressed in the 20 iCCA patients from cohort 1 (D) A specific strategy was used to measure the amount of circRNA, by designing three sets of primers: the first set targeted the overlapping junction between exon 16 and exon 12, the second set targeted the same junction but with additional random nucleotides as a negative control, and the third set targeted exon 1 which is not present in known circLTBP2 isoforms.

Figure S5

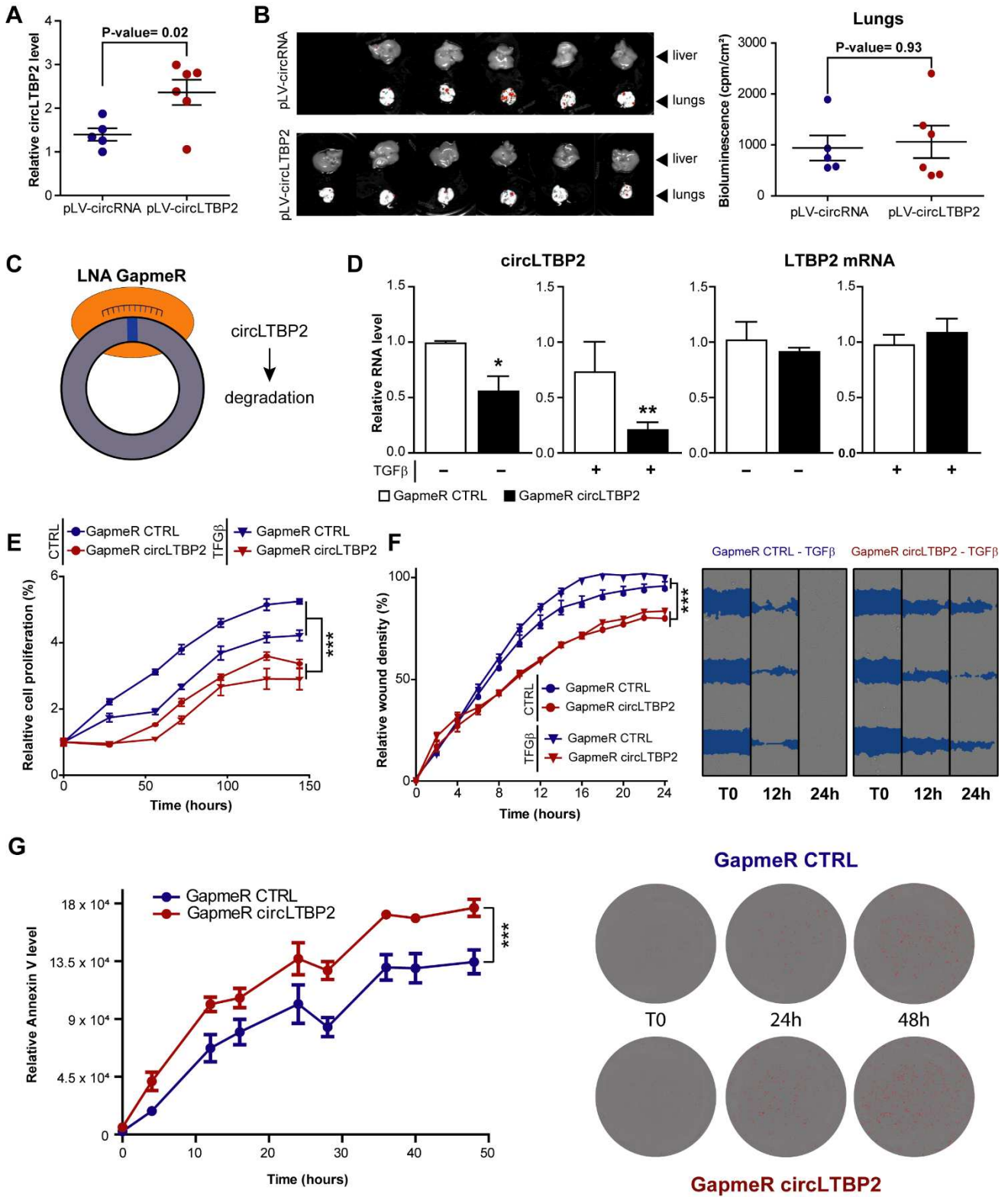


Figure S5. Gain and loss of function assays revealed circLTBP2 pro-oncogenic actions

(A) Bioluminescence measurements in the liver and lungs of mice that were subcutaneously injected with HuCC-T1 cells that had been genetically engineered to produce higher levels of circLTBP2 (pLV-circLTBP2) or infected with an empty vector (pLV-circRNA), showed no significant differences between the two groups after 90 days. (B) Relative expression levels of circLTBP2 evaluated by q-RT-PCR in tumors removed from mice in the pLV-circRNA and pLV-circLTBP2 groups. (C) Schematic representation of LNA gapmeRs targeting the circularization junction of circLTBP2 to degrade it. (D) Relative expression of circLTBP2 and its cognate mRNA in HuCC-T1 cells after being treated with LNA gapmeRs targeting circLTBP2 (50nM). (E) Relative confluency of cultured HuCC-T1 cells was evaluated for 150 hours (n=3 per group). The reduction of circLTBP2 expression in HuCC-T1 cells led to a decrease in cell proliferation, both in the presence and absence of TGF β treatment. (F) Cell migration was evaluated by wound healing assay performed on HuCC-T1 cells treated with circLTBP2 gapmeRs (50nM, n=3 per group). Relative wound density was assessed every 2 hours as a percentage of the initial wound surface, represented in blue on the right panel. HuCC-T1 cells were cultured in low FBS concentration medium (1% FBS) and with Actinomycin D (10 $\mu\text{g}\cdot\text{mL}^{-1}$) to inhibit cell proliferation. (G) HuCC-T1 cells resistance to gemcitabine-induced apoptosis (10 ng/mL) was evaluated every 4 hours using a red fluorescent Annexin V labelling. HuCC-T1 cells treated with gapmeRs targeting circLTBP2 showed a better sensibility to gemcitabine (50nM, n=3 per group).

Figure S6

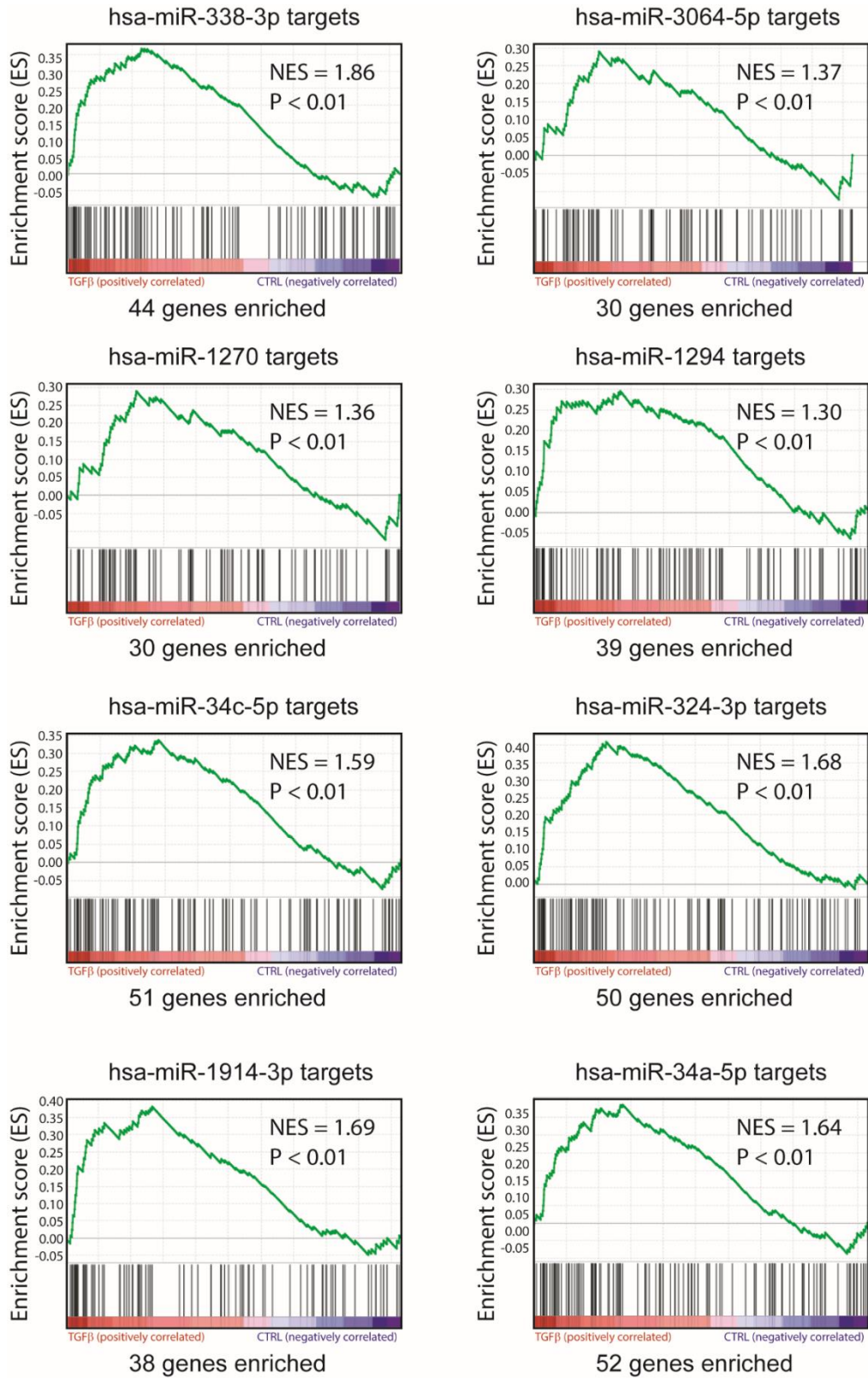


Figure S6. GSEA enrichment score of the targets of miRNA pulled down by circLTBP2 in HuCC-T1 TGF β signature

*The second criterion was evaluated using the Starbase v2.0 software, which predicts the linear targets of miRNA (Dudekula et al., 2016; Li et al., 2014). We then conducted a GSEA analysis to evaluate the enrichment of each miRNA targets in the HuCC-T1 TGF β signature that we previously described (GSE102109). The targets of miR-338-3p that displayed the most significant enrichment score in the HuCC-T1 TGF β signature are listed in the right part of **Figure 4C**.*

Table S1

(A) List of primers used in the study

circRNA name	Forward	Reverse
hsa-circ-0032603 (circLTBP2)	TACTGCACAGGAGGAACGTCTG	GGGGTCAAATTCCTCATCGGG
hsa-circ-0002720 (circEPHB2)	AACCAGGCAGGTTGTCCATC	TAGTAGCCATTGCGGCAGAC
hsa-circ-0008450 (circCMTM3)	CCACAAGACAGAAGGGTCTCT	AAGTACAAGGCCAGCAGGAAC
hsa-circ-0003893 (circCEMIP)	GGGACGAGGACTCAGGATGTA	CCAGGTAAAGAGGGTGGCTG
hsa-circ-0007138 (circPTPRK)	AGTTGCATTTCTTTAAACAGGTTCC	CCCGAAGCCAATCTCTACCC
hsa-circ-0075456 (circWRNIP1)	ATTACAACCTGCATCTCCGCC	TCCTCTATGAACATGGCGGG
hsa-circ-0004469 (circRBX1)	AAACACGACAGGTGTGTCCA	ACGCCTGGTTAGCTTGACAT
hsa-circ-0008993 (circACOT8)	TGTGAGACCCTCATTGACCA	AGATGGGCTTCCCATGTTG
hsa-circ-0010048 (circDDI2)	TCCGAAAGTTTTGGCCAAG	TGTGGTTGTCTGTGAGAGGT

Gene name	Forward	Reverse
LTBP2	TCACCCTGGCTCTCTTCGT	GCTGTTTGCTGTTGTCCATCC
SERPINE1	AAGCCTAATCAGCCACCAT	TAGCATTGACACCACCCCT
SMAD7	CCAACTGCAGACTGTCCAGA	GGGCTCCTGGACACAGTAGA
BIRC3	TTCCCGTGGCTCTTATTCAAACCT	GCACAGTGGTAGGAACCTTCTCAT
HNF1 α	CTCCAGCAGCCTGGTGCTGT	GAGGCCATCTGGGTGGAGAT
ADAM12	TCTCATTGCCAGCAGTTTCAC	CGTGTAAATTCGAGCGAGGG
KCJN6	CACATCAGCCGAGATCGGAC	GGTAGCGATAGGTCTCCCTCA
ABAT	TGGGTTGCTTAGCGACCAC	TCCAGAGGGTATTTAGCCCGT
HEWC2	AAATCCCAGATGCGGTACAC	CGGCTCTCAGAAGTCACCA
PI3KC2G	TTCAGCTAGTGGAAGTACCTCAA	ACGGAAATGCTGTAGTAGTGCT
MYO10	GAACCCCTACCAGCCATC	GTTTTACCTGCCCACTTTCA
HHAT	CGGGATGTGGAGGTATTTTGATG	CACCAGAGGTAGTCGTAGCC
ANHAK2	GTGCAGAAACGGAAGATGACC	GCCTCAGTCGTGTATTGCTAGA
NET1	GAGCCAAGCAATAAAAGAGTTCCG	TGGGACTGTTGACCTGCTAGA
FURIN	TCGGGGACTATTACCACTTCTG	CCAGCCACTGTACTTGAGGC
MN1	GACTCGCTGGAATACAATTACCC	ACCCGCTGCATAATGAGGC
CXCR4	GGAAAAGATGGGGAGGAGAG	CACTTCCAATTCAGCAAGCA
SOX4	CCAATCTTTTGGGGACTTT	CTGGCCCCTCAACTCCTC
SKIL	CAGATGCACCATCAGGAATG	TTGCCTCTGTCTTTGTGAGC
COL1A1	GGCCTCGGAGGAAACTTTG	TTTCTCATCATAGCCATAAGACAGCT
ZEB1	CCCACACGACCACAGATACG	TGAGGAGAACTGGTTGCCTG
ZEB2	CCCAACTCCGATGAACTGCT	GGAGGGTTACTGTTGGGAGC

(B) List of antibodies used in the study

Antibody	Host	Company
Actin (D68A)	rabbit	Cell Signaling
Vimentine (D21H3)	rabbit	Cell Signaling
E-cadherin (24E10)	rabbit	Cell Signaling
SMAD3 (9513)	rabbit	Cell Signaling
p-SMAD3 (C25A9)	rabbit	Cell Signaling

Supplementary Table S1. List of primers (A) and antibodies (B) used in this study

Table S2

UniqueID	Gene symbol	P-value	FC	CTRL (mean)	TGFβ (mean)
hsa_circRNA_103995	ADAM19	< 1e-07	10,00	85,44	820,17
hsa_circRNA_104495	PODXL	2,00E-07	4,55	804,9	3579,04
hsa_circRNA_052502	MBOAT2	1,50E-06	4,55	385,81	1752,18
hsa_circRNA_047235	LAMA3	< 1e-07	4,00	695,53	2745,68
hsa_circRNA_043637	KRT17	3,00E-07	4,00	60,34	239,76
hsa_circRNA_103855	ADAMTS6	1,01E-05	4,00	36,93	146,8
hsa_circRNA_038312	TMC7	1,00E-07	3,85	152,65	594,9
hsa_circRNA_052507	MBOAT2	4,70E-06	3,70	789,06	2939,81
hsa_circRNA_102327	LAMA3	2,00E-07	3,70	2868,39	10618,39
hsa_circRNA_102609	MBOAT2	2,00E-07	3,57	334,46	1203,48
hsa_circRNA_007719	CREB3L1	7,00E-07	3,57	30,65	110,92
hsa_circRNA_102326	LAMA3	3,00E-07	3,45	406,15	1408,2
hsa_circRNA_102608	MBOAT2	1,00E-07	3,33	145,63	490,8
hsa_circRNA_026129	TUBA1A	2,00 E-07	3,23	137,58	450,38
hsa_circRNA_103856	ADAMTS6	1,30E-05	3,23	88,96	291,19
hsa_circRNA_404879	PRR5L	9,40 E-06	3,23	76,31	249,05
hsa_circRNA_104260	SLC22A3	7,00 E-07	3,13	103,14	318,16
hsa_circRNA_102611	MBOAT2	1,00 E-06	3,13	180,26	561,11
hsa_circRNA_104185	PTPRK	1,40E-06	3,03	865,28	2625,15
hsa_circRNA_035619	TPM1	6,40E-06	3,03	179,81	542,15
hsa_circRNA_102168	SMURF2	6,10E-06	2,86	1309,76	3701,13
hsa_circRNA_100096	EPHB2	8,00E-07	2,78	1035,77	2863,77
hsa_circRNA_032603	LTBP2	2,20 E-06	2,78	49,53	137,81
hsa_circRNA_091578	GPC4	2,60 E-06	2,78	92,58	255,89
hsa_circRNA_402457	KLF7	8,50 E-06	2,78	304,88	849,65
hsa_circRNA_103854	ADAMTS6	2,30 E-06	2,70	40,78	109,08
hsa_circRNA_033571	AHNAK2	2,50 E-06	2,70	52,49	142,68
hsa_circRNA_004395	CD44	3,20 E-06	2,70	175,36	475,99
hsa_circRNA_104259	SLC22A3	8,00 E-07	2,63	105,21	280,56
hsa_circRNA_102170	SMURF2	5,70E-06	2,63	849,56	2210,32
hsa_circRNA_052973	TP53I3	6,50E-06	2,56	82,86	212,91
hsa_circRNA_405167	DNAJC3	1,54E-05	2,56	281,86	719,88
hsa_circRNA_406302	FOXP1	9,60E-06	2,56	44,5	112,7
hsa_circRNA_067938	SKIL	7,90E-06	2,56	111,3	286,07
hsa_circRNA_004520	VAV2	7,10E-06	2,50	91,61	231,77
hsa_circRNA_102838	ITGB6	2,50E-06	2,50	36,93	91,67
hsa_circRNA_104186	PTPRK	1,00E-06	2,50	1279,1	3176,2
hsa_circRNA_101622	CEMIP	0,0001499	2,44	106,96	259,57
hsa_circRNA_074595	ANXA6	4,20E-06	2,44	238,48	578,36
hsa_circRNA_062722	XBP1	3,10E-06	2,44	376,07	915,81

hsa_circRNA_103310	RBMS3	0,0001203	2,44	62,82	154,44
hsa_circRNA_102172	SMURF2	6,10E-06	2,44	452,76	1110,91
hsa_circRNA_071981	MYO10	2,80E-06	2,44	527,27	1276,52
hsa_circRNA_102469	TPM4	4,87E-05	2,38	559,67	1330,28
hsa_circRNA_405028	TRHDE	4,90E-06	2,38	80,59	191,85
hsa_circRNA_003665	TMCC1	9,61E-05	2,38	122,02	291,71
hsa_circRNA_102610	MBOAT2	1,80E-06	2,38	102,86	244,6
hsa_circRNA_103803	MYO10	1,90E-06	2,38	956,11	2281,88
hsa_circRNA_405495	CFDP1	0,0002704	2,38	36,2	85,86
hsa_circRNA_103745	INPP4B	1,24E-05	2,33	596,03	1380,7
hsa_circRNA_070216	ANTXR2	5,60E-06	2,33	118,94	273,44
hsa_circRNA_004875	CTBP2	0,0003841	2,33	45,55	106,73
hsa_circRNA_104258	SLC22A3	6,10E-06	2,33	85,31	198,44
hsa_circRNA_102169	SMURF2	5,50E-06	2,33	156,62	367,63
hsa_circRNA_008450	CMTM3	5,55E-05	2,27	268,34	616,77
hsa_circRNA_100963	PHLDB1	3,70E-06	2,27	38,94	88,71
hsa_circRNA_000558	FOXN3	0,0007204	2,27	158,19	356,33
hsa_circRNA_103804	MYO10	5,45E-05	2,27	180,25	413,2
hsa_circRNA_080887	SEMA3C	3,80E-06	2,27	412,7	929,18
hsa_circRNA_103743	INPP4B	3,11E-05	2,22	722,69	1602,97
hsa_circRNA_000689	MBOAT2	3,60E-06	2,22	54,41	121,36
hsa_circRNA_103801	MYO10	1,85E-05	2,22	1872,37	4134,79
hsa_circRNA_077816	PTPRK	1,34E-05	2,22	434,67	969,35
hsa_circRNA_007138	PTPRK	2,70E-06	2,22	380,62	840,58
hsa_circRNA_406591	MYO10	1,24E-05	2,17	393,48	852,11
hsa_circRNA_102879	SLC39A10	0,0002893	2,17	332,48	721,53
hsa_circRNA_078608	SLC22A3	2,12E-05	2,17	52,87	114,99
hsa_circRNA_103985	NDST1	3,24E-05	2,17	634,64	1380,5
hsa_circRNA_102895	BMPR2	9,70E-06	2,17	2199,52	4778,33
hsa_circRNA_401691	ZZEF1	0,0003325	2,17	135,01	294,17
hsa_circRNA_404693	SDCCAG8	4,00E-06	2,13	107,71	229,52
hsa_circRNA_007434	SMURF2	2,35E-05	2,13	210,88	453,35
hsa_circRNA_104372	GRB10	0,0006483	2,13	174,85	368,52
hsa_circRNA_403950	KDM7A	0,000336	2,13	52,31	110,79
hsa_circRNA_406709	FAM196B	0,0006012	2,13	113,74	243,91
hsa_circRNA_005078	BMPR2	0,0001993	2,13	1632,97	3461,73
hsa_circRNA_104066	ATXN1	0,0008042	2,13	163,53	348,68
hsa_circRNA_102552	LTBP4	6,70 E-06	2,13	103,7	222,44
hsa_circRNA_103858	ADAMTS6	0,0008477	2,08	264,63	545,94
hsa_circRNA_102797	LIMS1	1,92 E-05	2,08	239,54	501,46
hsa_circRNA_104555	DLC1	8,04 E-05	2,04	154,95	318,45
hsa_circRNA_103309	RBMS3	0,0001093	2,04	132,42	268,41
hsa_circRNA_011413	MARCKSL1	1,06E-05	2,04	56,96	117,38
hsa_circRNA_000646	HDGFRP3	0,0001038	2,00	1924,76	3866,05
hsa_circRNA_404449	PINK1	3,30E-05	2,00	150,67	303,72

hsa_circRNA_075501	SLC22A23	0,0001627	0,50	84,26	42,11
hsa_circRNA_406988	FBXL13	2,56E-05	0,50	137,86	68,44
hsa_circRNA_404819	FAM53B	0,0001073	0,50	75,95	37,53
hsa_circRNA_101627	PDE8A	0,0001027	0,49	123,1	60,67
hsa_circRNA_102312	FAM210A	2,43 E-05	0,49	154,29	76,12
hsa_circRNA_102660	SLC30A6	0,000419	0,49	710,31	344,85
hsa_circRNA_401445	DIS3L	0,000138	0,49	130,75	63,38
hsa_circRNA_104197	MAP3K5	2,66 E-05	0,48	176,97	85,49
hsa_circRNA_102051	TADA2A	0,0001173	0,47	6487,45	3062,27
hsa_circRNA_087964	LPAR1	9,45 E-05	0,47	355,1	166,39
hsa_circRNA_055894	CNOT11	6,30 E-06	0,47	81,15	37,84
hsa_circRNA_101626	PDE8A	7,44 E-05	0,47	140,61	65,51
hsa_circRNA_075320	SQSTM1	3,00 E-05	0,47	504,95	234,6
hsa_circRNA_404894	FOSL1	1,09 E-05	0,46	1927,02	892,15
hsa_circRNA_087962	LPAR1	1,00 E-05	0,46	4604,79	2126,53
hsa_circRNA_089866	WWC3	6,50 E-06	0,46	135,93	62,06
hsa_circRNA_002375	KITLG	8,40 E-06	0,45	127,47	57,3
hsa_circRNA_104126	GCLC	2,70 E-06	0,45	425,26	189,42
hsa_circRNA_401969	HDHD2	0,0004894	0,44	281,41	124,17
hsa_circRNA_104199	MAP3K5	1,21E-05	0,42	241,21	101,5
hsa_circRNA_104196	MAP3K5	4,50E-06	0,42	120,2	50,06
hsa_circRNA_104562	MTUS1	1,22E-05	0,41	343,51	142,6
hsa_circRNA_007407	CSRP2BP	8,00E-06	0,41	260,86	107,16
hsa_circRNA_403725	UST	9,00E-07	0,38	86,95	33,12
hsa_circRNA_102216	ENGASE	0,000119	0,37	261,91	97,76
hsa_circRNA_104200	MAP3K5	2,50E-06	0,34	435,78	148,09
hsa_circRNA_061482	EVA1C	5,03E-05	0,33	91,25	30,06
hsa_circRNA_001729	ZNF646	4,70E-06	0,33	13020,09	4270,41
hsa_circRNA_104198	MAP3K5	0,0001253	0,32	362,82	116,68
hsa_circRNA_000367	SIAE	2,00 E-07	0,31	17963,83	5522,24
hsa_circRNA_104270	FAM120B	2,00 E-07	0,27	10467,61	2872,07
hsa_circRNA_004239	C17orf53	1,77E-05	0,27	9118,66	2478,65
hsa_circRNA_404686	GPR137B	2,20E-06	0,27	2437,72	659,99
hsa_circRNA_008026	CAMK2D	3,40E-06	0,27	148,25	39,99

Supplementary Table S2. List of circRNAs differentially expressed in HuCC-T1 cells after 16 hours of TGF β treatment

Table S3

miRNA	CTRL-input	circLTBP2-IP	FC
hsa-miR-7974	0,59	4,80	8,12
hsa-miR-4677-5p	0,59	4,20	7,10
hsa-miR-193b-3p	6,80	45,92	6,75
hsa-miR-34c-5p	66,27	421,40	6,36
hsa-miR-3198	2,96	17,71	5,99
hsa-miR-551b-5p	1,78	10,51	5,92
hsa-miR-4741	1,78	9,60	5,41
hsa-miR-4783-3p	2,07	10,81	5,22
hsa-miR-324-5p	303,55	1525,33	5,03
hsa-miR-873-5p	4,73	19,21	4,06
hsa-miR-449c-5p	21,89	76,54	3,50
hsa-miR-34a-5p	2386,96	7440,28	3,12
hsa-miR-3180	8,58	26,71	3,11
hsa-miR-3180-3p	8,58	26,71	3,11
hsa-miR-146b-3p	1,78	5,40	3,04
hsa-miR-196b-3p	0,89	2,70	3,04
hsa-miR-6720-5p	10,65	31,22	2,93
hsa-miR-1301-3p	454,73	1275,01	2,80
hsa-miR-1229-3p	7,40	20,71	2,80
hsa-miR-615-5p	10,95	30,61	2,80
hsa-miR-3174	1,78	4,80	2,71
hsa-miR-6789-5p	0,89	2,40	2,71
hsa-miR-1914-3p	3,55	9,30	2,62
hsa-miR-6882-3p	2,07	5,40	2,61
hsa-miR-1468-5p	5,03	12,91	2,57
hsa-miR-210-3p	59,76	150,67	2,52
hsa-miR-1249-3p	48,82	123,06	2,52
hsa-miR-5583-3p	3,55	8,70	2,45
hsa-miR-185-5p	400,59	972,77	2,43
hsa-miR-194-3p	3,55	8,40	2,37
hsa-miR-769-3p	118,64	276,73	2,33
hsa-miR-1295a	5,03	11,41	2,27
hsa-miR-1292-5p	17,75	39,92	2,25
hsa-miR-6797-5p	1,48	3,30	2,23
hsa-miR-1911-5p	1,48	3,30	2,23
hsa-miR-561-3p	1,48	3,30	2,23
hsa-miR-4467	89,35	193,59	2,17
hsa-miR-671-3p	123,37	267,13	2,17
hsa-miR-6777-5p	2,66	5,40	2,03

hsa-miR-3605-3p	9,76	19,81	2,03
hsa-miR-6514-5p	1,18	2,40	2,03
hsa-miR-1287-5p	19,23	38,72	2,01
hsa-miR-338-3p	38,76	77,74	2,01
hsa-miR-4326	13,02	25,81	1,98
hsa-miR-197-5p	13,31	26,11	1,96
hsa-miR-3176	18,05	34,82	1,93
hsa-miR-1275	22,49	42,92	1,91
hsa-miR-1293	11,54	21,91	1,90
hsa-miR-3074-5p	8,88	16,81	1,89
hsa-miR-30c-1-3p	7,40	13,81	1,87
hsa-miR-137	1,78	3,30	1,86
hsa-miR-6793-5p	0,00	2,40	1,82
hsa-miR-3064-5p	2,66	4,80	1,80
hsa-miR-193a-5p	85,50	151,27	1,77
hsa-miR-1299	13,31	23,11	1,74
hsa-miR-18a-3p	378,70	654,91	1,73
hsa-miR-628-3p	63,02	108,65	1,72
hsa-miR-3651	0,00	2,40	1,70
hsa-miR-6857-5p	0,00	1,20	1,69
hsa-miR-1914-5p	5,92	9,90	1,67
hsa-miR-2110	89,94	149,77	1,67
hsa-miR-1322	0,00	2,10	1,66
hsa-miR-1237-3p	7,69	12,61	1,64
hsa-miR-615-3p	449,70	730,85	1,63
hsa-miR-324-3p	122,19	198,40	1,62
hsa-miR-7108-5p	0,00	1,80	1,62
hsa-miR-6511b-5p	2,37	3,75	1,59
hsa-miR-4461	1,18	1,80	1,52
hsa-miR-1285-5p	1,18	1,80	1,52
hsa-miR-193b-5p	77,22	116,46	1,51
hsa-miR-3943	0,00	2,70	1,50
hsa-miR-6783-5p	0,00	1,50	1,50
hsa-miR-6847-5p	0,00	3,30	1,50
hsa-miR-3130-5p	0,00	1,50	1,50
hsa-miR-6817-3p	0,00	1,50	1,50
hsa-miR-1270	0,00	1,20	1,50
hsa-miR-152-5p	0,00	2,10	1,50
hsa-miR-4660	0,00	2,40	1,50
hsa-miR-6851-5p	0,00	7,20	1,50
hsa-miR-4689	0,00	3,30	1,50
hsa-miR-627-3p	0,00	0,30	1,50
hsa-miR-3691-5p	0,00	0,30	1,50
hsa-miR-5001-5p	0,00	1,50	1,50
hsa-miR-6735-3p	0,00	1,50	1,50

hsa-miR-4656	0,00	1,50	1,50
hsa-miR-1294	0,00	3,00	1,50
hsa-miR-219a-1-3p	0,00	5,10	1,50
hsa-miR-6770-3p	0,00	2,10	1,50
hsa-miR-149-3p	0,00	1,50	1,50
hsa-miR-4525	0,00	6,00	1,50
hsa-miR-3622a-5p	0,00	2,70	1,50
hsa-miR-3188	0,00	4,80	1,50
hsa-miR-181d-3p	0,00	3,30	1,50
hsa-miR-4747-5p	0,00	18,31	1,50

Supplementary Table S3. List of miRNA interacting with circLTBP2 identified by pulldown assay

Table S4

Gene symbol	Rank in gene list	Rank metric score	Running ES	Core enrichment
CHST3	5	5	0,014	Yes
MYO10	7	5	0,0301	Yes
TPM1	20	5	0,0406	Yes
COL6A1	31	5	0,0521	Yes
FRMD6	38	5	0,0657	Yes
FURIN	40	5	0,0817	Yes
ULK1	41	5	0,0983	Yes
CREB3L2	44	5	0,1139	Yes
JUNB	46	5	0,13	Yes
TTPAL	51	5	0,1446	Yes
LUZP1	54	5	0,1602	Yes
SOX4	56	5	0,1763	Yes
CDKN1A	68	5	0,1872	Yes
SIK1	71	5	0,2028	Yes
PDIA4	82	5	0,2143	Yes
TRIB1	109	4,712	0,2167	Yes
ARFGAP1	112	4,675	0,2312	Yes
RHOB	122	4,589	0,2418	Yes
SLC39A10	127	4,504	0,2547	Yes
STK38L	136	4,322	0,265	Yes
SESN2	145	4,164	0,2748	Yes
SEC24D	167	4,025	0,2774	Yes
EDEM1	179	3,975	0,285	Yes
CLSTN1	206	3,796	0,2843	Yes
TBC1D2B	231	3,651	0,2842	Yes
ACSL4	235	3,623	0,2947	Yes
HERPUD1	243	3,548	0,3029	Yes
SLC39A13	257	3,47	0,3078	Yes
ANKRD11	287	3,305	0,3039	Yes
GFOD1	293	3,273	0,3122	Yes
FOXP1	294	3,268	0,3231	Yes
MANF	331	3,065	0,3149	Yes
CDC42EP2	332	3,056	0,325	Yes
ETS1	361	2,974	0,3206	Yes
NCOR2	364	2,951	0,3294	Yes
B4GALT1	367	2,934	0,3381	Yes
MSN	376	2,894	0,3436	Yes
ITGB1	400	2,788	0,3411	Yes
ZBTB10	419	2,699	0,3409	Yes

HYOU1	428	2,65	0,3456	Yes
AKAP12	438	2,593	0,3496	Yes
DBN1	450	2,568	0,3525	Yes
FLRT2	455	2,554	0,359	Yes
CDC42SE1	459	2,54	0,3659	Yes

Supplementary Table S4. GSEA analysis showing hsa-miR-338-3p target genes significantly enriched in HuCC-T1 TGF β signature

Supplementary Methods

circLTBP2 quantification

To understand the function of circLTBP2 in iCCA, we developed a method for accurately measuring its expression in CCA samples. We identified stable housekeeping circRNA that were consistently present in all CCA samples and not affected by TGF β or RNase R treatment. We selected the circRNA with minimal isoforms (circRBX1, circACOT8, circDDI2 and circWRNIP1) (**Figure S4 A**). Q-RT-PCR analyses revealed that circWRNIP1 was the most stable among the identified housekeeping circRNAs and was therefore chosen as the reference for the rest of the study (**Figure S4 B**). CircWRNIP1 was also used as housekeeping circRNA to quantify circLTBP2 in tissues and sera from iCCA patients (**Figure 1F-H, Figure 6F-H, Figure S3B**).

A specific method for quantifying the expression of circLTBP2 was developed by designing a set of primers that target the unique sequence of the circRNA. The first set of primers (divergent primers) was designed to overlap the circularization junction (exon 16 - exon 2) and specifically amplify the circLTBP2 isoform (hsa-circ-0032603). The second set of primers, which contained random nucleotides instead of those specific to exon 16, served as a negative control. Finally, a third set of primers (convergent primers) was designed to target exon 1 of the LTBP2 gene, in order to specifically amplify the linear form, as none of the circLTBP2 isoforms contain exon 1 (**Figure S4C**). The accuracy of our method for distinguishing circLTBP2 from its linear counterpart was evaluated in HuCC-T1 cells using q-RT-PCR, by applying each set of primers after RNase treatment with and without TGF β . (**Figure 2C**.) The specific amplification of hsa-circ-0032603 was further confirmed by using the first set of primers which produced a single fragment of 203 nucleotides as verified by Sanger sequencing and gel electrophoresis (**Figure 2A-B**).

Discussion

These results are discussed more thoroughly in French in the next chapter.

In this study we identified by microarray the first signature of circRNA regulated by TGF β in iCCA, focusing on the role and underlying mechanisms of the induced circLTBP2 in iCCA progression. We demonstrated that an increased expression of circLTBP2 was involved in iCCA cell proliferation and migration, as well as gemcitabine-induced apoptosis resistance. Mechanistically, circLTBP2 exerts pro-oncogenic actions through the sponging of miR-338-3p, a well-known tumor suppressor. We also found that circLTBP2 expression in tissues and sera was associated with a worse prognosis for iCCA patients.

LTBP2, which is also termed Latent-TGF β binding protein 2, is a protein that is part of a complex involved in the availability of TGF β on the cell surface. The regulation of LTBP2 expression is crucial as it modulates the activity of the TGF β pathway (Robertson *et al.*, 2015). Many studies have reported increased expression of LTBP2 in several types of cancers, including iCCA, and that its overexpression contributes to tumor progression by potentiating the response to TGF β (Abudayyeh *et al.*, 2017). A study has shown the prognostic value of LTBP2 whose expression is associated with a poor OS in patients with HCC (da Costa *et al.*, 2015).

Here we showed that the induction of its circular counterpart also participates to iCCA progression by protecting pro-oncogenic actors from miR-338-3p degradation. This miRNA plays a crucial role in tumor progression and its activity is finely regulated. Many pathways depend on its activity, including WNT, MAPK, and PI3K/AKT, all of which are involved in the progression of iCCA. Thus, miR-338-3p plays a role in many pathological processes by inhibiting cell proliferation signals, inducing cell death, or decreasing angiogenesis (Moghbeli, 2021). MiR-338-3p has also a strong prognostic value for biliary tract cancers. Low levels of miR-338-3p have been found in several advanced stage (TNM3) biliary tract tumors associated with significant lymph node infiltration. MiR-338-3p has also been identified in a signature of 3 miRNA that predict survival in patients with iCCA. Patients with low levels of miR-338-3p had the worst OS and RFS (Zhang *et al.*, 2015).

We also showed that miR-338-3p targeted SOX4, another key gene in mesenchymal-to-epithelial transition. Studies have shown that SOX4 can induce many transcription factors that play a central role in tumor progression, such as TWIST1, SNAI1, and ZEB1 (Niu *et al.*, 2019). The regulation of SOX4 by miR-338-3p has already been described in several cancers

(e.g. breast cancer, kidney cancer) and associated with cell proliferation and migration (Jin et al., 2015; Tong et al., 2017). Only few studies have also described circRNA participating in iCCA oncogenesis by sponging tumor suppressor miRNA. For example, circACTN4 induction participates to iCCA progression by sponging miR-424-5p which results in an increased expression of its target, the Yes-associated protein 1 (YAP1) and the activation of the Wnt/ β -catenin pathway (Chen *et al.*, 2022).

We demonstrated the clinical value of circLTBP2 as prognostic biomarker by using three independent cohorts of resected iCCA. However, further validation is needed to confirm the link between circLTBP2 expression and survival using a larger sample size.

The diagnosis of CCAi represents a real clinical challenge due to the many anatomomorphological similarities between tumors developing along the hepatobiliary tree (Bragazzi *et al.*, 2018). In this study, we showed a differential expression of circLTBP2 between CCA and HCC, supporting that circRNA could be used as biomarker helping to differentiate these two types of tumors. We also demonstrated that circLTBP2 was expressed and induced by TGF β in eCCA. Thus, further research is needed to determine whether circLTBP2 play a tissue-specific role in tumor progression.

CCA is a heterogeneous group of tumors and applying a systematic and effective therapeutic approach for all patients is challenging. The current first-line reference treatment is a combination of gemcitabine and cisplatin. However, the benefit of this treatment is modest, and iCCA remains a poor prognosis cancer (Oh et al., 2022; Valle *et al.*, 2010). Our data also suggest that circLTBP2 inhibitors could be used to sensitize iCCA cells to gemcitabine used as first therapy for iCCA treatment. Translational studies supporting this point are required.

In summary, this study suggests that circRNA may inhibit the growth and spread of iCCA and serve as a predictive marker for patients with iCCA who have undergone surgery. Additionally, the study provides preliminary evidence of the circLTBP2/miR-338-3p pathway's regulatory role in iCCA. These findings provide a basis for further exploration of circRNA in iCCA, including their functions, diagnostic and prognostic potential, as well as therapeutic implications.

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Authors' contributions

CC and JE conceived the project, provided funding and supervision. CC, JE and CL designed the experiments. CL, MD, RP, PP, DL and RL acquired the data. CL, MD, RP and DL analyzed the data. CC, JE, CL, MD, RP interpreted the data. All authors critically revised the article.

Conflict of interest

J. Edeline received honoraria from MSD, Roche, AstraZeneca, and BMS. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership, or options, expert testimony, grants or patents received or pending, or royalties.

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Chapitre 4 : Discussion

I. Identification de l'oncogène circLTBP2 induit par le TGFβ dans le CCAi

Les ARNcirc ont longtemps été considérés comme des artefacts sans fonctions spécifiques. Néanmoins, depuis 2003, leur rôle dans l'initiation et la progression tumorale a été rapporté dans plusieurs cancers et ils sont aujourd'hui considérés comme des candidats prometteurs pour la découverte de nouvelles cibles thérapeutiques et de biomarqueurs innovants (Jeck and Sharpless, 2014). L'identification d'ARNcirc régulés par le TGFβ nous est apparue particulièrement pertinente dans le CCAi. En effet, ce type de tumeur évolue dans un contexte pro-inflammatoire marqué par un stroma abondant et une importante sécrétion de cytokines, telles que le TGFβ ou l'IL-6. Cette assertion est soutenue par une étude récente qui a identifié la première signature d'ARNcirc régulés par l'IL-6 dans le CCAi. Li et al. ont ainsi identifié l'ARN circGGNBP2 induit par l'IL-6 et participant à la prolifération cellulaire et au développement de métastases *in vivo* (Li et al., 2022a).

Dans cette étude, nous avons établi pour la première fois une signature de 119 ARNcirc régulés par le TGFβ dans le CCAi. Nous avons ainsi identifié circLTBP2, un ARNcirc induit par le TGFβ dont nous avons démontré les actions pro-oncogéniques dans une lignée cellulaire de CCAi. Nous avons observé que l'expression de circLTBP2 et de son ARNm associé LTBP2 étaient toutes les deux induites par un traitement au TGFβ. Ces deux ARN proviennent du même gène parental *LTBP2* codant pour une protéine qui intervient très en amont de la voie du TGFβ. LTBP2 est une protéine qui fait partie d'un complexe impliqué dans la disponibilité du TGFβ à la surface des cellules. La régulation de l'expression de LTBP2 est donc cruciale puisqu'elle module l'activité de la voie du TGFβ dans son ensemble (Robertson et al., 2015). De nombreuses études ont rapporté une augmentation de l'expression de LTBP2 dans plusieurs types de cancers, dont le CCAi, et que sa surexpression contribuait à la progression tumorale en potentialisant la réponse au TGFβ (Zhao et al., 2022). Une étude a notamment démontré la valeur pronostique de LTBP2 dont l'expression est associée à une mauvaise OS chez des patients atteints de CHC (da Costa et al., 2015).

Nous avons ici démontré que la surexpression de son homologue circulaire favorisait également la progression tumorale en induisant la prolifération et la migration de cellules humaines de CCAi. De plus, nous avons découvert que la surexpression circLTBP2 conférait

aux cellules de CCAi une résistance accrue contre la *gemcitabine*, un traitement utilisé en première ligne pour les CCAi de stade avancé ou métastatique (Valle *et al.*, 2010). CircLTBP2 vient ainsi s'ajouter aux quelques ARNcirc déjà décrits pour leur participation dans la progression du CCA. Par exemple, l'ARNcirc MBOAT2 induit dans le CCAi a récemment été décrit pour son rôle dans la régulation du métabolisme des lipides et dans la régulation du stress redox, favorisant ainsi la prolifération et l'invasion cellulaire (Yu *et al.*, 2023). (Yu *et al.*, 2023). Nous avons également retrouvé plusieurs isoformes de circMBOAT2 induits par le TGF β dans notre signature de 119 ARNcirc. Ces résultats corroborent l'idée que des ARNcirc régulés par le TGF β jouent un rôle important dans le développement de CCAi.

II. Les fonctions de circLTBP2 dans le CCAi

CircLTBP2 est un ARNcirc particulièrement long composé de 14 exons. Ces longs ARNcirc exoniques sont souvent qualifiés de « supers éponges ». Ils présentent généralement de nombreux sites de fixation pour des RBP et participent à la progression tumorale en séquestrant une grande quantité de miARN, les empêchant ainsi de dégrader leurs cibles naturelles. L'ARNcirc HIPK3, un ARN surexprimé dans de nombreux cancers, présente typiquement ces caractéristiques. Une des premières études ayant identifié circHIPK3 rapporte la présence de 18 sites de liaison pour 9 miARN antitumoraux différents (Zheng *et al.*, 2016). Dans le CCAi, plusieurs études rapportent des ARNcirc jouant ce rôle d'éponge à miARN. Par exemple, l'ARN oncogénique circDR1as favorise la progression, la migration et l'invasion des cellules de CCA en séquestrant le miRNA antitumoral miR-641, l'empêchant ainsi de cibler les gènes AKT3 et mTOR dont l'expression est régulièrement altérée dans ce type de tumeur (Li *et al.*, 2020a).

Nous avons donc fait l'hypothèse que circLTBP2 pouvait également assurer ce type de fonctions dans le CCAi. Ainsi, grâce à une approche de pull down, nous avons pu identifier par séquençage 94 miARN qui interagissent avec circLTBP2. Par exemple, miR-34a-5p et miR-34c-5p ont déjà été mesurés dans le sérum de patients atteints de CCA. Ces miARN étaient significativement moins exprimés comparé aux valeurs retrouvées dans le sérum d'individus sains. De plus, leur expression a été associée à des actions anti-prolifératives dans le CCA (Shen *et al.*, 2019; Shi *et al.*, 2021). Nous avons néanmoins sélectionné miR-338-3p, un miARN dont le rôle de suppresseur de tumeur est déjà largement décrit. Ce miARN joue un rôle pivot dans la progression tumorale et son activité est finement régulée.

De nombreuses voies dépendent de son activité, notamment WNT, MAPK et PI3K/AKT qui sont toutes impliquées dans la progression du CCAi. MiR-338-3p participe ainsi à de nombreux processus pathologiques en inhibant les signaux de prolifération cellulaire, en induisant la mort cellulaire ou encore en diminuant l'angiogenèse (Moghbeli, 2021).

MiR-338-3p possède également une forte valeur pronostique pour les cancers des voies biliaires. Des niveaux faibles de miR-338-3p ont notamment été retrouvés dans plusieurs tumeurs des voies biliaires de stade avancé (TNM3) associées à une infiltration ganglionnaire importante. MiR-338-3p a également été identifié dans une signature de 3 miARN prédictifs de la survie de patients atteints de CCAi. Ainsi, les patients qui présentaient des niveaux d'expression faible de miR-338-3p étaient aussi ceux avec la OS et à RFS la moins favorable (Zhang *et al.*, 2015). Ce miARN est également décrit pour son potentiel en tant que biomarqueur circulant. Les niveaux d'expression de miR-338-3p ont notamment été détectés dans le sérum de patients atteints de cancer du poumon à des niveaux beaucoup plus faibles que dans les sérums provenant d'individus sains (Tian *et al.*, 2021). MiR-338-3p est également impliqué dans la réponse aux traitements. Une étude démontre qu'il permet d'augmenter la sensibilité de tumeurs des ovaires au *cisplatine*, une option thérapeutique également utilisée en première ligne pour le CCAi, en ciblant WNT2B (Niu *et al.*, 2019).

Nous avons utilisé le site de prédiction *Circular RNA Interactome* pour identifier sur la séquence de circLTBP2 5 sites de fixation pour miR-338-3p. Ces données suggèrent que circLTBP2 participe de façon importante à la régulation de l'activité de ce miARN qui joue un rôle central dans la régulation de processus tumoraux. Nous avons notamment démontré que lorsque les niveaux de miR-338-3p étaient artificiellement augmentés, les processus pro-oncogéniques induits par circLTBP2 étaient rétablis. Nous avons plus précisément réussi à diminuer la migration cellulaire et à augmenter la sensibilité des cellules de CCAi à la *gemcitabine* induites par la surexpression de circLTBP2.

Nous avons ensuite cherché à déterminer quelles étaient les cibles de miR-338-3p qui pourraient expliquer son rôle dans l'inhibition de la progression du CCAi. Plusieurs études rapportent que les fonctions régulatrices de miR-338-3p passent par la dégradation de plusieurs oncogènes clés. Ce miARN est notamment décrit pour cibler des gènes clés de la TEM, tel que *ZEB2*. Des travaux décrivent qu'une diminution de l'expression de miR-338-3p conduit à une accumulation de *ZEB2*, ce qui déclenche des signaux pro-oncogéniques *via* l'activation des voies NF- κ B et PI3K/AKT (He *et al.*, 2020). Nous avons également démontré

que miR-338-3p ciblait SOX4, un autre gène clé de la TEM. Des études démontrent notamment que SOX4 est capable d'induire de nombreux facteurs de transcription jouant un rôle central dans la progression tumorale, tels que TWIST1, SNAI1 et ZEB1 (Niu *et al.*, 2019). La régulation de SOX4 par miR-338-3p a déjà été décrite dans plusieurs cancers (*e.g.* cancer du sein, cancer rénal) et associée à la prolifération et à la migration cellulaire (Jin *et al.*, 2015; Tong *et al.*, 2017).

Nous avons également démontré que miR-338-3p se fixait à LTBP2. Nous pouvons supposer que circLTBP2 participe donc également à l'induction de son ARN linéaire associé, en le protégeant de la dégradation par miR-338-3p. Plusieurs études ont également décrit ce type d'action coordonnée entre un ARNcirc et un ARNm provenant du même gène parental. Par exemple, l'ARN circNOTCH1 induit dans le cancer gastrique possède lui aussi un rôle d'éponge à miARN et séquestre le miARN antitumoral miR-449c-5p. Or, miR-449c-5p est connu pour cibler c-MYC, un ARN pro-tumoral responsable de l'activation de *NOTCH1*. Ainsi, circNOTCH1 contribue, *via* une boucle de rétrocontrôle positif, à induire l'expression de son gène parental participant ainsi à la progression tumorale (Zhao *et al.*, 2020b).

Le phénotype pro-tumoral observé suite à la surexpression de circLTBP2 pourrait donc s'expliquer par une activation de l'axe circLTBP2/miR-338-3p/SOX4. Nous avons néanmoins identifié d'autres miARN se fixant à circLTBP2. Or, chaque miARN possède une multitude de cibles. Les fonctions régulatrices opérées par les ARNcirc ne seraient donc pas si linéaires. Il faudrait plutôt considérer le réseau d'ARN dans son ensemble et intégrer des signatures complètes d'ARNm, de miARN et d'ARNcirc dont l'expression est altérée dans un contexte pathologique. Le nombre d'algorithmes bio-informatiques visant à identifier ce type de réseaux d'ARN et leurs rôles dans des processus pathologiques ne cesse d'augmenter. Par exemple, Bai *et al.* ont identifié des réseaux d'ARN participant à la progression du cancer rénal en s'appuyant sur les différentes bases de données disponibles. Ils ont procédé de la manière suivante : plusieurs signatures d'ARNcirc différentiellement exprimés dans ce type d'adénocarcinome ont été récupérées depuis la plateforme *Gene Expression Omnibus* (GEO). Puis, deux bases de données prédisant les interactions entre les miARN et les ARNcirc ont été utilisées pour identifier les miARN potentiellement séquestrés par ces ARNcirc (*circ Interactome* et *Cancer-specific circRNA database*). Enfin, la liste des cibles de ces miARN a été croisée avec des signatures de gènes dont l'expression est effectivement altérée dans ce type de tumeurs (base de données du TCGA). Une analyse d'enrichissement de gènes a ensuite permis d'identifier 10 gènes centraux régulés à plusieurs niveaux par le

réseau d'ARNcirc et de miARN. Ce travail a ainsi permis d'avoir une vision globale sur les perturbations du réseau d'ARN impliquées dans la tumorigenèse, et donc d'avoir une idée plus précise des leviers activables pour le développement de nouvelles stratégies thérapeutiques (Bai et al., 2020).

Dans cette étude, nous avons concentré nos efforts sur le rôle de circLTBP2 en tant qu'éponge à miARN. Néanmoins, d'autres fonctions sont assurées par les ARNcirc. En effet, bien que les ARNcirc aient longtemps été considérés comme des ARNnc, plusieurs d'entre eux sont traduits en protéines qui possèdent des fonctions spécifiques. Certaines protéines, lorsqu'elles sont exprimées de façon aberrante, peuvent participer à la cancérogenèse. Des outils spécifiques permettent d'établir le potentiel codant des ARNcirc, notamment en repérant les cadres de lecture potentiellement présents sur leurs séquences (Lei et al., 2020).

Ainsi, plusieurs ARNcirc traduits en protéines ont été décrits pour leur rôle dans la progression tumorale. Une étude récente a par exemple caractérisé l'ARN circAKT3. Cet ARNcirc est généré à partir de l'exon 3 à 7 du gène AKT3 (long de 524 nucléotides) et traduit en une protéine de 174 acides aminés (AKT-173aa). Cette protéine tronquée agit comme suppresseur de tumeur en servant d'appât pour la *phosphoinositide-dependent kinase* (PDK1), limitant ainsi la phosphorylation et l'activation d'AKT3. AKT-173aa inhibe ainsi la voie PI3K/AKT et limite la progression tumorale (Xia et al., 2019). Un seul ARNcirc traduit en protéine a jusqu'alors été décrit dans le CCAi. L'ARN pro-tumoral circGGBP2 produit une protéine de 184aa qui se fixe et phosphoryle STAT3, activant ainsi la voie IL-6/STAT3 et participant ainsi au développement du CCAi (Li *et al.*, 2022a).

L'exploration des fonctions assurées par ces protéines issues d'ARNcirc n'en est encore qu'à son commencement. L'identification de signatures d'ARNcirc régulés par le TGF β et traduits en protéines est une des thématiques que notre équipe va approfondir au cours des prochaines années. La technique de fractionnement des polysomes sera notamment adaptée pour les ARNcirc et permettra ainsi d'identifier les ARNcirc dont la traduction est régulée par le TGF β . Outre le fait d'identifier des nouvelles cibles thérapeutiques et des biomarqueurs innovants, cette approche permettra d'évaluer si circLTBP2 est traduit en protéine et participe à la progression du CCAi au travers de plusieurs fonctions.

Cette idée qu'une seule isoforme d'ARNcirc peut agir *via* plusieurs modes d'action a été confirmée dans plusieurs études, notamment dans le CCAi. Par exemple, l'ARN circACTN4 participe à la progression du CCAi par deux mécanismes. Dans le cytoplasme, il agit comme

une éponge pour miR-425-5p qui ne peut plus cibler l'activateur co-transcriptionnel YAP1. Dans le noyau, il recrute le facteur de transcription YBX1 qui initie la transcription de FZD7, un régulateur positif de la voie Wnt/ β -caténine. CircACTN4 favorise également l'interaction entre YAP1 et la β -caténine et leur accumulation dans le noyau, contribuant ainsi à la progression du CCAi (Chen *et al.*, 2022). Cette étude soulève l'importance de la localisation des ARNcirc dont leur fonction dépend, et des mécanismes qui permettent leur transport entre le noyau et le cytoplasme. Un des prochains objectifs de l'équipe sera donc de déterminer la localisation de circLTBP2 dans des cellules de CCAi et d'établir si cet ARNcirc possède d'autres fonctions que celle d'éponger des miARN.

III. CircLTBP2 comme marqueur prédictif de la survie dans le CCAi

Les ARNcirc ont récemment été identifiés comme des biomarqueurs potentiels pour plusieurs types de cancer. Une étude a notamment démontré que circSMARCA5 était un marqueur pronostique indépendant dans de nombreux cancers, incluant le cancer du pancréas, le cancer du foie et le cancer colorectal. Ainsi, les patients présentant des niveaux d'expression élevés de cet ARNcirc présentent un pronostic plus favorable, avec des tumeurs mieux différenciées et peu métastatiques (Chao *et al.*, 2021). Le CCAi étant encore associé à un pronostic très sombre, l'identification de nouveaux biomarqueurs pronostiques serait la bienvenue. Quelques études décrivent déjà des ARNcirc dont l'expression est associée à la OS de patients atteints de CCAi (*e.g.* circGGNBP2, circHMGCS1-016, circCCAC1) (Liao *et al.*, 2023).

Le critère principal qui a motivé le choix de circLTBP2 comme candidat pour cette étude s'est basé sur son potentiel en tant que biomarqueur pronostique. Afin d'évaluer sa valeur pronostique, l'expression de circLTBP2 a été mesurée dans 20 échantillons de CCAi. Bien que cette première cohorte ait démontré une forte corrélation de l'expression de circLTBP2 avec une mauvaise OS et une mauvaise RFS, les cohortes de validation ont confirmé une valeur pronostique de circLTBP2 uniquement pour la OS.

Cette étude représente uniquement une preuve de concept et la valeur pronostique de circLTBP2 devra être validée dans des cohortes de taille plus conséquente. Nous avons notamment accès aux échantillons tumoraux des 122 patients inclus dans l'essai randomisé de phase III PRODIGE-12, qui inclue des patients avec des CCA réséqués (R0/R1). Ces

patients sont randomisés en deux bras : i) les patients reçoivent une chimiothérapie de type GEMOX comprenant de la *gemcitabine* et de l'*oxiplatine*, ii) les patients ont une simple surveillance. Cette cohorte nous permettra ainsi de confirmer ou d'infirmer nos premières observations qui suggèrent que circLTBP2 pourrait être un facteur pronostique indépendant du CCAi. De plus, forts de nos résultats préliminaires qui suggèrent un rôle de circLTBP2 dans la résistance à la *gemcitabine*, nous pourrions évaluer si cet ARNcirc pourrait représenter un marqueur de réponse au traitement GEMOX.

L'utilisation de cohortes prospectives incluant des patients avec des CCAi de stade plus avancé et métastatiques pourra renforcer les résultats obtenus dans cette étude préliminaire. L'association de l'expression de circLTBP2 avec plusieurs facteurs pronostiques, tels que le stade TNM ou le niveau d'invasion ganglionnaire pourra potentiellement permettre de renforcer la valeur pronostique de cet ARNcirc dans le CCA.

IV. CircLTBP2 pour le diagnostic différentiel entre les CCAi et les CHC

Le diagnostic du CCAi représente un véritable défi clinique du fait des nombreuses similitudes anatomo-morphologiques entre les tumeurs se développant le long de l'arbre hépatobiliaire. Des erreurs de diagnostic peuvent être réalisées entre les CCAi et les CHC, ou encore entre les CCAp se développant dans le parenchyme hépatique et les CCAi des voies biliaires larges (Bragazzi *et al.*, 2018). Les ARNcirc pourraient représenter un nouvel outil diagnostique visant à limiter ces erreurs. Certaines études ont notamment démontré que les ARNcirc pouvaient être spécifiquement exprimés dans un type de tumeur. Une étude pionnière dans le domaine a évalué la spécificité des ARNcirc dans 17 types de cancers différents. Le CCA faisait partie des 3 tumeurs présentant le moins d'ARNcirc spécifiques (Vo *et al.*, 2019). Ces résultats sont donc peu encourageants concernant le potentiel des ARNcirc en tant qu'outils diagnostiques dans le CCA.

Néanmoins, nous avons voulu vérifier si circLTBP2 était différentiellement exprimé entre les différents sous-types de CCA. Nous avons ainsi démontré que circLTBP2 était exprimé et induit par le TGF β dans plusieurs lignées cellulaires de CCA, incluant des lignées d'origine intrahépatique et extrahépatique (i.e. Hu-CCT1, HuH-28, TFK-1, Mz-ChA-1 et SK-ChA-1). Néanmoins, nous n'avons pas mesuré de différences de niveaux d'expressions entre les

différents sous-types de CCA. Seule HuCC-T1 présente une expression de circLTBP2 particulièrement élevée.

Ces résultats sont soutenus par les données obtenues dans la cohorte de CCA de Salamanque comprenant des CCAi, des CCAp et des CCAAd. Ici encore, bien que le faible nombre d'échantillons ne nous permette pas de conclure avec certitude, nous n'avons pas relevé de différence d'expression de circLTBP2 entre les différents sous-types de CCA. En revanche, les CHC inclus dans cette cohorte présentaient des niveaux d'expression de circLTBP2 significativement inférieurs à ceux retrouvés dans les CCA. Ces données sont encourageantes quant à l'utilisation de circLTBP2 pour aider à distinguer les CCAi des petits CHC. Cette hypothèse devra néanmoins être validée dans une cohorte incluant davantage de patients.

Les différences d'expression de circLTBP2 observées entre les CHC et les CCA nous ont conduits à nous demander si cet ARN était spécifique des CCA, ou s'il était également présent dans d'autres types de tumeurs. Pour répondre cette question, nous avons utilisé la base de données MiOncoCirc qui répertorie les ARNcirc différentiellement exprimés dans 17 types de tumeurs solides (Vo *et al.*, 2019). Ainsi, nous avons pu confirmer que circLTBP2 était bien exprimé dans le CCA et non détecté dans les CHC. Nous avons également relevé quatre autres tumeurs marquées par des niveaux anormaux de circLTBP2 : le cancer rénal, le cancer ovarien, le cancer de la tête et du cou et le cancer des glandes salivaires. Ces données suggèrent que circLTBP2 n'est pas spécifique du CCA. En revanche, des données suggèrent que la voie du TGF β participe à la progression de chacune de ces tumeurs (Bostrom *et al.*, 2013; Pang *et al.*, 2018; Tauriello *et al.*, 2018; Zhang *et al.*, 2020). On peut donc faire l'hypothèse que circLTBP2 serait plutôt spécifique de la signalisation du TGF β , et que l'induction de son expression pourrait être responsable de l'activation de voies pro-oncogéniques impliquant le TGF β dans de nombreux cancers.

V. CircLTBP2 en tant que biomarqueur prédictif pour la *gemcitabine*

Les CCA sont un ensemble hétérogène de tumeurs et il est donc compliqué d'appliquer une approche thérapeutique systémique et efficace pour tous les patients. Le traitement de référence utilisé en première ligne actuellement est une combinaison de *gemcitabine* et de

cisplatine. Le bénéfice de ce traitement est néanmoins modeste et le CCAi reste un cancer de mauvais pronostic (Valle *et al.*, 2010). Dans cette étude, nous avons démontré que circLTBP2 était impliqué dans la résistance du CCAi à la *gemcitabine* via la séquestration de miR-338-3p. Nous avons identifié une cible de miR-338-3p, SOX4, qui est protégée par la surexpression de circLTBP2. C'est une des pistes que nous souhaiterions explorer pour expliquer la résistance à la *gemcitabine* induite par circLTBP2. Néanmoins, SOX4 n'est pas le seul ARNm ciblé par miR-338-3p et il est fort probable que d'autres soient également impliqués dans ces processus résistance aux traitements. Notre objectif sera donc d'identifier une signature d'ARN régulés par l'axe circLTBP2/mir-338-3p et associés à la résistance à la *gemcitabine*. Nous avons déjà identifié quelques candidats intéressants, tel que l'ARNm BAMBI qui est une cible de miR-338-3p et qui a également été identifié dans une signature génique associée à la résistance à la *gemcitabine* dans le cancer du poumon (Tooker *et al.*, 2007).

Nos résultats préliminaires suggérant un rôle joué par les ARNcirc dans la sensibilité des cellules tumorales à la *gemcitabine* sont soutenus par plusieurs études. Shao *et al.* ont notamment identifié une signature d'ARNcirc impliqués dans la résistance des cellules tumorales d'adénocarcinome canalaire pancréatique (PDAC) à la *gemcitabine*. Cette signature a ainsi permis d'identifier deux ARNcirc retrouvés dans le plasma de patients atteints de PDAC qui ont montré une mauvaise réponse à la *gemcitabine*. Ces résultats suggèrent que les ARNcirc pourraient être de bons marqueurs prédictifs dans le cancer.

Les données que nous avons obtenues sur circLTBP2 suggèrent également que l'utilisation de GapmeRs dégradant les ARNcirc qui participent à la résistance aux thérapies pourrait améliorer l'efficacité des traitements et, *de facto*, le pronostic des patients.

VI. CircLTBP2 en tant que biomarqueur sérique

L'utilisation d'approches -omiques pour identifier de nouveaux biomarqueurs, tels que circLTBP2, pourrait changer le paradigme de la prise en charge du CCAi. Il y a plus particulièrement un intérêt croissant pour l'utilisation de méthodes alternatives et non invasives. Les biopsies liquides, obtenues par prélèvement sanguins, représentent notamment une approche très attrayante qui permet un profilage individuel et un suivi dans le temps des patients atteints de cancer. Ces biopsies liquides sont riches en informations puisqu'elles contiennent des cellules tumorales circulantes (CTC), de l'ADN tumoral circulant

(ctDNA), des ARN circulants et des VE provenant de tumeurs (Byron et al., 2016). Ces types d'échantillons biologiques ont particulièrement attiré notre attention puisqu'ils contiennent également des ARNcirc. Certaines études suggèrent qu'ils seraient particulièrement enrichis dans des VE circulantes sécrétées par la cellule tumorale (Li et al., 2015a).

Nous avons démontré que l'ARN circLTBP2 était en effet présent dans les VE provenant de cellules de CCAi. Nous avons également observé qu'un traitement au TGF β conduisait à une internalisation plus importante de circLTBP2 dans les VE. Nous ne savons néanmoins pas si cela est simplement la conséquence d'une présence plus importante de circLTBP2 dans le cytoplasme des cellules ou si cette induction est le résultat d'un mécanisme actif régulé par le TGF β favorisant son internalisation. Plusieurs études suggèrent que l'entrée des ARNcirc dans les VE est médiée par des RBP présentes à la surface des ARNcirc. La RBP AGO2, connue pour réguler l'entrée des miARN dans les VE, présente des sites de fixation sur un grand nombre d'ARNcirc. Certaines études suggèrent donc qu'elle jouerait également un rôle important dans l'entrée préférentielle de certains ARNcirc dans les VE. Le fait de limiter l'entrée d'ARNcirc oncogéniques, tel que circLTBP2, pourrait ainsi limiter la progression tumorale en freinant la propagation des signaux pro-tumoraux aux cellules avoisinantes (Xu et al., 2020b).

Nous avons également isolé les VE contenues dans le sérum de patients avec des CCAi de stade avancé. Le niveau de circLTBP2 mesuré dans ces sérums varie grandement d'un patient à l'autre. Il était surtout bien supérieur à celui retrouvé dans le sérum d'individus sains. Nous avons également confirmé la valeur pronostique de circLTBP2 dans le sérum de ces patients, puisque ceux de très mauvais pronostic présentaient une expression élevée de circLTBP2. Ces données suggèrent une potentielle utilité de circLTBP2 en tant que marqueur sérique pronostique pour le CCAi.

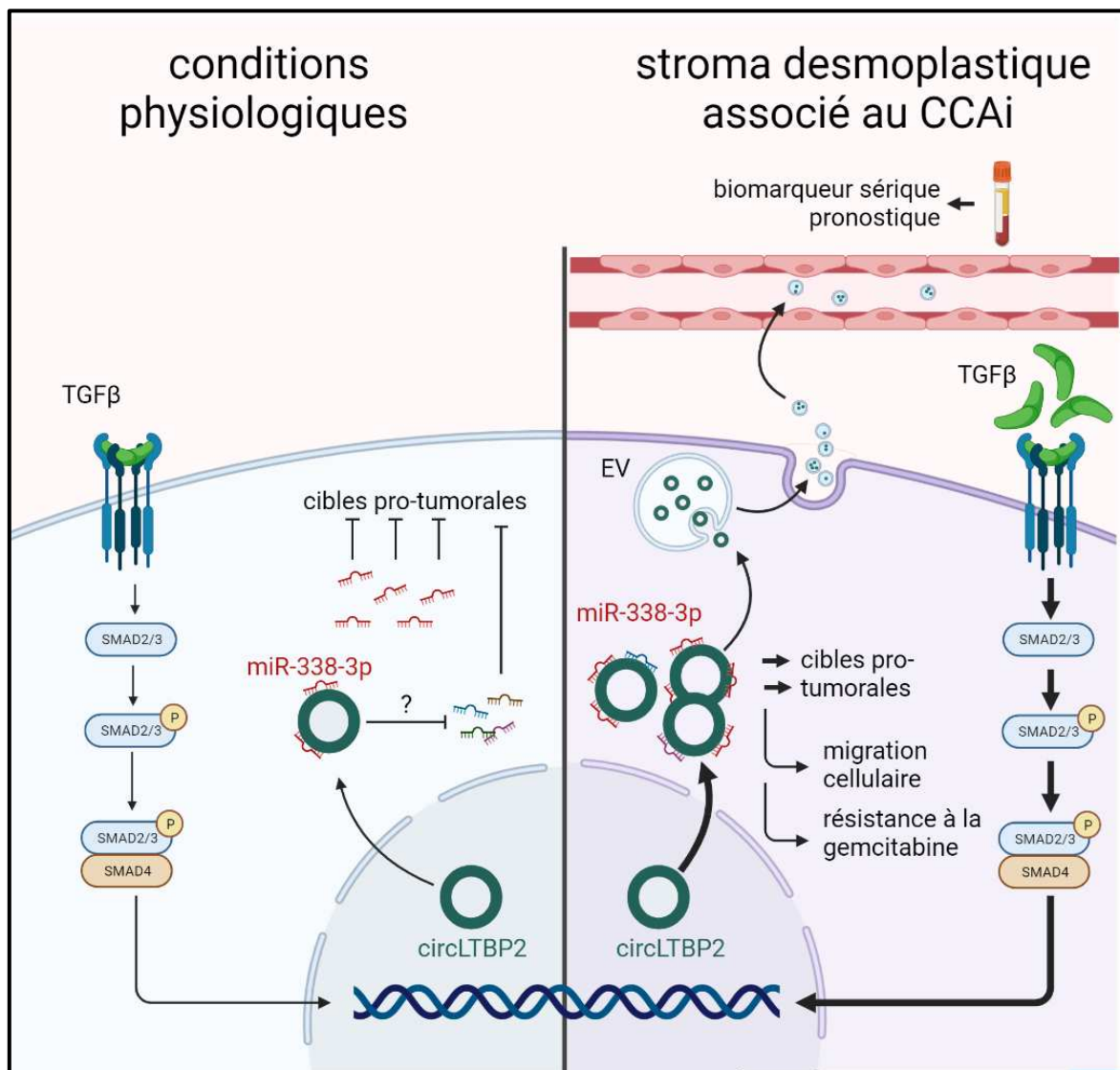
De plus, avec l'objectif d'utiliser ce nouveau biomarqueur en clinique, il serait nécessaire d'évaluer sa valeur pronostique dans le sérum et dans le sang total plutôt que dans les VE. En effet, bien que les ARNcirc soient enrichis dans les VE, il est possible que les différences d'expression de circLTBP2 soient détectables sans isoler les VE, simplifiant ainsi grandement cette approche sur le plan opérationnel.

Les deux marqueurs sériques de référence utilisés pour le diagnostic et le pronostic du CCA sont CA19-9 et CEA. Bien qu'ils présentent une utilité clinique, une grande variation de la sensibilité (50-90%) et de la spécificité (54-98%) a été mesurée pour ces deux biomarqueurs

sériques. De plus, 10% des individus ne présentent pas l'antigène Lewis et ne produisent donc pas de CA19-9. Les niveaux sériques de CA19-9 sont également élevés chez les patients atteints de pathologies biliaires non malignes telles que la cholangite sclérosante primitive ou l'obstruction biliaire pure. Ainsi, CA-19-9 ne serait pas le marqueur idéal pour la surveillance des patients atteints de CCA (Jaklitsch and Petrowsky, 2019). En nous appuyant sur des cohortes bien annotées, nous pourrions corréliser l'expression sérique de circLTBP2 avec celles de ces deux biomarqueurs de référence. L'objectif serait ainsi de renforcer nos premières conclusions sur la valeur pronostique de circLTBP2 et faire un premier pas vers pour l'utilisation de ces biomarqueurs innovants pour la prise en charge du CCAi.

Chapitre 5 : Conclusion et perspectives

Avec ce projet, nous répondons à la stratégie décennale nationale qui vise à concentrer nos efforts sur les cancers de mauvais pronostic. Nous avons notamment cherché à identifier de nouvelles cibles thérapeutiques et des biomarqueurs innovants pour le traitement du CCAi. Les ARNcirc représentent une nouvelle classe de biomarqueur avec une valeur pronostique, diagnostique et prédictive prometteuses. Ainsi, nous avons identifié par une approche non supervisée une signature de 119 ARNcirc régulés par le TGF β , une cytokine jouant un rôle important dans la progression du CCAi. Nous avons notamment démontré les fonctions pro-tumorales de l'ARN circLTBP2 induit par le TGF β . Les résultats préliminaires de cette étude suggèrent un bon potentiel clinique pour cet ARNcirc, dont l'expression est associée à la survie globale et à la résistance des cellules tumorales à la *gemcitabine* (Figure 18).



La valeur pronostique de circLTBP2 est d'autant plus intéressante que cet ARNcirc est quantifiable dans les biopsies liquides, et pourrait donc représenter une alternative peu invasive pour la détection de CCAi. Néanmoins, le chemin pour une application clinique reste long et l'ARN circLTBP2 devra répondre aux normes de référence avant d'être adopté (Figure 19). En effet, bien que les ARNcirc répondent à beaucoup de ces critères notamment sur le plan analytique (spécificité, sensibilité et stabilité), il faudra valider notre capacité à détecter avec fiabilité ce nouveau biomarqueur. La sensibilité, la spécificité et la valeur prédictive de circLTBP2 devront également être confirmées dans des cohortes de plus grand effectif. Enfin, l'intérêt clinique de l'utilisation de circLTBP2 pour le CCAi devra être démontré à long terme. Des essais cliniques incluant la mesure d'ARNcirc avec une valeur pronostique et des cohortes prospectives évaluant leur potentiel sur plusieurs années seront nécessaires pour que leur utilisation clinique se démocratise (Byron *et al.*, 2016).

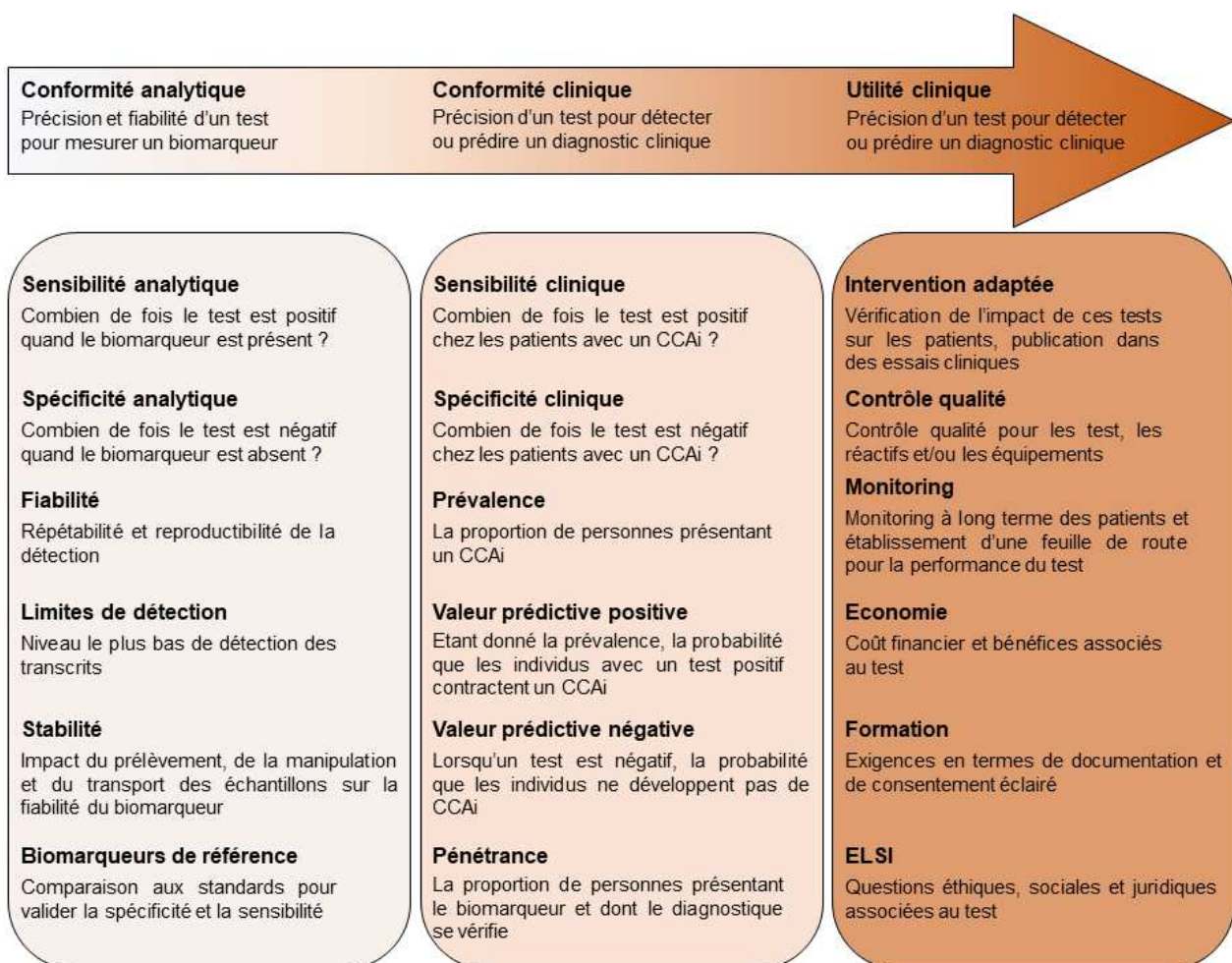


Figure 19 : Critères pour le développement et l'adoption de tests cliniques. Adapté de (Byron *et al.*, 2016).

Chapitre 6 : Références

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Chapitre 7 : Annexes

Emerging roles of circular RNAs in liver cancer

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Summary

Hepatocellular carcinoma and cholangiocarcinoma are the most common primary liver tumours, whose incidence and associated mortality have increased over recent decades. Liver cancer is often diagnosed late when curative treatments are no longer an option. Characterising new molecular determinants of liver carcinogenesis is crucial for the development of innovative treatments and clinically relevant biomarkers. Recently, circular RNAs (circRNAs) emerged as promising regulatory molecules involved in cancer onset and progression. Mechanistically, circRNAs are mainly known for their ability to sponge and regulate the activity of microRNAs and RNA-binding proteins, although other functions are emerging (e.g. transcriptional and post-transcriptional regulation, protein scaffolding). In liver cancer, circRNAs have been shown to regulate tumour cell proliferation, migration, invasion and cell death resistance. Their roles in regulating angiogenesis, genome instability, immune surveillance and metabolic switching are emerging. Importantly, circRNAs are detected in body fluids. Due to their circular structure, circRNAs are often more stable than mRNAs or miRNAs and could therefore serve as promising biomarkers – quantifiable with high specificity and sensitivity through minimally invasive methods. This review focuses on the role and the clinical relevance of circRNAs in liver cancer, including the development of innovative biomarkers and therapeutic strategies.

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Introduction

Circular RNAs (circRNAs) belong to a family of recently rediscovered RNA molecules. They are produced during the maturation of RNA transcripts. Structurally, circRNAs are covalently closed by a phosphodiester linkage between downstream donor and upstream acceptor RNA splice domains. Mainly considered as splicing errors during the past decade, circRNAs are now accepted as functional RNA molecules. They display tissue- and cell-specific expression patterns and are encoded from thousands of genes.¹ Emerging evidence demonstrates that circRNAs are involved in biological processes contributing to the onset and progression of cancer.^{2–4} In addition, due to their circular structure, circRNAs are resistant to the action of exoribonucleases and therefore exhibit an expanded half-life compared to their parental linear counterparts, allowing detection even when expressed at a low level.^{5,6} It is estimated that exonic circRNAs are very stable in cells, with most species exhibiting a half-life of over 48 hours,⁷ compared to an average half-life of 10 hours for mRNAs.⁶ These properties suggest that circRNAs could represent clinically relevant biomarkers for the management of patients with cancer. This review specifically highlights recent discoveries on the functions and clinical relevance of circRNAs in liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA).

CircRNA discovery, biogenesis and function

CircRNA discovery

CircRNAs were first discovered in 1976 on viroid particles.⁸ Then, circular structures were identified by electron microscopy in the cytoplasm of eukaryotic cells, notably in HeLa cells.⁹ Nevertheless, it was only in 1993 that circRNAs (circSry, a 1.3 kb circRNA derived from a single exon of the sex-determining region Y transcript in mice) were molecularly identified by dedicated experiments, including RNase H digestion followed by northern blotting, reverse-transcription PCR and sequencing.¹⁰ Afterward, several circRNAs previously described as “scrambled exon RNAs” were reported, including a circRNA from the rat cytochrome P450 2C24 gene.¹¹ However, no specific biological function was reported for circRNAs at that time.

Next-generation sequencing (NGS) ushered in the beginning of the genome-wide identification of circRNAs. Traditional RNA-sequencing pipelines remove non-conventional reads that do not map with the reference genome, such as reads that arise from gene fusion or those originating from back-splicing events. New bioinformatic algorithms based on back-splice junction overlapping reads recognition made it possible to efficiently detect *de novo* circRNAs and to differentiate them from their

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linear counterparts.^{12–15} In addition, novel specific protocols for NGS library preparation were established to enrich the RNA fraction with circRNAs, including ribosomal and polyA⁺ RNA depletion and RNase R treatment to degrade linear RNAs.¹⁶ Accordingly, the number of circRNA libraries drastically increased and repository databases (e.g. circBase) were established to annotate circRNAs.¹⁷ Recently, Xin *et al.* used rolling circle amplification followed by nanopore long read sequencing to decrypt full-length circRNA sequences, thereby increasing the roster of known circRNA isoforms.¹⁸ It is now accepted that circRNAs are the most abundant RNA isoforms, originating from thousands of human genes,¹⁹ and that their expression is conserved in eukaryotes.²⁰

CircRNA biogenesis

Contrary to their linear counterparts, circRNAs are generated by a non-canonical splicing mechanism, called back-splicing. A downstream 5' splice donor site from a pre-RNA transcript reacts with an upstream 3' splice acceptor site, which results in the 3' extremity of a downstream exon joining to the 5' extremity of an upstream exon. Therefore, the circularisation junction is formed of 2 mis-ordered exons (according to their genomic location). CircRNA back-splicing is an active process that can be promoted through various mechanisms (Fig. 1). For instance, many studies

Key points

- circRNAs contribute to liver cancer by regulating cellular processes involved in cancer onset and progression.
- circRNAs are currently mainly described as sponges regulating the activity of microRNAs but more diverse functions are emerging.
- circRNAs represent promising biomarkers due to the stability linked to their intrinsic circular structure.
- circRNAs are detected in body fluids, freely or embedded into vesicles.
- circRNA-based therapeutics represent a promising approach in cancer.

demonstrated that complementary inverted sequences localised into introns flanking the circularised exon junction could promote RNA circularisation.^{21,22} By hybridising to each other, inverted complementary sequences form a loop bringing distant splice sites into proximity, and thus facilitating back-splicing events. Further studies reported that circRNA biogenesis is driven by RNA-binding proteins (RBPs) located upstream and downstream of the circularised exons, promoting back-splicing by direct interaction or dimerisation. For example, by binding to specific intronic motifs, the RBP Quaking (QKI) induces circRNA biogenesis during epithelial-to-mesenchymal transition (EMT).²³ It was further demonstrated that artificial insertion of QKI motifs was sufficient to induce back-splicing. Altogether,

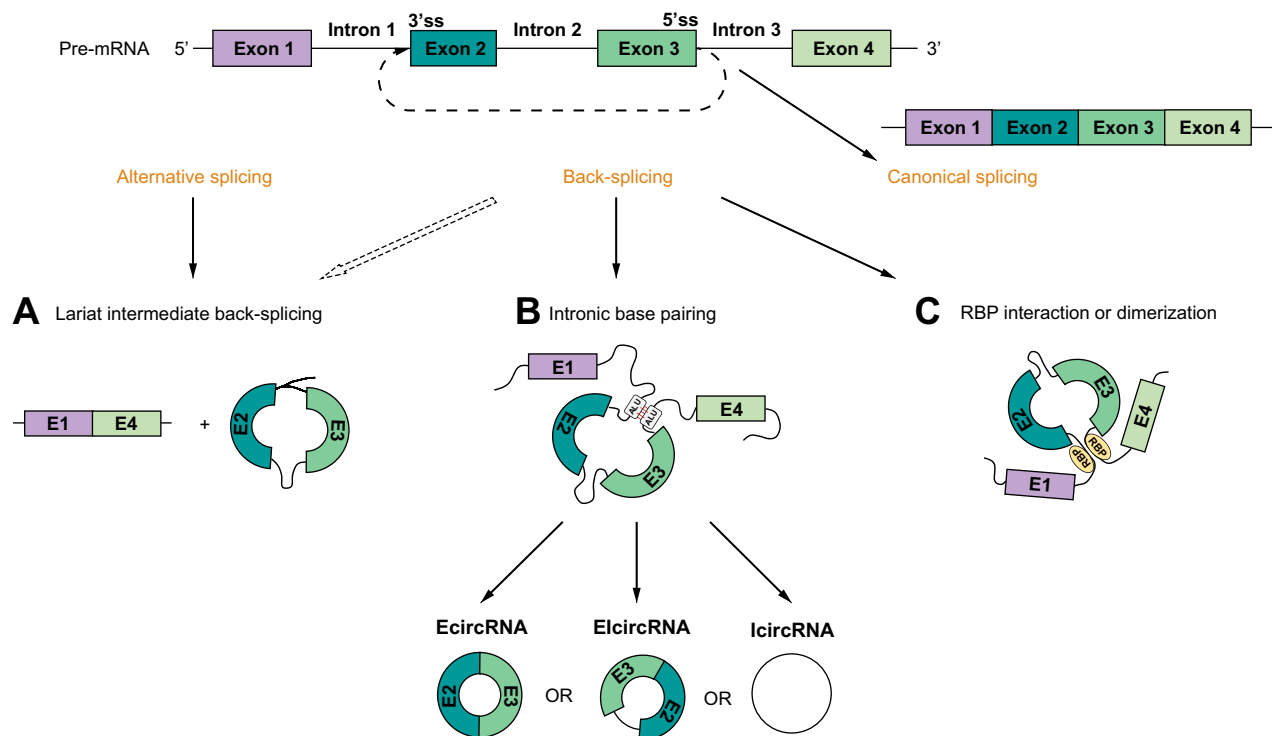


Fig. 1. The biogenesis of circRNA. CircRNAs are generated by a non-canonical back-splicing, where a 5' donor splice site (5'ss) of a downstream exon (here, exon 3, E3) will react with a 3' acceptor splice site (3'ss) of an upstream exon (here, exon 2, E2), resulting in the formation of a unique back-splice junction that does not exist in linear RNA (here, the 5' extremity of E2 with the 3' extremity of E3). So far, 3 main mechanisms spearhead the RNA circularisation. (A) Lariat intermediate back-splicing. The back-splicing process occurs after alternative splicing generates an intermediate structure called a lariat, which includes the excluded exons (here, E2 and E3) and which can be secondarily spliced. (B) Intronic base pairing. A direct physical proximity between distant splice sites allows the back-splicing process. This proximity is mediated by a complementary base pairing between the introns flanking the circRNA. (C) RBP interaction or dimerisation. Here, the physical proximity between 5'ss and 3'ss is mediated by RBP interaction/dimerisation located on the exons flanking the circularisation junction. These 3 main mechanisms result in the formation of either EcircRNA, ElcircRNA or IcircRNA. CircRNA, circular RNA; EcircRNA, exonic circRNA; ElcircRNA, exonic-intronic circRNA; IcircRNA, intronic circRNA; RBP, RNA-binding protein.

these data suggest that a combination of *cis*-acting elements and *trans*-acting factors bring into proximity a downstream 5' donor splice site with an upstream 3' splicing acceptor site, facilitating back-splicing events. Another so-called "lariat splicing" mechanism has also been reported to promote circRNA formation. During alternative splicing events, excluded exon(s) are incorporated into an intermediate circular lariat containing intron-exon structures, which could be spliced again to generate circRNAs^{7,11} (Fig. 1). While the majority of circRNAs are only comprised of exonic sequences (EcircRNA), a substantial portion of circRNAs also include retained intronic sequences. The so-called exonic-intronic circRNAs (EicircRNAs) have been reported to interact with U1 small nuclear ribonucleoproteins (snRNP), thereby regulating the transcription of their parental gene.²⁴ Likewise, some circRNAs consisting of only intronic sequences (IcircRNAs; lariat-derived circRNAs) have been reported.²⁵

CircRNA functions

Although circRNAs were discovered decades ago, defining their role remains a major challenge because of their low abundance and their sequence similarity with parental linear RNAs. To date, the most frequently reported mechanism of action of circRNAs is their capacity to sponge microRNAs (miRNAs) although other functions are emerging (e.g. transcriptional and post-transcriptional regulation, protein scaffolding) (Fig. 2).^{26,27} MiRNAs are small non-coding RNAs regulating gene expression at the post-transcriptional level. By binding the 3' untranslated regions of mRNAs, miRNAs repress mRNA translation and/or induce their degradation.²⁸ Accordingly, miRNA abundance and activity are tightly regulated. Notably, miRNAs can be sequestered by RNA sponges, including circRNAs.²⁹ Thus, CDR1 antisense circular RNA (circCDR1as) contains more than 70 miR-7 responsive elements. Through its sponging capability, circCDR1as acts as a competitive inhibitor of the tumour suppressor

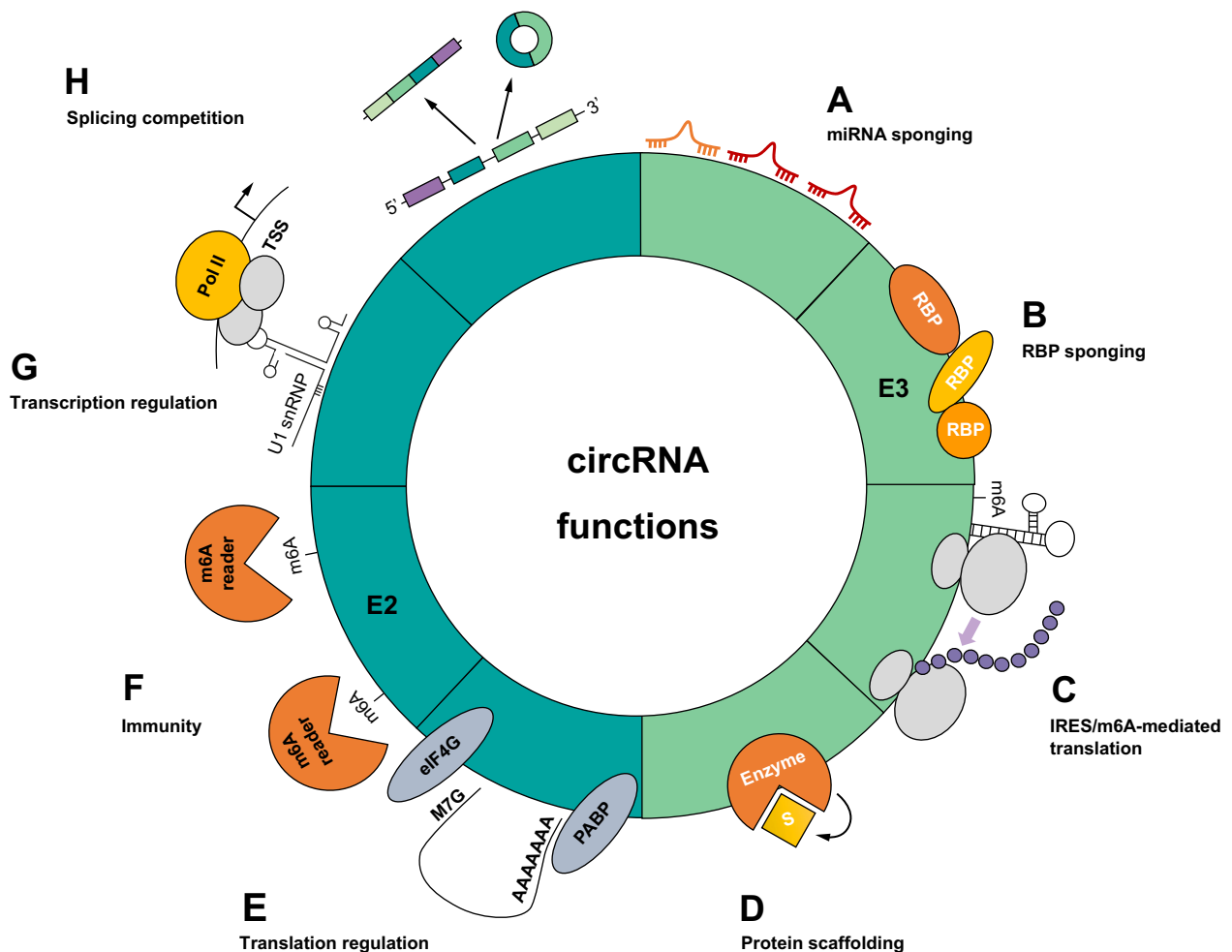


Fig. 2. CircRNA functions. (A) miRNA sponging: Numerous circRNA harbour miRNA response elements. Therefore, by sponging miRNA, circRNA act as competitive endogenous RNA, preventing miRNA post-transcriptionally binding to and repressing their natural targets. (B) RBP sponging: CircRNA display specific protein binding motifs offering them the capability to sequester RBP, regulate their activity and influence their localisation. (C) IRES/m6A-mediated translation: CircRNA containing IRES or with m6A epitranscriptomic modifications can be translated in a cap-independent manner. (D) Protein scaffolding: Proteins can be recruited by circRNA, facilitating enzymatic reactions. (E) Translational regulation: The translation initiation complex factors PABP and eIF4G are able to bind to circRNA. In this case, the interaction negatively regulates the translation initiation process. (F) Immunity: The endogenous m6A modification is necessary to distinguish self and non-self circRNA like those coming from viruses. (G) Transcription regulation: EicircRNA regulate transcription by interacting with U1 snRNP and promoting the transcription of their parental genes. (H) Splicing competition. Spliceosome machinery can foster circRNA biogenesis under specific conditions leading to the reduction of linear mRNA production. CircRNA, circular RNA; EicircRNA, exonic-intronic circRNA; IRES, internal ribosome entry sites; miRNA, microRNA; RBP, RNA-binding protein.

miR-7, preventing it from binding to and repressing its natural targets involved in tumour promotion.^{27,30} However, this feature is uncommon. Indeed, an expanded identification and characterisation of circRNAs in mammals reported that circCDR1as is only 1 of 2 circRNAs with an over-representation of binding sites for a single miRNA.³¹ In addition, it should be noted that circCDR1as is detected in circular but not in linear form, complicating interpretations of its role as a sponge for miR-7.²⁷ circRNA databases (e.g. CircInteractome, starBase) also predict the presence of circRNA/miRNA networks based on bioinformatics and/or experimental cross-linking immunoprecipitation (CLIP)-sequencing data.^{32,33} These databases also include information on RBP-circRNA interactions. Indeed, emerging evidence demonstrated that circRNAs can sponge RBPs, with functional consequences for their subcellular localisation and activity.³⁴ For example, it was recently demonstrated that circFOXK2 interacts with YBX1 and hnRNPK to promote the expression of NUF2 and PDXK oncogenes in pancreatic cancer.³⁵ In some cases, back-splicing also competes with canonical splicing. A typical example concerns muscleblind (*MBL* or *MBNL1* in humans) RNA which contains several intronic motifs which the MBL protein can bind to. When overexpressed, MBL binds to these specific intronic RNA motifs, thus promoting an exon 2 back-splicing event and reducing its own protein expression as a feedback mechanism.³⁶ Another study described a regulatory network involving YAP circRNA (circYAP) and its associated linear RNA. In breast cancer cell lines, it was demonstrated that circYAP interacts with YAP mRNA, PABP and the translation initiation protein eIF4G. This interaction inhibits the assembly of the translation initiation complex on YAP mRNA, resulting in the inhibition of translation.³⁷ Interestingly, circRNAs can promote transcription of their parental gene by interacting with U1 snRNP and the RNA polymerase II complex.²⁴ It is also important to emphasise that circRNAs may be coding, as demonstrated in *Drosophila* by ribosome footprinting sequencing specifically focused on reads that overlap with specific circRNA circularisation junctions.³⁸ Likewise, translation of circRNAs can be mediated by internal ribosome entry sites (IRES).^{39–41} In addition, emerging data suggest that circRNA translation could be driven by epitranscriptomic modifications, including N⁶-methyladenosine (m⁶A), in a cap-independent manner.⁴² Recent studies also demonstrated that the m⁶A modifications could distinguish self-circRNAs from immunogene foreign circRNAs (*i.e.* those coming from viruses).⁴³ Although the functions of circRNAs have not been fully elucidated, it is clear that circRNAs contribute to the regulation of gene expression. Thus, one can assume that the deregulation of circRNAs under pathological conditions will greatly affect fundamental processes required to maintain cellular and tissue homeostasis.

Overview of circRNAs in cancer: mechanisms and clinical relevance

As reviewed in 2020, circRNAs are involved in physiological processes by regulating gene expression and protein activity.⁴⁴ Growing evidence demonstrates that circRNAs are deregulated in cancer.^{2,4,44} Indeed, up- or downregulation of specific circRNAs correlates with clinical features like TNM stage, differentiation or survival.⁴⁵ In addition, the ratio between the expression of circRNAs and the corresponding linear RNAs can be modified, suggesting that back-splicing is an actively regulated process involved in cancer. In 2019, a loss-of-function screen using small-

hairpin RNAs (shRNAs) directed against highly expressed circRNAs indicated that 11.3% of circRNAs are necessary to promote cell growth in V16A prostate cancer cells. In contrast, among the related linear counterpart transcripts, 91.8% were not essential to fulfil this function.⁴⁶ Thus, by exhibiting tumour suppressive or oncogenic activities, circRNAs may contribute to cancer onset and progression. As mentioned above, a well-described function of circRNAs is their capability to sponge miRNAs, thereby promoting or suppressing tumour progression, depending on the nature of the miRNA targets.^{47,48}

Given that the field is still in its infancy, there is currently little overlap between studies,³ similar to what we observed years ago for miRNAs. Although the current literature has not yet enabled the identification of master oncogenic or tumour suppressive circRNAs, such as *RAS* or *TP53* for coding genes, several circRNAs have been detected in multiple cancers (e.g. circHIPK3, ciRS-7, circFOXO3, circMTO1). One archetype of a possible oncogenic circRNA is circHIPK3, which harbours 18 binding sites for sponging 9 tumour suppressor miRNAs involved in cell growth in several cancers, including breast cancer, colorectal cancer and HCC.⁴⁹ This observation has revealed new therapeutic opportunities, such as using target site blocker oligonucleotides to inhibit the sponging activity of circHIPK3. However, circHIPK3 was also reported to be downregulated in bladder cancer and involved in cell growth and metastasis inhibition, suggesting that circRNA function could be highly context-dependent, making it difficult to define a particular circRNA as oncogenic or tumour suppressive.^{3,50} Interestingly, circRNAs have also been shown to derive from master oncogenic or tumour suppressive genes, including circTP53, which was reported to promote colorectal cancer by sponging miR-876-3p and subsequently increasing cyclin-dependent kinase-like 3 expression.⁵¹ An exome capture RNA-sequencing project was recently conducted in more than 2,000 cancer samples, including cell lines, tumours from diverse organs and non-pathological tissues. Data were compiled into MiOncoCirc, the first international cancer-specific circRNA database.⁵

CircRNAs and the hallmarks of liver cancer

Liver cancer ranks sixth among the most prevalent cancers worldwide. Dramatically, both its incidence and associated mortality have steadily increased over the last decade. HCC and intrahepatic cholangiocarcinoma (iCCA) account for ~80% and ~15% of primary liver tumours, respectively.^{52–54} Both are associated with limited therapeutic options as they are frequently diagnosed late.^{53,54} Although our understanding of liver carcinogenesis has improved, notably through functional genomic studies, effective long-term targeted therapies are still lacking. By integrative genomics based on gene expression, the core transcriptional hallmarks of human HCC were previously highlighted, identifying potentially targetable signalling pathways that were commonly altered.⁵⁵ In 2011, Hanahan and Weinberg updated the hallmarks of cancer and described 10 hallmarks that govern the evolution of normal cells to a neoplastic state in most cancers.⁵⁶ Defining circRNAs that are involved in the hallmarks of liver cancer could provide new insights into liver carcinogenesis and open avenues for the development of new therapeutic options. However, to what extent circRNAs contribute to liver carcinogenesis still requires experimental investigations. In order to determine how circRNAs could contribute to liver cancer, we reviewed the current

literature on cancer hallmarks impacted by circRNAs (Fig. 3 and Table 1).

Hepatocellular carcinoma

Data from the recent literature suggest that circRNAs are relevant molecules involved in regulatory networks governing HCC onset and progression. While the role of circRNAs in cell proliferation, apoptosis, migration and invasion is rather well described, their role in angiogenesis, immune surveillance and metabolic switching is still under investigated. The involvement of circRNAs in enabling replicative immortality, another hallmark of cancer cells, has not been investigated in HCC.

CircRNAs in cell proliferation and resistance to cell death

Sustained proliferation is one of the distinguishing features of cancer cells. Some circRNAs exhibit tumour suppressor activities and display low expression in HCC (Table 1). For instance,

circMTO1 was downregulated in HCC and was reported to sponge miR-9, a well-known oncogenic miRNA. Reduced expression of circMTO1 in HCC results in increased miR-9 activity, leading to reduced expression of its natural targets, such as the cell cycle inhibitor p21. Accordingly, circMTO1 suppresses HCC progression and low expression of circMTO1 predicts poor overall survival.⁴⁷ Deregulation of cell proliferation pathways in HCC is also mediated by pro-oncogenic circRNAs. Among them, SCD-circRNA 2 was upregulated in HCC tissues. A gene regulatory network was proposed, in which the RBP RBM3 promotes HCC cell proliferation in a SCD-circRNA 2-dependent manner.⁵⁷ Oncogenic circRHOT1 was also related to sustained HCC cell proliferation by recruiting TIP60 (also known as KAT5), a member of the MYST sub-family of histone acetyltransferases, to the promoter of NR2F6 and subsequently enhancing its transcription.⁵⁸ Interestingly, NR2F6 has also been reported as a central intracellular immune checkpoint for cancer immune surveillance that suppresses adaptive anti-cancer immune responses.⁵⁹ An

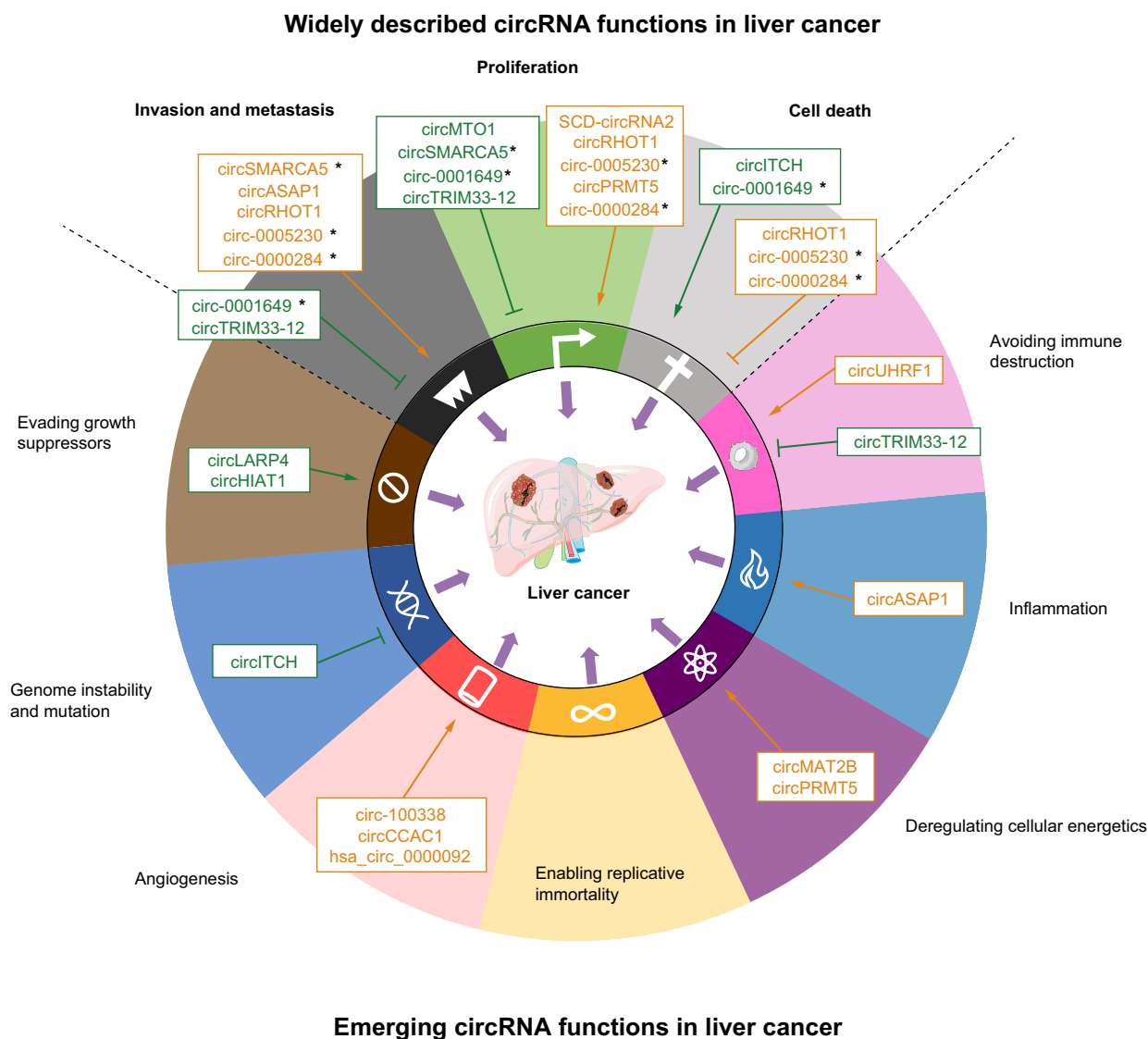


Fig. 3. CircRNA functions in liver cancer onset and progression. The upper part gathers the most described functions driven by circRNAs in liver cancer, while the lower part pinpoints potential emerging regulatory functions of circRNAs. Pro-oncogenic circRNAs are indicated in red and tumour suppressor circRNAs are indicated in green. CircRNAs deregulated in iCCA are marked by an asterisk. CircRNA, circular RNA; iCCA, intrahepatic cholangiocarcinoma.

Table 1. Reported functions of circRNAs in liver carcinogenesis.

Liver cancer	circRNA ID	circRNA function	Sponged molecules	Regulated targets	Reported functions
circRNA induced in HCC					
HCC	SCD-circRNA2*	RBP interaction	N/A	p-ERK ↑	Sustaining proliferation and metastasis
HCC	circRHOT1*	transcription regulation	NRF26 TIP60	NRF26 ↑	Sustaining proliferation, cell death, invasion and metastasis
HCC	circ-100338*	RBP interaction	N/A	MIMP9 ↑	Sustaining proliferation and metastasis, invasion and metastasis
HCC	circ-0000092*	miRNA sponging	miR-338-3p ↓	HN1 ↑	Sustaining proliferation and metastasis, invasion and metastasis
HCC	circPRMT5*	miRNA sponging	miR-188-5p ↓	HK2 ↓	Sustaining proliferation and metastasis energetics
HCC	circMAT2B*	miRNA sponging	miR-338-3p ↓	PKM2 ↑	Sustaining proliferation and metastasis energetics
HCC	circSAP1*	miRNA sponging	miR-326 ↓ miR-532-5p ↓	MAPK1 ↑ CSF-1 ↑	Sustaining proliferation and metastasis inflammation
HCC	circβ-catenin*	translated, decoy	N/A	β-catenin ↑	Sustaining proliferation and metastasis
HCC	circUHRF1*	miRNA sponging	miR-449c-5p ↓	TIM3 ↑	Sustaining proliferation surveillance escape
circRNA repressed in HCC					
HCC	circTRIM33-12*	miRNA sponging	miR-191 ↑	5hmc ↓	Sustaining proliferation surveillance escape
HCC	circH1AT1*	miRNA sponging	miR-3171 ↑	PTEN ↓	Sustaining proliferation suppresses growth
HCC	circLARP4*	miRNA sponging	miR-761 ↑	RUNX3 ↓ p53 ↓ p21 ↓	Sustaining proliferation suppresses growth
HCC	circMTO1*	miRNA sponging	miR-9 ↑	p21 ↓	Sustaining proliferation and metastasis
HCC	circITCH	miRNA sponging	miR-224-5p ↑	maf1 ↓	Sustaining proliferation cell death, genon
circRNA induced in CCA					
iCCA	circ-0000284*	miRNA sponging	miR-637 ↓	LY6E ↑	Sustaining proliferation cell death, invasion and metastasis
iCCA	circ-0005230*	miRNA sponging	miR-1238 ↓ miR-1299 ↓	N/A	Sustaining proliferation cell death, invasion and metastasis
iCCA	circACTN4*	miRNA sponging transcription regulation	miR-424-5p ↓	YAP ↑ FZD7 ↑	Sustaining proliferation invasion and metastasis
circRNA repressed in HCC and CCA					
HCC iCCA	circ_0001649*	miRNA sponging	miR-127-5p ↑ miR-612 ↑ miR-4688 ↑	SHIPR ↓	Sustaining proliferation cell death, invasion and metastasis
HCC iCCA	circSMARCA5*	miRNA sponging	miR-17-3p ↑ miR-181b-5p ↑	TIMP3 ↓	Sustaining proliferation and metastasis

circRNA, circular RNA; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; miR/miRNA, microRNA.

* *In vivo* functional confirmation in preclinical mouse models.

original mode of action of circ β -catenin, a circRNA derived from β -catenin, a well-characterised oncogene in liver cancer, has recently been reported.⁶⁰ Indeed, circ β -catenin is translated into a 370-amino acid β -catenin isoform and acts as a decoy. This shorter isoform can stabilise full-length β -catenin by antagonising GSK3 β -induced β -catenin phosphorylation and degradation, leading to activation of the Wnt pathway, and promoting tumour cell growth.⁶⁰

Another well-described feature of tumour cells is their ability to evade anti-proliferative signals governed by tumour suppressor genes (*i.e.* quiescence, cell cycle arrest, senescence, cell death).⁵⁶ Such properties are essential to maintain a pool of active transformed cells, notably by repressing or inactivating cell cycle regulators. For instance, a network involving circHIAT1, miR-3171 and the *PTEN* tumour suppressor gene was shown to contribute to HCC progression (Table 1). Thus, circHIAT1 downregulation in HCC was responsible for miR-3171 release, thereby promoting *PTEN* repression.⁶¹ Similarly, it was demonstrated that miR-671 activation, as a consequence of circLARP4 sponge downregulation, drives the silencing of master tumour suppressor genes RUNX3, CDKN1A and TP53 in HCC.⁶²

Programmed cell death by apoptosis is responsible for the removal of damaged cells. Notably, apoptosis serves as a bulwark against the propagation of transformed cells. Investigations into pro- and anti-apoptotic programmes will be critical to elucidate the paths whereby tumours succeed in progressing to states of high-grade malignancy and resistance to therapy.⁵⁶ Lately, circRNAs were reported for their entailment in signalling tracks governing the apoptotic machinery in liver cancer. Several studies identified circRNAs that can regulate apoptosis (Table 1). Pro-oncogenic circRNAs such as circRHOT1, circ-0005230, or circ0000284 have all been shown to exert anti-apoptotic actions on HCC and iCCA cell lines. Through intrinsic miRNA sponging properties, these circRNAs promote tumourigenesis by preserving anti-apoptotic factors, such as *NRF26* and *LY6E*, from degradation.^{58,63,64} Meanwhile, a decreased expression of circITCH and circ-0001649 contributes to cell death resistance by loosening the sponging of specific anti-apoptotic miRNAs. Thus, pro-apoptotic natural targets (*e.g.* MAFF and SHRPB) lack circRNA protection against miRNA degradation and are unable to counteract tumour cell spreading.^{65–67} Remarkably, most of these circRNAs are also involved in sustained abnormal cell proliferation, resulting in unbalanced cell homeostasis and cancer progression.

CircRNAs in invasion, metastasis and angiogenesis

Invasion and metastasis are tightly associated with EMT, a critical cellular process governing the plasticity of epithelial cells which lose adherent junctions, apico-basal polarity and acquire the abilities to resist apoptosis, to invade, and to disseminate.^{56,68} However, to what extent circRNAs are involved in acquiring mesenchymal features and a metastatic phenotype has not been extensively explored in liver cancer (Table 1). RNA-sequencing identified a signature of circRNAs that were significantly deregulated in HCC tissues, including circSMARCA5. Low expression of circSMARCA5 in HCC was attributed to the regulation of DEXH-box helicase 9 (DHX9), an abundant nuclear RNA helicase, and was shown to promote HCC cell growth and metastasis. One mechanism of action resulting from the reduced expression of circSMARCA5 involved a decreased expression of the metalloproteinase inhibitor TIMP3 due to the increased activity of miR-17-3p and miR-181b-5p, 2 miRNAs naturally

sponged by circSMARCA5. Further highlighting its importance in cancer, it was recently reported that circSMARCA5 can bind to its parent gene locus, forming an R-loop, which results in the downregulation of tumour-promoting SMARCA5 in breast cancer.⁶⁹ In glioblastoma multiform, circSMARCA5 is also a tumour suppressor and acts as a decoy for SRSF1 (serine and arginine rich splicing factor 1) through a GAUGAA binding motif, thus controlling cell migration and angiogenesis, notably by regulating *VEGFA* mRNA splicing.⁷⁰ In HCC, circSMARCA5 downregulation was associated with poor outcomes, including shorter overall and recurrence-free survival.⁴⁸ The role of circRNAs in HCC metastasis was further evaluated by circRNA expression profiling in 15 patients who displayed pulmonary metastasis and 15 patients that did not show any metastasis or recurrence. Hence, circASAP1 was specifically upregulated in HCC with a high metastatic potential. Gain and loss of function experiments demonstrated that circASAP1 promotes HCC cell metastasis by acting as a competitive endogenous RNA for miR-326 and miR-532-5p, leading to the induction of their common targets, the kinase MAPK1/ERK2 and CSF1, also known as macrophage colony-stimulating factor (M-CSF). By regulating these 2 targets, circASAP1 modulates HCC cell proliferation, invasion and tumour-associated macrophage (TAM) infiltration.⁴⁸ More recently, an original mechanism of action has been reported for circPABPC1, a tumour suppressive circRNA downregulated in HCC.⁷¹ Although circPABPC1 is predominantly localised in the cytoplasm, its small size (91 bases) makes it unlikely to be a miRNA sponge. Indeed, circPABPC1 functions as a protein scaffold for ITGB1, a key integrin involved in HCC metastasis. Frequent loss of circPABPC1 correlates with increased ITGB1 level in patients with advanced HCC. Mechanistically, circPABPC1 physically links ITGB1 to the proteasome 26S and promotes its degradation in a ubiquitin-independent manner, thereby reducing tumour cell adhesion, migration, and metastatic spreading.⁷¹

Tumour cell spreading requires a tailored vascular system to ensure its operational needs. Hence, during tumour progression, a new tumour-associated vasculature is generated by uncontrolled angiogenesis.^{56,72} *In silico* analysis suggested that circRNAs, including hsa_circ_0000092, could contribute to the tumour-associated angiogenic switch occurring during liver carcinogenesis (Table 1). Hsa_circ_0000092 is upregulated in HCC and significantly associated with shortened overall survival. A comprehensive analysis of both coding and non-coding RNAs suggested that hsa_circ_0000092 interferes with the negative regulation of haematopoietic- and neurologic-expressed sequence 1 (HN1) by miR-338-3p. Increased expression of oncogenic HN1 not only promotes HCC cell invasion and migration, but also angiogenesis.⁷³

CircRNAs in metabolic switching

An important hallmark of cancer is the adjustment of energy metabolism to support cancer cell proliferation. During carcinogenesis, cells classically switch towards aerobic glycolysis to meet their energy demands. This typical glycolytic reprogramming is associated with well-known oncogenes.^{56,74} However, there is still little evidence regarding the role of circRNAs in this process (Table 1). Meta-analysis of gene expression datasets demonstrated that circMAT2B was upregulated in HCC (compared to paired adjacent normal tissues) and strongly associated with glycolysis. More specifically, circMAT2B promotes glycolysis-dependent cell proliferation and migration

under hypoxic conditions both *in vitro* and *in vivo*. A regulatory axis was proposed in which circMAT2B positively regulates the miR-338-3p target gene pyruvate kinase M2, a key enzyme involved in glycolysis.⁷⁵ CircMAT2B is also clinically relevant since its elevated expression is associated with shortened overall survival.⁷⁵

CircRNAs in immune system surveillance escape and inflammation

It has long been recognised that tumour onset and progression are continuously monitored by the immune system. Thus, proliferating tumour cells have somehow developed the capability to evade immune surveillance.^{56,76} A recent study pinpointed that circTRIM33-12 downregulation in HCC was correlated with poor clinical features, suggesting its potential utility as an independent HCC risk factor (Table 1). Mechanistically, circTRIM33-12 expression was negatively associated with cell proliferation, migration and invasion through the miR-191-dependent positive regulation of TET1 demethylase. In addition, circTRIM33-12 was shown to participate in immune evasion via the regulation of activating receptor NKG2D (natural-killer group 2 member D) ligands. Thus, circTRIM33-12 regulates natural killer cell-, $\gamma\delta$ +T cell- and CD8+ T cell-mediated immune responses to cancers.⁷⁷

Inflammation is an essential mechanism to protect the body against injuries and pathogenic microorganisms. Nevertheless, in the context of carcinogenesis, substantial evidence indicates that the immune system could exert tumourigenic properties.⁷⁸ For instance, by releasing cytokines, infiltrated immune cells indirectly promote tumour growth and survival.⁷⁹ One study reported the involvement of circRNAs in tumour-promoting inflammation in HCC (Table 1). Hence, it was highlighted that an enhanced expression of circASAP1 indirectly induces CSF1 expression, a macrophage mitogenic factor, by sponging miR-532 and miR-326, and therefore promotes TAM recruitment.⁸⁰ There is growing evidence that TAM infiltration is strongly associated with tumour onset, progression and aggressiveness.⁷⁹ Thus, circASAP1 could promote HCC development by participating in TAM infiltration.⁸⁰

Intrahepatic cholangiocarcinoma

Only few studies have reported on the role of circRNAs in iCCA so far (Table 1). One study focused on circSMARCA5, a tumour suppressor circRNA already largely described for its role in the progression of several cancers, including HCC. The expression of circSMARCA5 was reduced in iCCA tissues and negatively correlated with advanced TNM stage and overall survival. Overexpression of circSMARCA5 was further associated with decreased iCCA cell proliferation.⁸¹ Very recently, circACTN4 has been shown to be induced in iCCA and to promote tumour cell growth and metastasis by regulating the Hippo and Wnt signalling pathways. Interestingly, circACTN4 has been reported i) to act as a sponge for miR-424-5p which could target YAP in the cytoplasm and ii) to recruit YBX1 to initiate FZD7 transcription in the nucleus. This study implies that circACTN4 acts as a signalling nexus allowing for the coordinated activation of Hippo and Wnt pathways in iCCA.⁸² However, the dynamic regulation of circRNA shuttling between the nucleus and the cytoplasm, in relationship to their function, is an important feature which remains to be elucidated. A couple of other studies identified aberrantly expressed circRNAs in both CCA cell lines and resected

iCCA tumours. Thus, circ-0001649 and circ-0005230 were respectively down- and upregulated in iCCA. These circRNAs were associated with cell proliferation, migration and invasion.^{63,67} Very recently, upregulation of circHMGCS1-016 was also reported to contribute to iCCA development and immune tolerance by sponging miR-1236-3p to regulate CD73 and GAL-8 expression.⁸³ Several circRNAs derived from CASC15, a transforming growth factor beta-induced long non-coding RNA, have also been associated with an inflammatory microenvironment in iCCA.⁸⁴ Most of the studies reported so far have focused on circRNAs within iCCA tumours. Nevertheless, circRNA are also found circulating in body fluids (e.g. embedded in extracellular vesicles [EVs]) and could therefore provide promising clinical opportunities. For instance, the expression of circ-0000284 was increased in CCA cell lines, tissues and plasma EVs. The circ-0000284-enriched EVs produced by CCA cells were able to enhance migration, proliferation and invasion of the normal surrounding cells both *in vitro* and *in vivo*.⁶⁴ These results not only highlight circRNAs as mediators of cellular communication but also as potentially promising biomarkers.

Limitations and challenges in interpreting data on circRNAs

While studies reporting on the functions of circRNAs in liver cancer are promising, it is important to point out that circRNAs' sponge activity is the major mechanism investigated so far (Table 1). Many of these studies rely on the cytoplasmic location of the circRNA of interest as a rationale to investigate miRNA activity, although this is where most exon-encoded circRNAs are located.^{7,19} Most studies also look for evidence of sponge activity by searching for any predicted miRNA binding sites in the circRNA and correspondingly expressed miRNAs and/or looking for interaction of the circRNA with proteins from the RNA-induced silencing complex (RISC). Although these strategies suggest interactions, they do not demonstrate function and should be complemented with loss of function or depletion studies to validate a functional effect on miRNAs. It is also important to consider that essentially all of the sequences attributed to circRNAs are contained in the parental mRNA, so unless the circRNA is more abundant than the mRNA or is somehow locking the miRNA into the RISC, it is hard to understand the stoichiometry to explain how circRNAs can effectively act as sponges in many cases. Similarly, a circRNA contains the same mRNA sequence encoded by the same exons (with the exception of the back-splice) so that ectopic expression of circRNA and mRNA from the same gene could have the same effect. This point should be addressed in functional studies given that in many cases, the expression of circRNAs tends to follow the expression of the mRNAs, so increases in expression of circRNAs are usually associated with increased expression of mRNA. However, given that the median half-life of circRNAs is estimated to be at least 2 to 5 times higher than that of linear mRNA,^{6,7,85} one can hypothesise that the contribution of circRNA to RNA sponging activity is greater than that of their linear counterpart. Thus, the dynamic of mRNA splicing and translation, the half-life of circRNA vs. mRNA, as well as their sub-cellular location, should be considered, as these features may contribute to the differential sponge activity of circRNA vs. mRNA. Additional studies, including Ago-CLIP followed by long read sequencing without RNase R treatment, will be needed in the future to better understand the stoichiometry of each

Table 2. CircRNA as biomarkers for liver cancer diagnosis and prognosis.

Liver cancer	circRNA ID	Biological sample	Biomarker relevance	Ref.
Upregulated circRNA				
HCC (HBV related)	circRNA-100338	HCC tissue	Prognostic	114
HCC (HBV related)	hsa_circ_0000976	plasma	Diagnostic	115
	hsa_circ_0007750 hsa_circ_0139897			
HCC (HBV related)	hsa_circ_0027089	plasma	Diagnostic	116
HCC (early stage)	circ-CDYL	HCC tissue	Surveillance biomarker, early diagnostic	95
HCC	circRNA ciRS-7 (Cdr1as)	HCC tissue	Microvascular invasion	117
HCC	circ_0008450	HCC tissue	Prognostic	118
HCC	circRHOT1	HCC tissue	Prognostic	58
HCC	circBIRC6	HCC tissue	Prognostic	119
HCC	hsa_circ_0000517	HCC tissue	Prognostic	120
HCC	hsa_circ_0128298	HCC tissue	Diagnostic, prognostic	121
HCC	hsa_circ_0091579	HCC tissue	Diagnostic, prognostic	122
HCC	hsa_circ_0016788	HCC tissue	Diagnostic, prognostic	123,124
HCC	circPCNX	HCC tissue	Prognostic	125
HCC	hsa_circ_0056836	HCC tissue	Diagnostic, prognostic	126
HCC	hsa_circ_0005075	HCC tissue	Diagnostic, prognostic	127,128
HCC	hsa_circ_0091581	HCC tissue	Prognostic	129
HCC	hsa_circ_0128298	HCC tissue	Diagnostic, prognostic	121
HCC	circ-10720	HCC tissue	Prognostic	130
HCC	circRNA-101368	HCC tissue	Prognostic	131
HCC	circMAT2B	HCC tissue	Prognostic	75
HCC	circ_0000267	HCC tissue	Prognostic	132
HCC	circ_001569	HCC tissue	Prognostic	133
HCC	circRNA-104718	HCC tissue	Prognostic	134
HCC	hsa_circ_0067934	HCC tissue	Prognostic	135
HCC	SCD-circRNA 2	HCC tissue	Prognostic	57
HCC	circRNA_0000502	HCC tissue	Prognostic	136
HCC	circPTPRM	HCC tissue	Prognostic	137
HCC	circPVT1	HCC tissue	Prognostic	138
HCC	circRNA MYLK	HCC tissue	Prognostic	139
HCC	circABCB10	HCC tissue	Prognostic	140
HCC	circ-ZNF652	HCC tissue	Prognostic	141
HCC	hsa_circ_0103809	HCC tissue	Prognostic	142
HCC	circ_0021093	HCC tissue	Prognostic	143
HCC	hsa_circ_0000673	HCC tissue	Prognostic	144
HCC	hsa_circRNA_103809	HCC tissue	Prognostic	145
HCC	circZFR	HCC tissue	Prognostic	146
HCC	circMET	HCC tissue	Prognostic	147
HCC	circ-HOMER1	HCC tissue	Prognostic	148
HCC	circFBXO11	HCC tissue	Prognostic	149
HCC	circ-TCF4.85	HCC tissue	Diagnostic	150
HCC	circBACH1	HCC tissue	Diagnostic, prognostic	151
HCC	circRNA-SORE	HCC tissue	Sorafenib resistance	99
HCC	hsa_circ_0003998	HCC tissue, plasma	Diagnostic, prognostic	152
HCC	circASAP1	HCC tissue, plasma, plasma exosomes	Prognostic	80
HCC	circ-ZEB1.33	HCC tissue, serum	Diagnostic, prognostic	153
HCC	circRNA_101237	HCC tissue, serum	Prognostic, cisplatin resistance	154
HCC	circ-FOXP1	HCC tissue, serum	Diagnostic, prognostic	155
HCC	circ_0000798	Peripheral blood mononuclear cells	Diagnostic, prognostic	156
HCC	circ_104075	Serum	Diagnostic	94
HCC	hsa_circ_000224	Serum	Diagnostic	157
HCC	circPTGR1	Serum exosomes	Prognostic	158
CCA	circCdr1as	CCA tissue	Prognostic	159
Downregulated circRNA				
HCC	hsa_circ_0001649	HCC tissue	Prognostic, diagnostic	160
				161
HCC	circMTO1	HCC tissue	Prognostic	47
HCC	circZKSCAN1	HCC tissue	Diagnostic	162
HCC	circSETD3	HCC tissue	Prognostic	97
	hsa_circ_0000567			
HCC	hsa_circ_0028502	HCC tissue	Diagnostic	163
HCC	hsa_circ_0076251	HCC tissue	Diagnostic, prognostic	163
HCC	circ-ITCH	HCC tissue	Prognostic	65
HCC	hsa_circ_0068669	HCC tissue	Diagnostic, prognostic	164
HCC	hsa_circ_0003570	HCC tissue	Diagnostic	165
HCC	hsa_circ_0078602	HCC tissue	Diagnostic, prognostic	166
HCC	hsa_circ_0004018	HCC tissue	Diagnostic	167
HCC	circC3P1	HCC tissue	Prognostic	168

(continued on next page)

Table 2 (continued)

Liver cancer	circRNA ID	Biological sample	Biomarker relevance	Ref.
HCC	circTRIM33-12	HCC tissue	Prognostic	77
HCC	hsa_circ_0091570	HCC tissue	Diagnostic, prognostic	169
HCC	circRNA_101505	HCC tissue	Prognostic cisplatin resistance	170
HCC	circHIAT	HCC tissue	Prognostic	61
HCC	circADAMTS13	HCC tissue	Prognostic	171
HCC	circ-EPHB4	HCC tissue	Diagnostic	172
	hsa_circ_0001730			
HCC	circSMARCA5	HCC tissue, plasma	Diagnostic, prognostic	48,173
HCC	circADD3	HCC tissue, plasma	Diagnostic, prognostic	174
HCC	hsa_circ_0001445	Plasma	Diagnostic	175
HCC	hsa_circ_0051443	Plasma exosomes	Diagnostic	176
HCC	hsa_circ_00156	Serum	Diagnostic	157
HCC	hsa_circ_000520	Serum	Diagnostic, prognostic	157

CCA, cholangiocarcinoma; CircRNA, circular RNA; HCC, hepatocellular carcinoma.

molecule implicated in the sponging of miRNAs. It should also be noted that even if the aforementioned studies performed experimental validations using animal models (subcutaneous injection of cancer-derived cell lines stably over- or under-expressing a specific circRNA), the readout of these experiments is mainly tumour growth. Therefore, even though *in vivo* experiments confirmed the tumour suppressive or pro-oncogenic role of circRNAs, further investigations are needed to validate their involvement in several of the hallmarks of cancer. This aspect could be critical if circRNAs' function is context-dependent and should be considered in view of emerging strategies for specific knockdown of circRNAs *in vivo*.⁸⁶

CircRNAs: clinical opportunities in liver cancer

CircRNAs are promising clinically relevant biomarkers

In addition to the new insights on their regulatory functions, circRNAs also provide good opportunities to improve the management of patients with liver cancer. Indeed, even if most circRNAs are expressed at a low level (roughly <10% of the expression of their linear counterpart),⁸⁷ their circular structure provides a higher resistance to exonuclease activity and therefore an expanded half-life. Such intrinsic physical features mean that circRNAs could be promising diagnostic and prognostic biomarkers. In addition, circRNAs are found circulating in liquid biopsies (e.g. blood, urine, saliva, bile),^{5,88} either freely or embedded into EVs.⁸⁹ A recent study demonstrated that circRNAs are more specifically enriched and stable in exosomes compared to host secretory cells.⁹⁰ Therefore, exosomal circRNAs could be useful minimally invasive biomarkers. They provide clear benefits over protein biomarkers which are prone to degradation and present limited organ specificity.⁹¹ In addition, methodologies used for circRNA detection are usually more specific and sensitive than the immunoassays commonly used for protein biomarkers.⁹² Indeed, as nucleic acids, circRNAs can be easily and specifically amplified by reverse-transcription quantitative PCR using specific primers overlapping the back-splice junction.³⁴ It was further demonstrated that circRNAs display tissue- and cell-specific expression patterns,¹ a feature which could be helpful for diagnosis. Thus, circRNAs appear as promising molecules for the management of patients with cancer, as they exhibit many of the features expected of a biomarker: they are often more stable than linear RNAs, including miRNAs, and require minimally invasive intervention to be detected in body fluids by specific and sensitive methods. Accordingly, several ongoing clinical trials are exploring circRNAs as

biomarkers in cancer, including biliary tract cancers (e.g. NCT03334708, NCT04584996, NCT04792437). However, it should be noted that a high level of circRNAs from serum/plasma may also only reflect a high level of circulating cells, as was reported for miRNAs.^{3,93}

CircRNAs for liver cancer diagnosis and prognosis

Recent profiling studies support the utility of circRNAs as potential diagnostic and prognostic biomarkers in patients with liver cancer (Table 2). However, these studies currently suffer from limited sample sizes; hence, a robust signature of circRNAs commonly deregulated in patients with liver cancer remains elusive. Despite this weakness, merging the results of 3 independent studies identified circ_104075 as a circRNA recurrently upregulated in tissues and sera of patients with HCC compared to healthy individuals.⁹⁴ Its expression is particularly high in HCC compared to other types of cancer and liver disease, suggesting that it could represent a biomarker for HCC diagnosis.⁹⁴ Further statistical analyses demonstrated that circ_104075 exhibited a better sensitivity and specificity than biomarkers commonly used to diagnose HCC, such as alpha-fetoprotein.⁹⁴ Such observations could be practice changing given that alpha-fetoprotein serum dosage and ultrasound, which are routinely used to diagnose early stage HCC, sometimes lack precision.⁵⁴ Thus, patients could benefit from these innovative specific biomarkers if they enable the early detection of HCC. Similarly, circCDYL was expressed during the early stages of HCC, and thus might be a clinically relevant biomarker for early diagnosis and surveillance in high-risk populations, including patients with hepatitis B or C infection and/or cirrhosis.⁹⁵

The prognostic potential of circRNAs has also been reported in liver cancer. A recent meta-analysis in HCC highlighted a tight correlation between an elevated expression of pro-oncogenic circRNAs and poor clinical outcomes such as reduced overall survival.⁹⁶ Two other studies reported that low expression of circMTO1 and circSETD3 in HCC was significantly associated with poor prognosis.^{47,97} In the same vein, it was shown that low levels of circSMARCA5 in iCCA correlate with poor overall survival. Interestingly, overexpression of circSMARCA5 in iCCA cell lines improves chemosensitivity to gemcitabine and cisplatin.⁸¹ Likewise, downregulation of circ-0003418 resulted in cisplatin resistance in HCC cells through activation of the Wnt/ β -catenin pathway.⁹⁸ Lately, circRNA-SORE was reported to mediate sorafenib resistance in HCC by stabilising YBX1, a master oncogenic factor. CircRNA-SORE acts by trapping YBX1 and thus, preventing its degradation by the E3 ubiquitin system. More interestingly,

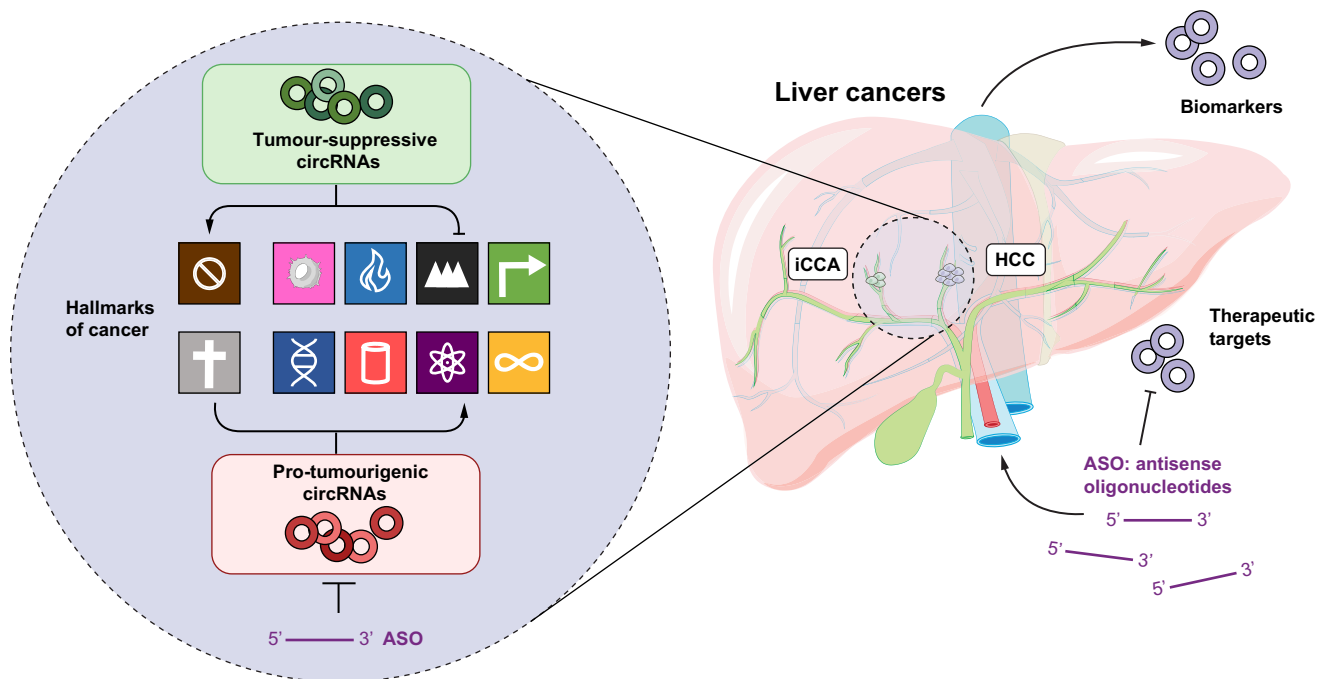


Fig. 4. CircRNAs as innovative biomarkers and therapeutic targets in liver cancer. The left panel highlights tumour-suppressive and pro-tumourigenic circRNAs acting on well-known cancer hallmarks. The right panel highlights circRNAs as innovative biomarkers and therapeutic targets using ASOs. ASO, antisense oligonucleotide; circRNA, circular RNA; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma.

circRNA-SORE-related features spread across cancer cells through exosomal communication.⁹⁹ These observations raise the possibility of developing innovative circRNA biomarkers to predict response to therapy.

CircRNAs as companion biomarkers for targeted therapies in liver cancer

Several targeted therapies are currently under clinical evaluation in liver cancer.¹⁰⁰ In this context, the development of robust diagnostic tools to select the most suitable personalised therapy could be valuable. In HCC, nivolumab, an immune checkpoint inhibitor targeting PD-1, was approved by the FDA in 2017. However, objective anti-tumour response rates were only 15-20%.^{54,101} Therefore, a circRNA signature predictive of anti-PD1 response might be relevant. This innovative concept involving the development of companion biomarker tests from circRNA signatures may represent a breakthrough to guide targeted therapies. So far, to the best of our knowledge, there is no published report of circRNAs as companion biomarkers and/or theranostic targets that can predict response to a specific therapy, either in HCC or in iCCA.

Future directions: circRNAs and therapeutic opportunities

As mentioned above, there is growing evidence showing that circRNAs are deregulated in cancer and contribute to the regulation of oncogenic processes (Fig. 3). Thus, circRNAs might be considered as relevant therapeutic targets in cancer. The singularity of circRNA regulatory functions offers various opportunities to regulate their action during tumour progression and therefore, to develop innovative therapeutic strategies.

The pro-oncogenic activity of numerous circRNAs relies on sponging of tumour suppressor miRNAs.^{47,48} Thus, several strategies could be developed to reduce this pro-oncogenic activity, including the development of specific target site blockers (TSBs). TSB antisense oligonucleotides (ASOs) could be designed to target miRNA response elements carried by circRNAs. Thus, by competitive binding, TSBs could restore the tumour suppressor activity of a given miRNA. Although this strategy has not been applied to circRNAs yet, it showed great efficacy when applied to linear RNA sponges *in vitro* and *in vivo*.¹⁰² Another strategy aiming at directly decreasing circRNA abundance could be even more efficient. For that purpose, antisense locked nucleic acid gapmers specifically targeting the back-splice junction of circRNAs could switch off the expression of a specific circRNA without affecting the expression of the parental linear RNA. Several studies already demonstrated the efficacy of this approach not only with gapmers, but also with small-interfering RNAs and shRNAs. Besides, it was shown that circHIPK3 silencing in mice *via* adeno-associated virus shRNA could alleviate diabetic proliferative retinopathy.¹⁰³ CRISPR/Cas13-based RNA editing systems¹⁰⁴ could also be used to specifically silence circRNA activity. In addition, the abundance of pro-oncogenic circRNAs could be modulated at an upstream level by interfering with the splicing machinery to control the occurrence of back-splicing events. Indeed, it was already reported that circRNA biogenesis during EMT is tightly regulated by the RBP splicing factor QK15.²³ Thus, one could hypothesise that modulating the expression level of a specific RBP could control the expression of circRNAs. Otherwise, as indicated above, there is strong evidence that circRNA biogenesis is mediated by hybridisation of complementary

intronic regions flanking circRNA sequences.^{21,22} Thus, one might anticipate that targeting these complementary sequences on pre-messenger RNAs using ASOs may block circRNA biogenesis by preventing the interaction of back-spliced regions. On the contrary, ASOs targeting key splice domains could be designed to enhance circRNA biogenesis. Recently, a base editing strategy targeting key back-splice domains showed great efficacy to specifically repress circRNA expression without affecting the linear counterpart RNAs (bioRxiv; <https://doi.org/10.1101/2021.08.05.455347>). This study provides an efficient method to deplete circRNAs for functional studies.

Nucleic acid-based therapeutics represent a promising approach in cancer. To date, several RNA-based drugs like ASOs are already FDA approved, and many are under clinical evaluation,¹⁰⁵ paving the way for circRNA-based therapeutics. Indeed, circRNAs are often more stable than mRNA or miRNA molecules, and methods have already been developed to produce synthetic circRNAs.¹⁰⁶ The advantage of this approach is the possibility of designing specific circRNAs. For example, a synthetic circRNA containing 5 binding sites for miR-21, an overexpressed onco-miRNA, was shown to effectively suppress miR-21 activity and to induce apoptosis in gastric cancer cell lines.¹⁰⁷ Similarly, synthetic circRNAs could be designed to sequester oncoproteins. Indeed, a specific artificial circRNA able to sequester and inactivate the RBP hnRNP has already been engineered.¹⁰⁸

Considering that RBPs participate in cancer progression, especially via splicing deregulation, this proof-of-concept study opens new avenues for promising circRNA-based therapeutics. In addition, it was demonstrated that exogenous circRNA can efficiently produce proteins *in vitro*.¹⁰⁹ Thus, circRNA carrying IRES could be specifically engineered to express tumour suppressor proteins.

Conclusions

A plethora of therapeutic strategies are being developed to target circRNAs in liver cancer. Although proof-of-concept studies have reported promising results, it must be taken into account that circRNAs are still newly described protagonists in cancer onset and progression. Indeed, a great deal of effort is needed to fully understand the physiological roles, the regulatory functions and the biogenesis of circRNAs. A better understanding of these molecular mechanisms will provide a deeper insight into the specific role of circRNAs in the whole RNA regulatory network governing cancer hallmarks. This knowledge could eventually pave the way for circRNA-mediated molecular therapies and for clinically relevant biomarkers in liver cancer (Fig. 4). Thus, as an emerging field, circRNAs represent a wealth of opportunities for future research.

Abbreviations

ASO, antisense oligonucleotide; CCA, cholangiocarcinoma; circRNA, circular RNA; CLIP, cross-linking immunoprecipitation; EMT, epithelial-to-mesenchymal transition; EVs, extracellular vesicles; HCC, hepatocellular carcinoma; HN1, haematopoietic- and neurologic-expressed sequence 1; IRES, internal ribosome entry sites; miRNA, microRNA; NGS, next-generation sequencing; QKI, Quaking; RBP, RNA-binding protein; RISC, RNA-induced silencing complex; shRNA, small-hairpin RNA; snRNP, small nuclear ribonuclear proteins; TAM, tumour-associated macrophage; TSB, target site blockers.

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Conflict of interest

The authors declare no competing interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

All the authors contributed to all aspects of the work. CL and DL contributed equally as co-first authors.

Supplementary data

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Author names in bold designate shared co-first authorship

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Targeting the tumor microenvironment in cholangiocarcinoma: implications for therapy

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REVIEW



Targeting the tumor microenvironment in cholangiocarcinoma: implications for therapy

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ABSTRACT

Introduction: Cholangiocarcinoma (CCA) is a rare, deadly cancer that is characterized by an abundant desmoplastic stroma. Late diagnoses and limited available effective treatments are major problems with this malignancy. Targeting of the tumor microenvironment (TME) has emerged as a potential therapeutic strategy.

Areas covered: In this review, we describe the role of the various compartments of the TME in CCA and focus on the preclinical rationale for the development of innovative therapies. Relevant literature was identified by a PubMed search covering the last decade (2010–2020).

Expert opinion: Low efficacy of surgery and cytotoxic chemotherapy emphasizes the need for new therapeutic strategies and companion biomarkers. Single-cell RNA sequencing of the stroma is yielding a critical functional characterization of TME in CCA and is paving the way for immunotherapies and cancer-associated fibroblast and extracellular matrix-oriented treatments. We believe that the development of treatments targeting the components of the TME will produce the best results if in combination with cytotoxic chemotherapy. Biomarkers should be developed to define the patient population of interest for each combination strategy.

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Angiogenesis; cancer-associated fibroblast; cholangiocarcinoma; combined therapy; extracellular matrix; immunotherapy; stroma; therapeutic targets; transcriptomics; tumor microenvironment

1. Introduction

Cholangiocarcinoma (CCA) is a deadly cancer worldwide with increased incidence and limited effective therapeutic options. CCA comprises different types of solid tumors arising along the biliary tree. Based on the anatomic location, intrahepatic (iCCA), perihilar (pCCA) and distal CCA (dCCA) are distinguished (Figure 1) [1,2]. The incidence of CCA is currently increasing in Western countries [1,3]. Treatment of CCA presents numerous challenges, from basic understanding of CCA heterogeneity to clinical aspects related to the silent nature of the disease [1]. Surgery remains the treatment of reference for curative intent. However, a majority of patients could not have their tumor removed as a result of locally advanced or metastatic disease at the time of diagnosis [4]. Adjuvant chemotherapy was proven effective, although the benefit was modest and the majority of patients still recur within 5 years [5–7]. In the advanced setting, chemotherapy offers palliation and increases modestly the survival. However, the median overall survival (OS) is still approximately only 1 year after first-line and only 6 months after second-line treatment [8,9]. Intensive chemotherapy regimen recently failed to improve results [10]. The existence of molecular alterations in biliary tract cancers allows the development of targeted therapies [11]. However, the development of drugs is rendered difficult by the multiplicity of the targets in a population of patients with relatively rare tumors [12].

As for many types of cancers, targeting the tumor microenvironment (TME) offers another attractive avenue for drug development, which is particularly relevant in CCA. Indeed, CCA is recognized as a tumor with an important desmoplastic reaction, due to the stromal cells, and the infiltration of the tumor by different types of immune cells (Figure 2). Changes in the TME of CCA were also associated with prognosis in multiple studies [13]. Importantly, targeting the TME could also be conducted in combination with other treatment modalities, including cytotoxic chemotherapy and tumor cells targeted therapies, with opportunities for synergies.

In this review, we will describe the role of different compartments of the TME in CCA, focusing on the preclinical rationale for the development of new therapies. We will finally propose our opinion on how the development of innovative treatments targeting the TME could be conducted in CCA.

2. TME is a clinically relevant histological hallmark of CCA

Growing evidence demonstrates that TME contributes to cancer onset and progression in various cancers, including CCA [14–16]. The presence of a complex desmoplastic stroma is a distinctive histological feature of CCA (Figure 2A–B). TME consists of an intricate ecosystem made of non-tumor cells interacting with components of the extracellular matrix (ECM) and soluble factors (Figure 2C). A wide variety of cells

Article highlights

- CCA is a deadly cancer with increasing incidence. It has limited effective therapeutic options and is characterized by a dense desmoplastic stroma.
- Targeting of the tumor microenvironment (TME) is emerging as a therapeutic strategy in cholangiocarcinoma
- Gene expression profiling of the stroma provides information on the role of TME in CCA onset and progression and is paving the way for the design of effective immunotherapies.
- Targeting genetic alterations of the cancer cells and TME represents a promising strategy for treating CCA and overcoming drug resistance.
- Targeting the TME should be conducted in combination with cytotoxic chemotherapy. Relevant biomarkers are needed to define the patient population of interest for each combination strategy.
- Cytotoxic chemotherapy will continue to play a role in the foreseeable future, especially in patients with no targetable molecular alterations. In contrast, targeting molecular alterations appears very promising and will probably become available in the next few years.

This box summarizes key points contained in the article.

populate the TME, including endothelial cells, pericytes, cancer-associated fibroblasts (CAFs) and immune cells [13]. The dynamic crosstalk between the TME and CCA tumor cells modulates cancer-associated processes, including cell death and proliferation, angiogenesis, invasion, metastasis, and chemoresistance [17]. Soluble factors such as cytokines, chemokines, growth factors, and extracellular vesicles play a key role in orchestrating these interactions [14,18,19].

The clinical relevance of the stroma has been reported in several solid tumors, including breast cancer, colorectal cancer, and CCA [20–22]. Importantly, these studies suggest that the stroma may exhibit a protective or an aggravating value depending on the cancer types. Thus, in breast cancer,

patients with a stroma-high tumor have a worse prognosis compared to patients with a stroma-low tumor. In addition, the prognostic value of the tumor–stroma ratio is most discriminative in patients with grade III or triple-negative breast cancer [21]. In resectable colorectal cancer, an artificial intelligence algorithm based on a convolutional neural network model using transfer learning demonstrated that the tumor–stroma ratio is an independent predictor of prognosis, a high amount of stroma being associated with a reduced OS [22]. Similarly, a nationwide population-based study in Denmark reported that a high amount of stroma is an independent prognostic marker of an adverse outcome in patients with stage II colon cancer [23]. However, in iCCA, the stromal to tumor area ratio was inversely correlated with vascular invasion and positively correlated with a well-differentiated tumor grade. Actually, iCCA with more fibrous and less cellular features were associated with a better outcome. Patients with high stromal area exhibit a better disease-free survival (DFS), suggesting that desmoplastic stroma is protective in iCCA, possibly through a limitation of tumor invasiveness by building a barrier against tumor diffusion [20]. In addition, a high collagen reticulation index, defined as the number of collagen fiber branches within the entire length of the collagen network, was associated with a poorer OS [20]. Thus, not only the abundance of the stroma but also its reticulation and stiffness may influence the properties of the stroma and ultimately its prognostic value.

3. Transcriptomic profiling highlights TME heterogeneity in CCA

In 2013, by combining laser capture microdissection and gene expression profiling, we highlighted transcriptomics alterations occurring in stromal cells of iCCA. A signature made of

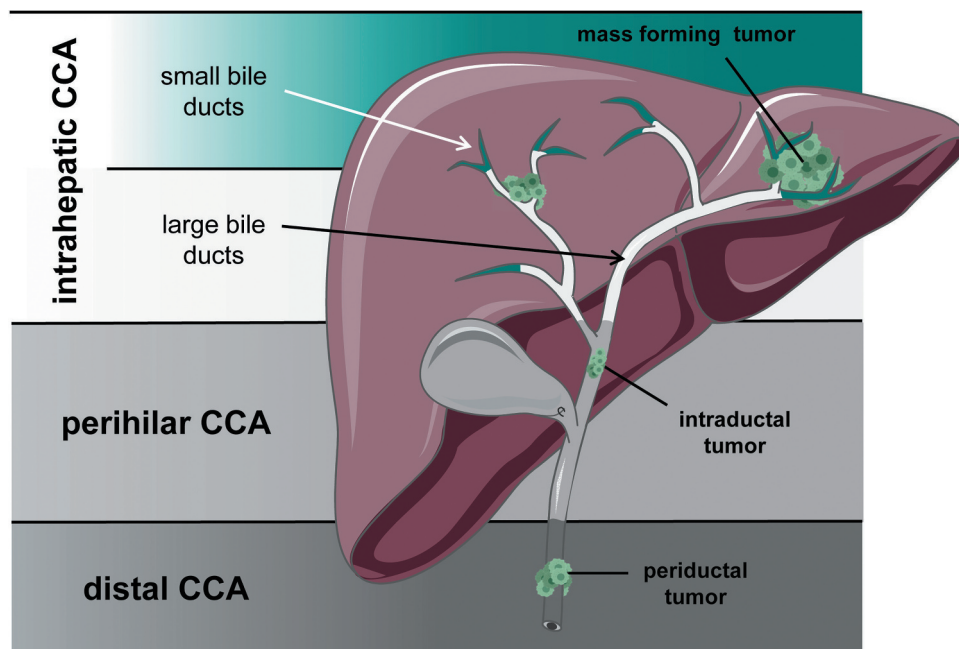


Figure 1. Anatomical classification of CCA malignancies along the biliary tract. Adapted with permission from Louis C, Papoutsoglou P, Coulouarn C. Molecular classification of cholangiocarcinoma. *Curr Opin Gastroenterol.* 2020 Mar;36(2):57–62.

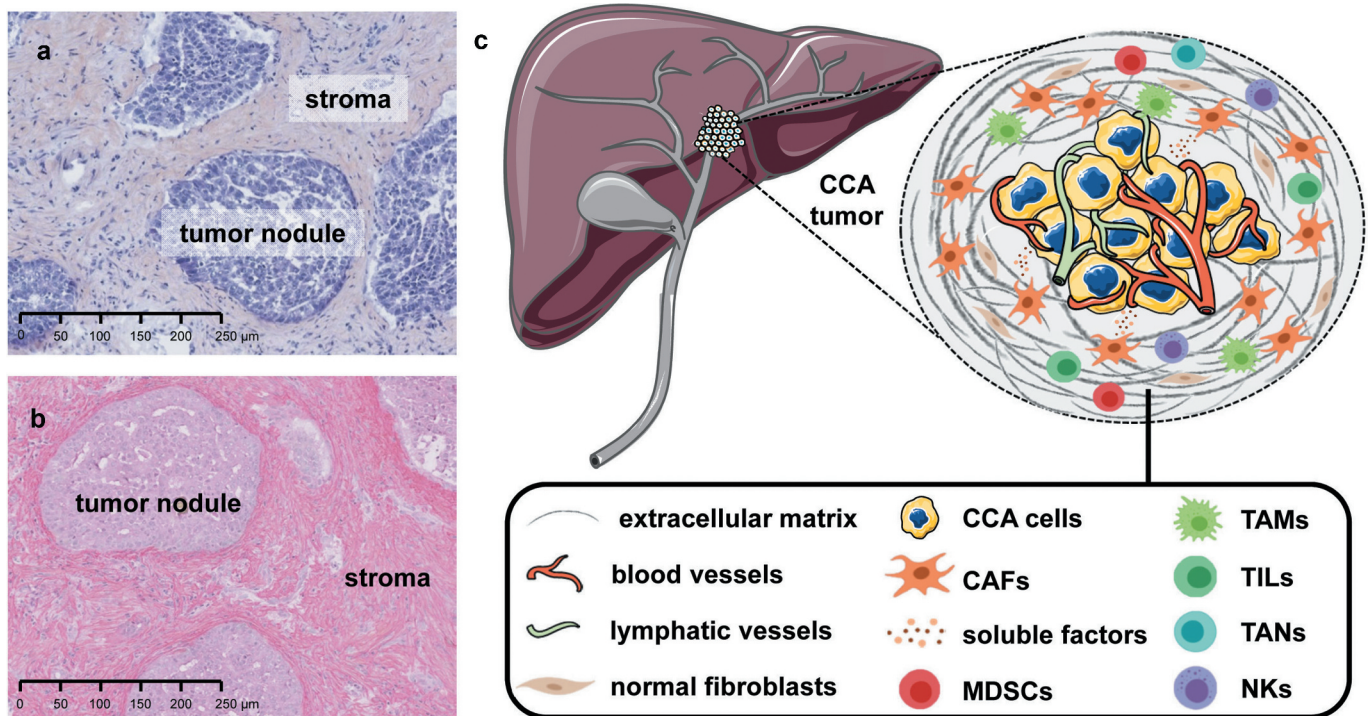


Figure 2. Overview of the tumor microenvironment (TME) in CCA. (A) Hematoxylin and Eosin staining of a representative human CCA tumor tissue. (B) Sirius red staining of a representative human CCA tumor tissue. Both staining highlights the abundant stroma in CCA. (C) Schematic overview of the intricate ecosystem constitutive of the TME. This environment is populated with a wide variety of cells including CCA cancer cells, cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), tumor-infiltrating lymphocytes (TILs), tumor-associated neutrophils (TANs) and natural killer cells (NKs). These cells interact together as well as with components of the extracellular matrix (ECM) and soluble factors within the TME to modulate CCA onset and progression.

1,073 genes was shown to discriminate the stromal compartment of iCCA tumors from the fibrous tissues in the adjacent non-tumor livers [24,25]. Notably, we demonstrated that osteopontin and TGF β 2 are both overexpressed in the stroma of iCCA. The expression of these two genes exhibited a heterogeneous expression and a high expression was demonstrated to correlate with a poor outcome, highlighting interesting therapeutic targets [24,25]. More recently, the same approach was used to pinpoint the functional features and diversity of stromal cells [26]. The study demonstrated that the TME of iCCA displays a large range of immunologic orientations and can be classified into four subtypes based on the cellular composition, including an immune desert subtype versus immunogenic, myeloid and mesenchymal subtypes. Importantly, this stromal classification is predictive of patient outcome [26]. Such immune-based classification might serve as a basis to design clinical trials evaluating specific targeted therapies, as we will develop in section 5.

These early transcriptomics studies mostly based on the microdissection of large fibrous areas highlighted the heterogeneity of the stroma in CCA and its complex transcriptional architecture. Deciphering the multifaceted nature of the stroma represents a great challenge, at both a basic and a translational level, to develop effective therapies. Addressing that particular issue, a recent study in iCCA took advantage of single-cell RNA-sequencing to unravel the complexity of the stroma at a cellular and molecular level [27,28]. This transcriptomic profiling at a single cell resolution highlighted a wide heterogeneity in the malignant, immune and

stromal cells. Notably, the study identified 6 distinct fibroblast subsets. The predominant vascular-associated fibroblast subclass displayed a signature enriched for the microvasculature and showed a massive secretion of IL-6. Through functional analyses, the fibroblast-mediated secretion of IL-6 was shown to affect CCA cells malignancy through epigenetic mechanisms [28].

In summary, these transcriptomic studies allowed a better characterization of the alterations occurring in the TME of CCA, highlighting a high degree of transcriptional heterogeneity. They also provided a better insight into the interactions between tumor cells and TME, and they pave the way for the development of innovative therapeutic options targeting several components of TME.

4. CAFs diversity offers various therapeutic orientations in CCA

4.1. CAFs are central protagonists in the TME of CCA

Once the desmoplastic process is well engaged, the most predominant cells in the stroma are fibroblast-like cells referred to as cancer-associated fibroblasts (CAFs). CAFs form a heterogeneous group of cells characterized by the expression of phenotypic markers, including alpha-smooth muscle actin (α -SMA) and platelet-derived growth factor receptor beta (PDGFR β) [29]. The origin of CAFs remains ambiguous in CCA. CAFs may derive from a wide variety of cells in the liver including portal fibroblasts, hepatic stellate cells (HSC) and bone marrow-derived

mesenchymal precursor cells [16]. CAFs are first recruited by CCA tumor cells which subsequently sustain their activity. Once activated, CAFs promote CCA progression through the secretion of various soluble factors. For instance, transforming growth factor beta (TGF β), stromal cell-derived factor 1 (SDF1), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF) and epidermal growth factor (EGF) are extensively secreted by CAFs [30]. CAFs are central protagonists in the TME (Figure 3). They are involved in multidimensional interactions and influence the activity of a wide variety of cells including immune cells, lymphatic endothelial cells (LECs) and CCA tumor cells. For instance, Cadamuro and colleagues described a paracrine loop within the tumor-reactive stroma of CCA, unleashed by PDGFD and involving CCA tumor cells, CAFs and LECs [29]. By secreting PDGFD, tumor cells induce the release of VEGFC and VEGFA by CAFs leading to the expansion of the lymphatic vasculature and tumor cell intravasation (Figure 3). This study highlights CAFs as potential targets in the TME for the treatment of CCA. Indeed, inducing CAFs apoptosis or targeting PDGFD-induced axis may represent an effective tool to block tumor-associated lymphangiogenesis and impede CCA tumor cell invasion [29].

CAFs are also involved in a reciprocal interplay with immune cells. Notably, CAFs expressing the fibroblast activation protein (FAP) are part of a specific fibroblast subset exhibiting a pro-inflammatory phenotype. Growing evidence show that FAP⁺ CAFs could mediate tumor-promoting inflammatory and immunosuppressive effects (Figure 3). More specifically, FAP⁺ CAFs are able to foster the recruitment of myeloid-derived suppressor cells (MDSCs) in a STAT3 signaling-dependent manner [31,32].

4.2. CAFs-oriented therapeutic strategies

CAFs phenotype is clinically relevant for CCA treatment and represents an independent prognostic factor for OS [33]. Over the last decade, targeting CAFs has therefore been considered as a robust therapeutic strategy in CCA. For instance, by antagonizing PDGFR β and VEGFR3 (through imatinib mesylate), or by neutralizing VEGFA and VEGFC (respectively through SU5416 and SAR131675), the trans-LEC migration of CCA cells was alleviated. Moreover, a navitoclax-induced CAFs depletion triggers the reduction of the tumor-associated lymphangiogenesis in a rat model of CCA [29]. A combined administration of gemcitabine and nintedanib, an antifibrotic drug, was recently shown to reduce iCCA aggressiveness in mice through the reduction of pro-oncogenic soluble factors secreted by CAFs such as IL-6 and IL-8. Thus, the growth of iCCA xenografted tumors was shown to be significantly reduced [34]. Photothermal depletion of CAFs was also shown to normalize tumor stiffness in a mouse model of desmoplastic CCA [35]. We also recently reported that tumors developed from CCA cells resistant to EGFR inhibition in mice exhibited a more prominent stromal compartment enriched in CAFs [36]. CAFs were shown to contribute to chemoresistance of CCA cells by producing IGF2. Thus, the study provides a rationale for testing combined therapies against EGFR and IR/IGF1R in CCA [36].

These recent studies contributed to better appreciate the diversity of CAFs and their respective functionalities in CCA, opening promising opportunities for clinical trials targeting CAFs (Figure 3).

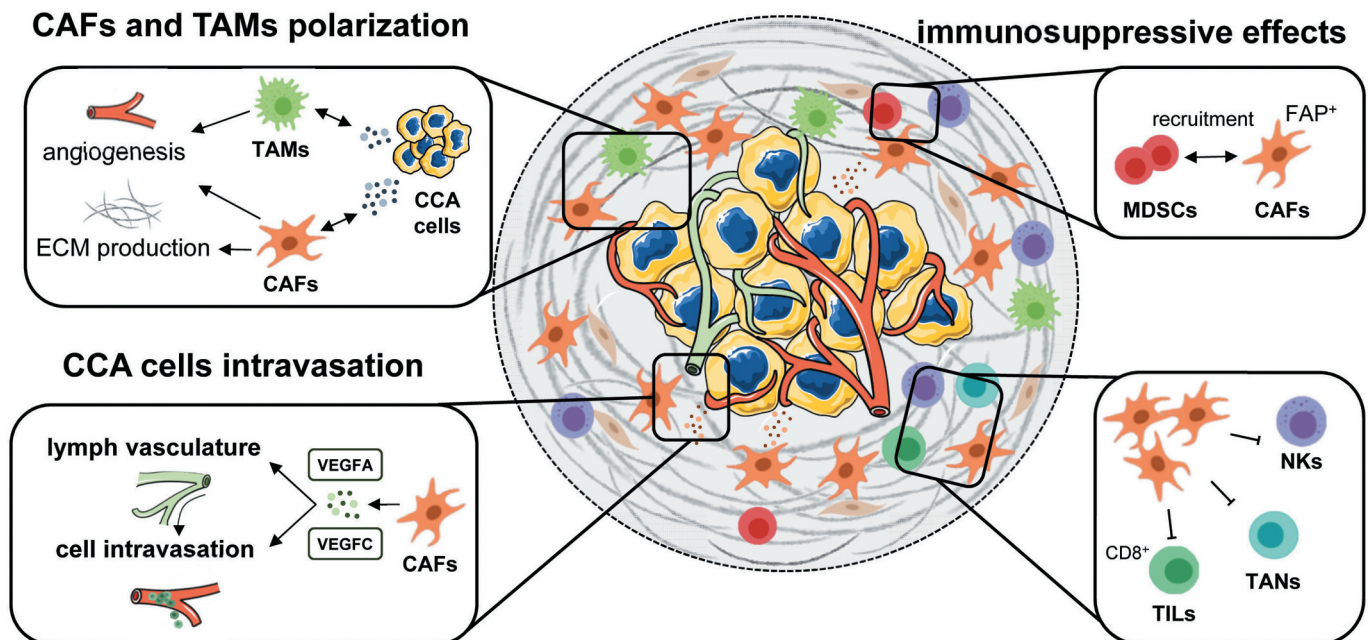


Figure 3. Examples of mechanisms by which the tumor microenvironment fosters CCA onset and progression. (A) Interactions of cancer-associated fibroblasts (CAFs) and/or tumor-associated macrophages (TAMs) with CCA tumor cells promote the secretion of various soluble factors to induce extracellular matrix (ECM) remodeling and angiogenesis. (B) By secreting platelet-derived growth factor D (PDGFD), tumor cells induce the release of vascular endothelial growth factor A (VEGFA) and C (VEGFC) by CAFs leading to the expansion of the lymphatic vasculature and tumor cell intravasation. (C) Mechanisms developed by tumors to escape immune surveillance. Notably, fibroblast activation protein-positive (FAP⁺) CAFs mediate tumor-promoting inflammatory and immunosuppressive effects through the recruitment of myeloid-derived suppressor cells (MDSCs) in a STAT3 signaling-dependent manner. Immunosuppressive effects of CAFs on tumor-infiltrating lymphocytes (TILs), tumor-associated neutrophils (TANs) and natural killer cells (NKs) are shown.

5. Targeting ECM in CCA

The abundant presence of CAFs in the TME is also responsible for an aberrant production of ECM. CCA progression, dissemination, and treatment are critically supported by ECM [37]. ECM is a highly reactive scaffold formed by a wide array of multifunctional molecules, encompassing collagens and non-collagenous glycoproteins, proteoglycans, glycosaminoglycans, and polysaccharides [37]. ECM remodeling is a key feature of CCA carcinogenesis, modulating signaling pathways involved in tumor progression. ECM is also a reservoir of soluble factors acting in the crosstalk between tumor cells and the stroma (Figure 2) [38]. Accordingly, the characterization of ECM composition and structure constitutes a promising line of investigation from a prognostic perspective [39]. As mentioned above, ECM proteins (e.g. osteopontin) are clinically relevant biomarkers associated with patient prognosis. In addition, growing evidence demonstrated that the overexpression of periostin (POSTN) and distinct mesothelin forms can predict a poor prognosis in a rat desmoplastic model of CCA [40]. Mechanistically, ECM proteins, including POSTN, tenascin-C and osteopontin were shown to modulate pro-tumorigenic functions by activating specific intracellular pathways, such as PI3K/AKT or MAPK, and thus promoting tumor cell proliferation, migration, and invasion [37]. An exhaustive characterization of ECM at the protein level was very recently emphasized by Carpino and colleagues who reported proteome profiles specific of the TME in iCCA. This matrisome unraveled increased levels of fibrillar collagens and POSTN. The stiffness of the desmoplastic stroma was also explained by the near absence of proteoglycans and elastic fibers. A wide range of proteins involved in the maintenance of the basement membrane were also found downregulated [41]. Notably, evidence was provided that COL3A1 promotes iCCA cells migration as a component of tumor-associated aligned collagen, supporting COL3A1 as a promising therapeutic target to hamper tumor expansion and metastatic dissemination [41]. Interestingly, COL3A1 expression was reported as a prognostic biomarker in early-stage ovarian carcinoma patients, being associated with a shorter overall survival [42]. Similarly, COL3A1 overexpression was shown to confer a poor prognosis (reduced OS and DFS) in patients with bladder cancer [43]. In a mouse model of bleomycin-induced pulmonary fibrosis associated with ECM deposition, histone deacetylase inhibitors were shown to decrease COL3A1 expression, opening interesting therapeutic opportunities targeting ECM components [44].

Changes in the composition and the structure of ECM within TME can also negatively impact the response to therapies [45]. Indeed, both the density and the stiffness of the stroma can create an impenetrable barrier, concealing tumor cells from therapeutic agents. Steric hindrance also leads to an impaired supply in nutrients, oxygen, and metabolites resulting in an increased hypoxia and metabolic stress, which could lead to the activation of anti-apoptotic and drug resistance pathways. In addition, modification of the ECM components have been reported to be involved in the response of tumor cells to radiotherapy [46]. Accordingly, addressing the TME in early drug discovery has been recommended as a strategy to

overcome drug resistance and identify novel targets for cancer therapy [45].

In the light of the functional impact of ECM components on tumor progression and resistance to treatment, it is natural to consider degrading this fibrous stroma as a therapeutic strategy to improve the care of patients with CCA. For instance, current therapies employed for CCA could be combined with a targeting of collagen or hyaluronan synthesis to improve their efficacy. The regulation of pathways upstream ECM synthesis could also represent an option. Notably, inhibition of the TGF β signaling pathway to reduce ECM stiffness could be a relevant strategy. However, given that the abundance of the desmoplastic stroma has been recently associated with a better outcome in iCCA [20], it seems crucial to consider not only the amount of ECM deposition but also the composition, organization, and post-translational modifications of ECM. Accordingly, unsatisfactory results were obtained with pegylated recombinant human hyaluronidase combined with chemotherapy in patients with metastatic pancreatic cancer in a phase 1b/2 clinical trial [47].

6. Targeting the immune microenvironment in CCA

The better understanding of the tumor immune microenvironment (TIME) led to the recent revolution of immunotherapy in oncology [48]. Mechanisms of the immune surveillance were described, and consequently also the mechanisms developed by tumors to escape this immune surveillance, focusing primarily on CD8 + T cells effectors [49–51]. The most widely immune checkpoints targeted to date for immunotherapy are programmed death-1 (PD-1) and its ligand PD-L1, as well as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), with numerous drugs targeting these pathways. Moreover, multiple other immune checkpoints are currently the focus of new drug development, and targeting of other effectors than CD8 + T cells is also studied. Here, we will describe the current knowledge about the role of TIME in CCA, starting from the type of cells then moving to functionality, in each case focusing on prognostic information related to the TIME.

6.1. Description of the tumor immune microenvironment in CCA

The study of all types of immune cells seems to be important to completely understand prognosis of CCA [52]. Regarding the adaptive immune system, lymphocytes are the main effectors of anti-tumor immune responses [53]. Tumor-infiltrating lymphocytes (TILs) have been described in CCA and are associated with the prognosis [54]. This is the case for both iCCA and eCCA [52,55]. Specifically, the presence of CD8+ lymphocytes seems to be associated with a better prognosis [51]. Regarding the innate immune system, Natural Killer (NK) cells are important components of tumor surveillance [56]. Epidemiological data suggest that polymorphisms of important genes related to NK cells functions (KIR and NKG2D) might be associated with the risk of developing CCA [57,58], suggesting an important role of these cells on the immune surveillance against CCA. Furthermore, the infiltration by NK

cells has been associated with better survival in resected iCCA [59]. Tumor-associated macrophages (TAMs) are important regulators of the tumoral immune response. In the liver, TAMs can be derived from Kupffer cells or from circulating bone-marrow-derived macrophages. More importantly, TAMs are divided between the M1 subtype, favoring anti-tumor immune response, and the M2 subtype, exerting immunosuppressive effects [60]. The presence of TAMs, and specifically of M2 TAMs has been associated with worse prognosis in CCA [61,62]. Moreover, CCA cells were shown in vitro to interact with macrophages, providing them with tumor-promoting capacities [63,64]. TAMs could also stimulate directly CCA tumor cells, notably through the Wnt/beta-catenin pathway [65]. Albeit usually considered of less importance, tumor-associated neutrophils could also play an important prognostic role in CCA [52].

6.2. Functional description

The importance of the immune infiltration in a subset of CCA could also be studied by gene expression analysis [66]. In a study of resected iCCA, Sia and colleagues described two classes of tumors, the proliferation and the inflammation classes. However, this analysis was done on the bulk of the tumors. As mentioned previously, a recent analysis focused on the stromal component, using laser microdissection [26]. The gene expression profile of the TME could then describe 4 TME subtypes. Subtype I1 displayed an immune desert, subtype I2 showed a reactive immunogenic pattern, subtype I3 displayed a myeloid-rich infiltrate, while subtype I4 was dominated by activated fibroblasts. These different subtypes were associated with clear differences in prognosis [26].

The functionality of the cells is of course of paramount importance, as we suggested when discussing the differences between M1 and M2 TAMs. One first evaluation could be done through the expression of immune checkpoints. Expression of PD-L1 was associated with infiltrations by TAMs and neutrophils [67]. There is conflicting evidence about the potential prognostic role of the expression of PD-L1 in CCA [68,69]. Technical issues and the selection of antibody might influence the results [70]. A meta-analysis suggested that PD-L1 expression was related to the tumor stage, which might explain discrepancies in results of various studies, and that overall PD-L1 expression was not associated with prognosis [71]. Another study suggested that PD-L1 expression depends on the etiology of iCCA [72]. Finally, the type of cells expressing PD-L1 might also influence the prognosis: TAMs expressing PD-L1 might be particularly important [73]. CTLA-4 expression might also have a prognostic role [74]. Expression on the adjacent tissue might be specifically important [75]. Finer evaluation of the functionality of the cells could be critical, using for example multiparametric flow cytometry or cytometry by time of flight (CyToF) but has rarely been performed in CCA. A study in eCCA using immunohistochemistry suggested that CD8+ or PD-L1+ cells were not associated with survival, but that focusing on CD8+ CD54RO+ memory cells, a clear prognostic value was demonstrated [76]. In conclusion, more insight may be gained using refined techniques to characterize the functionality of the TIME in CCA.

6.3. Consequences for drug development

The role of the TIME in CCA, but mainly the efficacy of immune checkpoint inhibitors (ICI) in other tumor types, led to the development of clinical trials. Despite early disappointing results when ICI were used as monotherapy, phase 3 trials were launched, and results are awaited. However, these trials did not use selection based on the immune contexture. In case of negativity, testing ICI in specific subgroups based on a biomarker might prove more interesting.

7. Targeting angiogenesis

Targeting angiogenesis through VEGF inhibition showed modest results in many tumor types. As in most solid tumors, angiogenesis plays an important role in the development of CCA, and was showed to have prognostic value [77]. Regorafenib, a multitarget tyrosine kinase inhibitor targeting notably VEGFR2, was showed to have modest activity in CCA [78]. However, recent focus changed to using inhibition of VEGF in combination with other treatments. The concept of vascular normalization partially explains possible synergy between VEGF inhibition and improved penetration of other treatments inside the tumor [79]. Moreover, VEGF also plays a regulating role for immune cells, favoring by numerous ways the immunosuppressive components of the TIME, such as Tregs and M2 TAMs [80]. Finally, other angiogenic cytokines, such as TIE-2, also act in interplay between angiogenesis and immune infiltration [81]. It is thus probable that targeting angiogenesis in CCA will play a role only in combination with other treatment strategies.

8. Past and ongoing clinical trials targeting TME in CCA

The better characterization of different components of the TME has only recently lead to clinical trials. The development of immune-oncology lead to the trials of different anti-PD-(L)1 inhibitors. However, results were overall disappointing when the drugs were used as monotherapy [82]. Nevertheless, recent results of combination with chemotherapy might prove more interesting [83]. Based on these results, different phase 3 trials were launched comparing standard chemotherapy to the combination of chemotherapy with anti-PD-(L)1 (clinicaltrials.gov identifiers NCT04003636, NCT03875235). The targeting of the TGF β pathway is currently being tested in the clinics. Specifically, the TGF β inhibitor galunisertib is currently under investigation for the treatment of metastatic pancreatic adenocarcinoma in combination with durvalumab (NCT02734160), and in different settings of hepatocellular carcinoma (as monotherapy NCT02240433 or in combination with sorafenib NCT02178358). However, no clinical trials were conducted in CCA. Finally, angiogenesis inhibition was tested in 2 trials using regorafenib, with interesting results [78,84]. Advantages and limitations of the current strategies targeting the TME in CCA are summarized in Table 1.

9. Conclusion

Studies performed over the last decade clearly highlighted the important role played by the TME in CCA, not only in tumor

Table 1. Current strategies used for targeting the TME in CCA.

Strategies	Advantages	Limitations
Anti-PD-(L)1 monotherapies	Validated in many tumor types Easily available Low toxicity	Low efficacy No predictive factors of response High costs
Anti-PD-(L)1 – chemotherapy combinations	Validated in many tumor types Easily available Acceptable toxicity	No predictive factors of response High costs
TGF β inhibition	Low toxicity	No predictive factors Efficacy in combination with anti-PD-(L)1 inhibitors understudied
Angiogenesis inhibition	Certain level of efficacy Toxicity of Regorafenib	Potential synergy in combination with anti-PD-(L)1 inhibitors

onset and progression, but also in the lack of efficacy of current treatments. Large-scale genomic approaches demonstrated the extreme heterogeneity of TME, at the molecular and the cellular level. Clinically relevant subtypes of CCA were delineated based on TME specific features and relevant cell types and/or signaling pathways were identified for possible therapeutic targeting.

10. Expert opinion

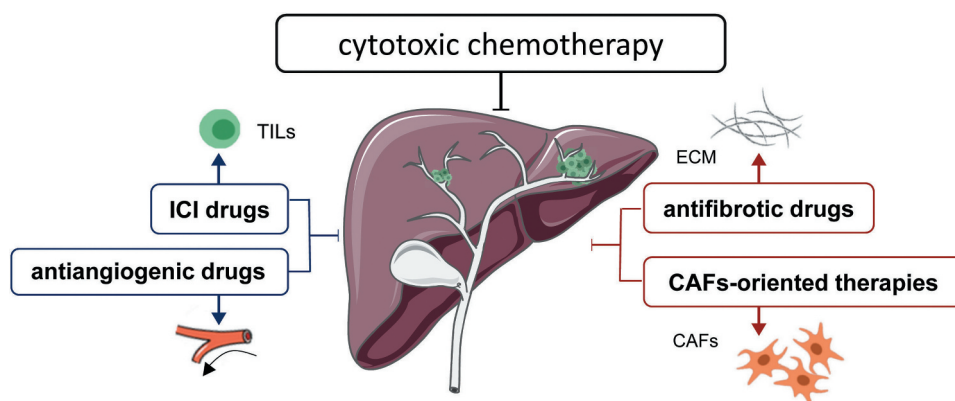
Cytotoxic chemotherapy remains the mainstay of the medical treatment of CCA. However, its limitations are numerous, and do not appear to be easily overcome by novel drugs. Cytotoxic chemotherapy will probably continue to play a role in the foreseeable future, especially in patients with no targetable molecular alterations. In contrast, targeting molecular alterations appears very promising and will probably become available in the next few years [11]. However, such treatments frequently have other limitations, such as resistance mechanisms, and difficulty to penetrate the desmoplastic stroma of CCA. Also, they can only be applied in the subgroup of patients whose tumor harbors specific alterations and for which a drug is available. Limitations of these strategies centered on the tumor cells could be mitigated by combination with treatments targeting the TME. Indeed, targeting the CAFs could help other treatments to exert direct cytotoxic effects. In return, targeting the TME could only produce significant clinical efficacy if another treatment refrains the proliferation of tumor cells.

Different components of the TME also show complex interplays. The TME interacts with endothelial cells and CAFs. Positive effects of combination of ICI and antiangiogenics

drugs were already demonstrated in multiple tumor types. Such combination of treatment targeting different components of the TME might thus be a promising approach for developing new treatment strategies in CCA.

Combination strategies of different modalities might also be an important mean to avoid the emergence of resistance mechanisms. Selection pressure with a monotherapy usually leads in clinics to the development of a clone of tumor cells resistant to the treatment. The emergence of a resistant clone would be mitigated if a treatment targeting an alternative pathway is combined. The emergence of a resistance clone could also be less probable if normalization of the vascularization occurs following use of an antiangiogenics drug, enabling for stronger inhibition in the whole bulk of the tumor.

Another important parameter for the development of alternative treatment strategies will be the discovery of relevant biomarkers. As the screening for molecular alterations guide the treatment with targeted therapies, similar strategies could be applied with other treatment modalities. We have seen that there is inter-patient heterogeneity in the immune infiltrate of CCA; this is thus probable that a 'one-fit-all' approach will not lead to the best results. If we apply this reasoning to the subtype of TME described by Job and colleagues, we might propose that the I1 subtype of tumors with an immune desert should receive a treatment favoring immune infiltration; subtype I2 showing a reactive immunogenic pattern could best be treated by ICI; subtype I3 displaying a myeloid-rich infiltrate might be best treated with ICI-antiangiogenics combination; finally, the subtype I4 dominated by activated fibroblasts should be treated with drugs inhibiting fibroblasts activation [26]. Thus, a rationale drug

**Figure 4.** Outlook on the therapies targeting the CCA tumor microenvironment in combination with cytotoxic chemotherapy.

development based on building adequate biomarkers for each drug is clearly an important prerequisite to success of targeting the TME.

Additional significant difficulty in the development of these strategies is the multiple actors involved (different pharmaceutical companies developing drugs; different academic groups focusing on different treatment strategies), sometimes competing for developing treatments at the same time for the same patient population. While the development of a biomarker might be the best option when focusing on patient's benefit, short-term development of a 'one-fit-all' approach is frequently easier to conduct. Coordination of different actors in a strategy trying to combine targeting of different pathways is also an important challenge. Finally, an important difficulty of the combinations is also the risk for increased, sometimes synergistic toxicities precluding effective application in the clinical setting.

Thus, we believe that the development of treatment targeting the TME will produce the best results if combined with cytotoxic treatments (chemotherapy and/or targeted therapies depending on the molecular alterations presented by the tumor), as well as a combination of treatments targeting different components of the TME (Figure 4). Positive results would also be clearer if biomarkers are developed to define the population of interest for each combination strategy.

Declaration of interest

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Molecular classification of cholangiocarcinoma

Corentin Louis*, Panagiotis Papoutsoglou*, and Cédric Coulouarn

Purpose of review

Cholangiocarcinoma (CCA) are heterogeneous tumors that arise from the malignant transformation of cholangiocytes along the biliary tree. CCA heterogeneity occurs at multiple levels and results in resistance to therapy and poor prognosis. Here, we review the molecular classification of CCA by focusing on the latest progresses based on genetic, epigenetic, transcriptomic and proteomic profiles. In addition, we introduce the emerging field of radiogenomics.

Recent findings

Genome-wide integrative omics approaches have been widely reported by using large cohorts of CCA patients. Morphomolecular correlations have been established, including enrichment of *FGFR2* gene fusions and *IDH1/2* mutations in iCCA. A specific *IDH* mutant iCCA subtype displays high mitochondrial and low chromatin modifier expression linked to *ARID1A* promoter hypermethylation. Examples of translation of these classifications for the management of CCA have also been reported, with prediction of drug efficacy based on genetic alterations.

Summary

Although there is currently no international consensus on CCA morphomolecular classification, the recent initiatives developed under the umbrella of The European Network for the Study of Cholangiocarcinoma (ENSCCA) should favor new collaborative research. Identifying distinct molecular subgroups and developing appropriate targeted therapies will improve the clinical outcome of patients with CCA.

Keywords

cholangiocarcinoma, genomics, molecular classification

INTRODUCTION

Cholangiocarcinoma (CCA) constitute a heterogeneous group of tumors that arise from the malignant transformation of cholangiocytes along the biliary tree. CCA is a deadly cancer whose management is currently a clinical challenge. Both incidence and mortality of CCA have been steadily increasing during the last decade [1]. In addition, most of the patients are diagnosed with advanced stage tumors, allowing very limited therapeutic options. Surgical resection has poor chances to result in a total recovery due to a high rate of tumor recurrence [2]. Guidelines based on diverse classifications have been established [3]. Reaching points of consensus is critical to improve CCA care and to estimate the incidence of different forms of CCA, which are associated with variable mortality trends [4]. Anatomical classification of CCA, that draws a distinction between intrahepatic (iCCA) and extrahepatic CCA (eCCA), has been widely accepted. Beyond this basic anatomical classification, efforts have been made to classify CCA at the cellular and molecular levels. Here, we review the recent advances in the molecular classification of CCA, allowing a better characterization of CCA

heterogeneity and providing insights into the molecular mechanisms underlying CCA pathogenesis. The ultimate goal of these classifications is to develop relevant targeted therapies based on personalized features.

ANATOMICAL AND HISTOLOGICAL CLASSIFICATION

Currently, CCA are classified according to their anatomical location and histological features in three broad categories: intrahepatic (iCCA), perihilar (pCCA), and distal CCA (dCCA) (Fig. 1) [5]. iCCA arise from the second-order bile ducts and represent approximately 20% of all CCA. eCCA are divided

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KEY POINTS

- CCA form a disparate group of tumors along the biliary tree. CCA are characterized by highly heterogeneous anatomical, histological and molecular features. Innovative morphomolecular classifications are being developed to take into account histological and molecular disparity.
- Recent omics-based integrative studies allowed to draw a global picture of CCA alterations at genetic, epigenetic and genomic levels and to identify clinically relevant CCA molecular subtypes.
- New classification based on noncoding RNA expression, proteomic profiles and radiogenomics are emerging. These newcomers in the field could lead to major advances in CCA management.
- Coordinated efforts are required to merge omic data from various sources and to reach an international consensus on CCA classification, as a first step toward a molecular personalization of the treatments.

into two subclasses: pCCA emerging from large hepatic hilar bile duct, and dCCA originating from Vater's ampulla [6]. However, this dogma is being questioned. As recently specified, the former classification does not take into account the differences between small and large duct iCCA that exhibit specific features [3]. The convergence of molecular-based phenotypes shared by pCCA and iCCA arising from the large bile ducts was rightly pointed out [3]. Histologically, eCCA are mostly mucin-producing tumors. In addition, iCCA display more heterogeneous features. Although small second-order bile ducts are largely mass forming, iCCA that originate from large bile ducts show growth pattern similar to pCCA and dCCA. They may develop either as mass forming (MF), periductal, or intraductal tumors (Fig. 1) [3]. Interestingly, this histological classification correlated with clinical features. For example, MF-iCCA are associated with chronic liver disease, such as cirrhosis or viral hepatitis, whereas mucinous iCCA are more likely correlated with primary sclerosing cholangitis [7].

MOLECULAR CLASSIFICATION

The last state-of-the-art review about CCA genomics in *Current Opinion in Gastroenterology* was published in 2012 and was focused on genetic profiling of iCCA [8]. With the tremendous progress in omic-based approaches and the growing interest of the liver community for CCA, distinct genetic, epigenetic and genomic patterns have been identified over the last few years. Integrative molecular analyses also

provided clinically relevant morphomolecular classifications of CCA. These classifications addressed CCA heterogeneity and identified molecular mechanisms underlying CCA pathogenesis. These findings are also of particular importance in identifying clinically relevant CCA subtypes together with targetable signaling pathways.

GENETIC CLASSIFICATION BASED ON MUTATIONS AND GENE FUSIONS

Several studies identified gene alterations specific to either iCCA or eCCA. Although the number of samples studied was limited, Putra *et al.* [9] reported that iCCA showed *IDH1/2* and *NRAS* mutations, whereas eCCA displayed mainly *TP53* (75%), *KRAS* (50%), and *BRAF* (12.5%) mutations. By analyzing 137 iCCA and 74 eCCA, Nakamura *et al.* [10] reported genetic alterations specific for iCCA (e.g. *IDH1/2*, *BAP1* mutations, *FGFR2* fusions), eCCA (e.g. *PRKACA*, *ARID1B* mutations) or shared between iCCA and eCCA (e.g. *KRAS*, *GNAS*, *TP53* mutations). Interestingly, this study identified actionable growth factor-mediated signals specific of iCCA or eCCA, opening personalized therapeutic opportunities [10]. A recent report that was focused on genomic alterations of a large cohort of 496 iCCA patients using three classifier genes (i.e. *IDH*, *KRAS*, *TP53*) identified unique mutational signatures, co-mutation profiles and enriched pathways for each subgroup [11]. More importantly, it was shown that such a molecular stratification of iCCA patients could predict therapeutic sensitivity in drug repositioning protocols [11]. Mutation-based classifications are also associated with etiologic and clinical-pathological features. For instance, *TP53*-defective iCCA patients were reported to be more likely positive for the hepatitis B surface antigen (HBsAg), whereas *KRAS* mutations are almost exclusively detected in HBsAg-seronegative iCCA patients [12].

Another idea brought forth was to connect the morphological CCA subclasses with specific molecular-based patterns, as recently reviewed [13]. Thus, two distinctive morphomolecular groups of iCCA can be distinguished. Large duct iCCA exhibit a mutational spectrum similar to eCCA, with a high mutation frequency for *KRAS* (15–30%) and *TP53* (10–40%), whereas small duct iCCA are typically mass forming and enriched for *IDH1/2* mutations and *FGFR2* gene fusions (Fig. 1) [5]. In CCA, most fusion genes involved fibroblast growth factor receptor 2 (*FGFR2*). *FGFR2* is a transmembrane receptor, with tyrosine kinase activity, which is a main component of the fibroblast growth factor-signaling pathway. This pathway is frequently activated in CCA, where it can promote migration and

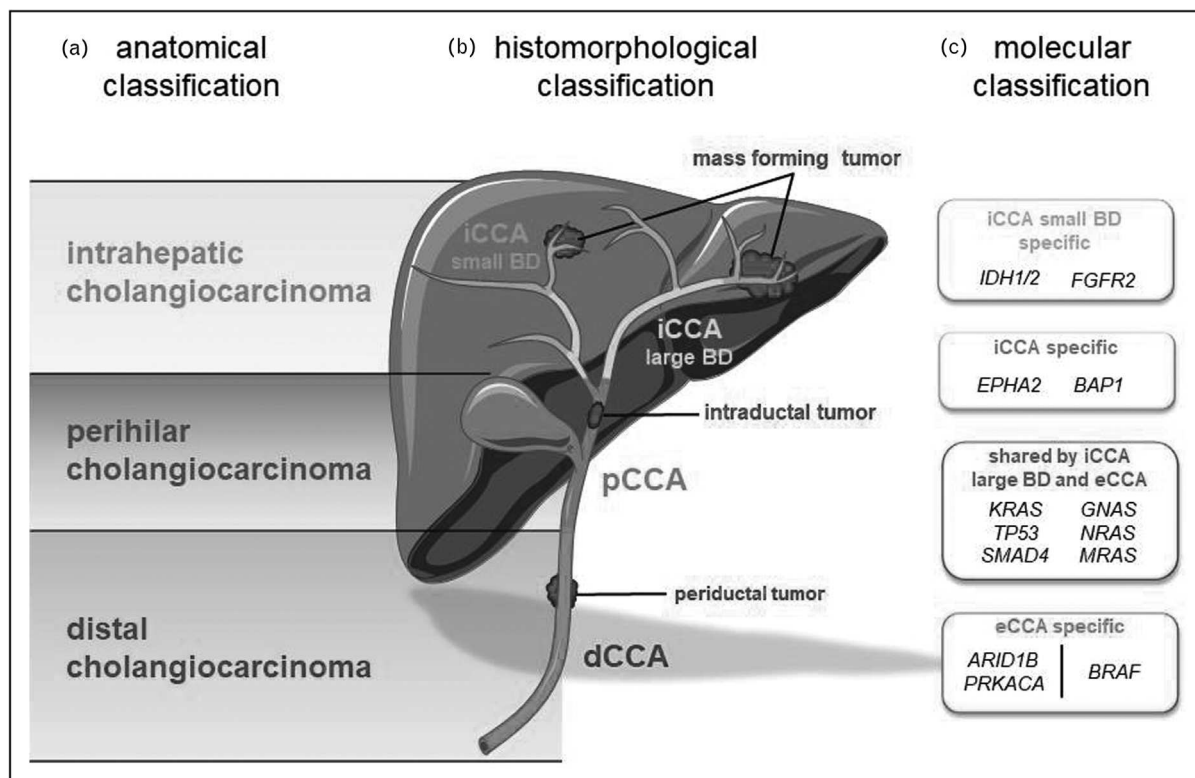


FIGURE 1. Classification of biliary tract malignancies according to their anatomical, histomorphological, and molecular features. (a) Cholangiocarcinoma (CCA) are broadly classified into three main entities based on their anatomical location: intrahepatic CCA (iCCA) that develop within the liver from the second-order bile ducts (BD) and proximal intrahepatic BD. Extrahepatic CCA (eCCA) include perihilar CCA (pCCA) arising from both left and right hepatic ducts, and distal CCA (dCCA) found further down the BD, closer to Vater's ampulla. (b) Three main growth patterns have been observed in CCA: mass forming (MF), intraductal (ID), and periductal infiltrating (PD). iCCA tumors exhibit mostly MF growth pattern, whereas eCCA are mainly PD and ID mucinous tumors. (c) Specific alterations at genetic, epigenetic and genomic levels allowed identifying clinically relevant molecular subtypes of CCA [5,9,10].

angiogenesis [14]. In iCCA, fusion genes consisting of *FGFR2* and a number of genes (e.g. *BICC1*, *AHCYL1*, *TACC1*, *MGEA5*, *PPHLN*) have been described. In addition, *FGFR2* pairs with *PRKACA* and *PRKACB*, in iCCA and eCCA, respectively. The formation of these chimeric genes results in the constitutive activation of *FGFR2* and its pro-tumorigenic functions. Additional fusion genes, such as *RABGAP1L-NTRK1* have also been observed in iCCA [15].

EPIGENETIC CLASSIFICATION BASED ON METHYLATION PROFILES

Epigenetics refers to modifications in chromatin (DNA or histones) without altering the DNA sequence. Differences in epigenetic signatures between cancer and normal cells are frequent and are usually associated with a loss of expression of tumor suppressor genes and a gain of function of oncogenes [13]. In CCA, hypermethylation at the

promoters of tumor suppressor genes (e.g. *DAPK*, *P14*, *ASC*) has been described [16]. The first comprehensive genome-wide investigation of DNA methylation patterns in CCA revealed differential methylation in GC-rich regions (CpG islands) both in iCCA and eCCA. Many of the genes that showed altered CpG methylation belonged to WNT, TGF β , PI3K, MAPK, and NOTCH signaling pathways [17]. In another study, a large number of CCA tumors collected from different countries, were categorized into four clusters according to somatic mutations, copy number alterations, gene expression, DNA methylation, and etiology, that is, fluke-positive or fluke-negative [18^{*}]. Tumors within Cluster 1 were fluke-positive and exhibited frequent mutations in *TP53*, *ARID1A*, *BRCA1/2* genes, as well as mutations in promoters of genes enriched for trimethylation on lysine 27 of histone 3 (H3K27me3). They exhibited high DNA methylation on CpG islands, located at promoters and were associated with a poor prognosis. In contrast, Cluster 4

included fluke-negative tumors with high incidence of mutations in *BAP1* and *IDH1/2*, alterations in *FGFR*, elevated FGF and PI3K signaling and better prognosis. In these tumors, CpG hypermethylation was also observed, but mainly at CpG promoter shores. Moreover, different groups of genes were hypermethylated between these two clusters. DNA hypermethylation in Cluster 1 resulted from the decreased expression of TET1 demethylase and the enhanced expression of EZH2 histone methyltransferase. On the other hand, DNA hypermethylation in Cluster 4 was connected to *BAP1* and *IDH1/2* mutations [18^{*}]. In a recent study, CpG island methylation profiling in 172 iCCA unraveled that methylation of the *DLEC1* locus was a good prognostic factor of small bile duct iCCA [19]. DNMT1-dependent hypermethylation at the promoter of the tumor suppressor gene *SOX17* resulted in its down-regulation in CCA and has been associated with poor survival [20].

INTEGRATIVE GENOMIC CLASSIFICATION

One of the first detailed molecular characterization of a large cohort of CCA ($n = 104$) identified distinct subclasses of patients with different clinical outcomes [13]. *KRAS* mutations were associated with a reduced survival. Importantly, the study highlighted the potential efficacy of tyrosine kinase inhibitors in patients with poor clinical prognosis [13]. By combining whole-gene expression data, chromosomal aberrations, and signaling pathway activation, two molecular subgroups were identified from another large series of 149 iCCA [21]. The so-called ‘inflammation class’ (38% iCCA) was characterized by the activation of inflammatory signaling pathways and was associated with the overexpression of Th2 cytokines (IL-10 and IL-4). A ‘proliferation class’ (62% iCCA) was characterized by the activation of oncogenic MET or RAS/RAF/MAPK pathways and *KRAS* mutations. Importantly, iCCA included in the proliferation class displayed aggressive clinical traits and correlated with a poor survival and a high rate of recurrence [21]. These two seminal studies highlighted the potential of integrative omic-based approaches to refine the molecular classification of iCCA and to provide novel therapeutic options specific for each subclass [13,21]. More recently, further studies supplemented this molecular classification, and brought a further step towards a growing understanding of CCA molecular pathogenesis and heterogeneity [18^{*},22,23]. Notably, a molecular classification scheme was reported by The Cancer Genome Atlas based on a multiplatform integrated analysis of transcriptomic, copy number and methylation profiles [23]. Hence, an *IDH*-mutant cluster of

iCCA was characterized by an enriched expression of genes involved in mitochondrial structure and function, and a low chromatin modifier expression signature [23]. A similar multiplatform approach integrating mutation, copy-number, gene expression and methylation data from a cohort of 489 CCA defined four molecular subtypes of CCA [18^{*}]. Notably, a better prognosis subtype was enriched in *BAP1* and *IDH1/2* mutations, and *FGFR* expression signature [18^{*}].

EMERGING NONCODING RNA-BASED CLASSIFICATION

Noncoding RNA (ncRNA) form a heterogeneous class of RNA molecules that play important roles in cancer. Gene expression profiling studies identified clinically relevant ncRNA signatures in CCA, as recently reviewed [24]. Thus, profiling studies identified microRNA (miRNA) with tumor-promoting (e.g. miR-21, miR-25, miR-26a, miR-181c and miR-191) or tumor-suppressing (e.g. miR-29, miR-200b/c, miR-203 and miR-214) functions. Among these miRNA, miR-21, miR-191 and miR-26a were identified as markers of poor prognosis CCA. Long noncoding RNA (lncRNA), form another subgroup of ncRNA and include noncoding transcripts longer than 200 nucleotides. Similar to mRNA and miRNA, lncRNA signatures were recently reported in CCA. The expression of numerous lncRNA (e.g. H19, MALAT1, NEAT1, PVT1, TP73-AS1, PANDAR, CASC15, HULC) are elevated in CCA and associated with enhanced metastasis and poor survival, as recently reviewed [25]. More focused studies recently identified clinically relevant lncRNA signatures in iCCA [26]. ANRIL lncRNA (also known as CDKN2B-AS1) was notably identified as a marker of poor prognosis [26]. In contrast, a reduced expression of tumor-suppressive NEF lncRNA was reported in poor prognosis iCCA [27].

MICROENVIRONMENT-BASED CLASSIFICATION

CCA are evolving in a highly desmoplastic microenvironment. Stroma homeostasis is orchestrated by a complex ecosystem involving cancer-associated fibroblasts (CAFs), tumor infiltrating lymphocytes (TILs), and numerous immune cells [such as T lymphocytes, natural killer (NK) cells, and macrophages] [28]. They are active players in tumor onset and progression by releasing a variety of cytokines, chemokines and growth factors. Such a reactive stroma enhances fibrosis, which limits the efficiency of drug delivery and chemotherapies [29]. Hence, a classification of CCA based on stroma features could have a

beneficial impact on disease management. A molecular profiling study identified genomic alterations specific of iCCA stromal compartment. Thus, the expression of 1073 genes was found to distinguish tumor stroma from nontumoral fibrous tissue. It allowed the identification of several biomarkers of clinical interest. For instance, a clear correlation has been established between iCCA aggressiveness and the expression of osteopontin in the stroma [30]. Recently, new therapies based on immune checkpoint inhibitors are being developed (e.g. PD-1 and PD-L1 inhibitors). These inhibitors aim at preventing cancer cells from escaping the immune system. Hence, a classification of CCA taking into consideration PD-L1 positivity could be beneficial for anti PD-L1 testing guidelines. A recent study investigated PD-L1 positivity in 170 CCA, using notably the PD-L1 antibody clone SP263 as a detection marker. The study revealed that 5% of iCCA, 4% of pCCA, and 3% of dCCA were PD-L1 positive. These results are of clinical relevance as PD-L1 positivity has been correlated with poor clinical outcome [31]. Another immunohistological study of 58 iCCA and 41 pCCA suggested that CCA with dense intratumoral lymphocytic infiltration might represent good candidates for PD-L1/PD-1-blocking agents [32].

PROTEOMICS-BASED CLASSIFICATION

A recent report highlighted the classification of CCA based on proteomic data [33^{*}]. The cohort included 17 iCCA [9 developed on normal (iCC_N) and 8 on cirrhotic (iCC_C) liver], 5 hilar CCA (CC_H) and 5 pancreatic CCA (CC_P). On the basis of proteomic criteria, the above anatomical subgroups were categorized into two clusters. The first cluster included the majority of iCC_C cases, whereas the second cluster was divided into one cluster containing iCC_N tumors and a second cluster containing CC_H and CC_P tumors. Comparisons of protein expression patterns between the iCC_N and iCC_C subgroups revealed that iCC_C tumors highly expressed proteins involved in cell adhesion and motility (ITGA4, ITGA7, Rho GTPase), cell growth (TRAF3IP3), inhibition of apoptosis (BCL2) and angiogenesis (PDGFR β , VCAM1). In contrast, iCC_N tumors were characterized by high levels of keratin 19 and PAI-1 proteins. When CC_H and iCC_N tumors were compared, ectopic expression of mucin proteins, secretion-related AGR family proteins and tumor microenvironment-associated factors, such as S100A4 was observed in CC_H. On the other hand, iCC_N tumors potently expressed proteins involved in RNA processing, nucleocytoplasmic shuttling and nuclear pore complex components. At last, CC_H tumors were enriched in inflammatory-related

proteins, when compared with CC_P tumors, whereas CC_P tumors predominantly expressed proteins involved in RNA processing and nuclear trafficking [33^{*}]. Overall, there are distinct protein expression profiles between different anatomical CCA subgroups and, also within iCCA tumors, depending on the liver background.

RADIOGENOMIC-BASED CLASSIFICATION

Radiogenomics rapidly emerged in cancer research and is likely to become of greater interest to improve patient outcomes [34]. This approach aims at appending cancer-imaging features with tissue-based genomic profiles in order to facilitate clinical decision-making, and define a more relevant prognosis. Thus far, only one radiogenomic iCCA study has been reported ($n=25$ patients) [35]. In this work, the correlation between quantitative imaging phenotypes, qualitative imaging features, and expression of hypoxia biomarkers (e.g. EGFR, VEGF, CD24, P53, GLUT1) was assessed. Three qualitative imaging features (entropy, correlation and homogeneity) were notably correlated with EGFR expression [35].

CONCLUSION

CCA is a heterogeneous disease at multiple levels. Ongoing efforts to gain a better insight into CCA heterogeneity allowed the emergence of innovative histological and molecular classifications. However, there is still no accepted international consensus that could be exploited to improve the management of patients with CCA, and to develop effective targeted and personalized therapies. Future research will require additional collaborative efforts to merge molecular data from different laboratories and to reach such a consensus. A better understanding of the intricate and coordinated network of coding and ncRNA will be also required to unravel novel aspects of CCA carcinogenesis. Coordinated multicentered studies developed under the umbrella of dedicated international networks, including The European Network for the Study of Cholangiocarcinoma (ENSCCA), will certainly boost research projects in the field of CCA and allow a better management of this devastating disease.

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Conflicts of interest

There are no conflicts of interest.

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Review

Transforming Growth Factor-Beta (TGF β) Signaling Pathway in Cholangiocarcinoma

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Abstract: Cholangiocarcinoma is a deadly cancer worldwide, associated with a poor prognosis and limited therapeutic options. Although cholangiocarcinoma accounts for less than 15% of liver primary cancer, its silent nature restricts early diagnosis and prevents efficient treatment. Therefore, it is of clinical relevance to better understand the molecular basis of cholangiocarcinoma, including the signaling pathways that contribute to tumor onset and progression. In this review, we discuss the genetic, molecular, and environmental factors that promote cholangiocarcinoma, emphasizing the role of the transforming growth factor β (TGF β) signaling pathway in the progression of this cancer. We provide an overview of the physiological functions of TGF β signaling in preserving liver homeostasis and describe how advanced cholangiocarcinoma benefits from the tumor-promoting effects of TGF β . Moreover, we report the importance of noncoding RNAs as effector molecules downstream of TGF β during cholangiocarcinoma progression, and conclude by highlighting the need for identifying novel and clinically relevant biomarkers for a better management of patients with cholangiocarcinoma.

Keywords: TGF β ; cholangiocarcinoma; liver cancer; signaling; noncoding RNA

1. Cholangiocarcinoma

1.1. Epidemiology and Risk Factors

Cholangiocarcinomas (CCAs) comprise heterogeneous hepatobiliary tumors with cholangiocyte differentiation features. CCA is the second most common hepatic malignant tumor after hepatocellular carcinoma (HCC). CCAs are classified as intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA), according to their anatomic location [1]. CCA subtypes differ by their epidemiology, etiology, pathogenesis, and thus, clinical management and targeted therapeutic options. Although challenges in the classification of CCA made it difficult to quantify, a gradual increase in CCA incidence was reported worldwide during the last few decades [2]. The discrepancy in incidence of sporadic CCA worldwide is associated with well-established risk factors. Higher incidence of CCAs in Eastern countries reflects a geographical disparity in the prevalence of risk factors [1]. For instance, hepatobiliary flukes such as *Clonorchis sinensis* and *Opisthorchis viverrini* are both common risk factors in Southeast Asia, where CCA is recognized as a non-rare cancer [3,4]. Hepatitis B and C have also been identified as risk factors for CCAs, especially for iCCA. While hepatitis C is prevalent in Western countries, hepatitis B is strongly associated with CCAs in Asia [5]. Furthermore, the association between primary sclerosing cholangitis (PSC) and CCA is well-established. Studies from Western countries reported that PSC patients developed CCAs with a prevalence ranging from 5% to 15% and a yearly developmental rate of CCAs ranging from 0.5% to 1.5% [6]. Other risk factors including hepatolithiasis, metabolic

syndrome, alcohol, smoking, and diabetes are also suspected to be involved in CCA onset, all of these factors contributing to generate a pro-inflammatory environment in biliary tracts [5,7]. As a result of its silent nature, CCA is generally diagnosed at the advanced stage, when therapeutic options are limited. Surgery is currently the best option for CCA treatment, even though tumor size, metastasis, and lymph node invasion make it unfeasible in more than 65% cases [8]. For the resectable early stage CCAs, survival at five years ranges from 15% to 40%, but is associated with a high risk of recurrence [9]. However, for unresectable advanced CCAs, overall survival (OS) is below 15 months [9]. The lack of a clear and global picture of cellular and molecular alterations, which occur in aggressive CCAs, might account for this unfavorable clinical outcome. Improvement in CCA outcome relies on efforts toward a better understanding of cholangiocarcinogenesis mechanisms to develop efficient targeted therapies, as well as the identification of reliable biomarkers for early detection.

1.2. Molecular Pathogenesis

CCA is frequently associated with drastic changes in the tumor microenvironment, including intense extracellular matrix remodeling and inflammation, which modulate the activity of signaling pathways involved in tumor onset and progression [10,11]. These alterations lead to an aberrant expression and/or activation of key cytokines, tyrosine kinases, and ultimately transcription factors which control cell fate [12–14]. As an example, an increase of interleukin 6 (IL6) secretion by CCAs and desmoplastic stromal cells lead to the activation of STAT3, a latent cytoplasmic transcription factor. IL6 binds to the dimerized GP130 receptors associated with Janus family kinases (JAK) including JAK1, JAK2, and TYK2, leading to STAT3 phosphorylation and activation [15]. STAT3 acts not only as an activator of transcription but also as a signal transducer. Its activation modulates a variety of genes involved in cell survival, proliferation, and migration. An elevated expression of STAT3 in CCA tumor tissues has been identified as an independent prognostic factor for OS and disease-free survival (DFS) [16]. There is also evidence demonstrating that epidermal growth factor receptor (EGFR) contributes to CCA progression by disturbing cell–cell adhesion and cell motility, triggering epithelial to mesenchymal transition (EMT) and thus promoting a pro-metastatic process [17]. EGFR activation by its ligands (e.g., EGF, TGFA, AREG) initiates several signal transduction cascades, including extracellular signal-regulated kinases (ERK) 1/2 and serine/threonine kinase 1 (AKT1), which are both implicated in cell proliferation and migration [18].

Developmental pathways are well-conserved axes required for biliary tract cell differentiation and proliferation. Unsurprisingly, dysregulations of these pathways have been described in CCAs. Recent evidence showed that a persistent activation of Notch signaling is associated with iCCA [19]. A study using a mouse model of iCCA revealed that the Notch axis was critical in hepatocyte conversion into biliary lineage, and therefore, an enhanced activity of this pathway may contribute to malignant conversion of hepatocytes into CCAs [20]. Hedgehog (HH) is another developmental pathway involved in critical cell fate decision, including apoptosis, stem cell maintenance, and wound healing [21]. HH pathway was identified as a key player in tumor initiation in several cancers, including CCAs [22,23]. El Khatib et al. investigated the effects of blocking the HH pathway using cyclopamine in vitro on human CCA cells, and in vivo using xenograft of CCA cells in mice. Cyclopamine is a steroidal alkaloid isolated from *Veratrum californicum*. It plays a critical role in embryonic development by hindering the HH pathway. Such a muting of the HH pathway resulted in a blockage of CCA cell migration and invasion. In CCA xenografts, cyclopamine treatment led to a significant inhibition of tumor growth, highlighting the importance of the HH pathway in CCAs and the clinical relevance of its inhibition [24].

High throughput strategies identified numerous genetic, epigenetic, and genomic alterations in CCA, and highlighted specific targetable signaling pathways. Among them, mutations in isocitrate dehydrogenases genes (*IDH1* and *IDH2*) and chromatin-remodeling genes, including AT-rich interaction domain 1A (*ARID1A*), as well as gene fusions involving fibroblast growth factor receptor 2 (*FGFR2*) were frequently detected in CCAs [25–28]. Mutations of the tumor suppressor *TP53* are also commonly

found in CCAs (44% of cases), so are mutations of *KRAS* (17% of cases) and *SMAD4* (17% of cases) [29]. According to the same study, seven genes (*TP53*, *SMAD4*, *KRAS*, *RNF43*, *NDC80*, *ROBO2*, and *GNAS*) scored as the top mutated genes in *Opisthorchis viverrini*-associated CCAs, with false discovery rates ranging from 2.1×10^{-5} to 0.29. More recently, protein tyrosine phosphatase non-receptor 3 (*PTPN3*) was reported to be frequently mutated in iCCA and associated with tumor recurrence [30].

1.3. Targeted Therapies

Although significant progress in understanding the molecular basis of CCA pathogenesis has been achieved, there is no approved molecular targeted therapy that significantly improves patient survival. Thus, there is a critical need of designing innovative therapeutic strategies and biomarkers for a better management of patients with CCA, which remains a deadly cancer with a very poor prognosis [9]. Previous genomic characterizations of the stroma in iCCA highlighted clinically relevant biomarkers predictive of patient survival, some of them being related to the transforming growth factor β (TGF β) signaling [11,31,32]. Several clinical trials are still in progress based on genetic alterations observed in CCAs. For example, the therapeutic effect of the multi-targeted tyrosine kinase inhibitor, ponatinib, has been tested in two patients with advanced CCA tumors associated with activating *FGFR2* gene fusions. This trial resulted in an efficient anti-tumor response characterized notably by shrinkage of metastatic lymph nodes [33]. Promising results using IDH1 and IDH2 inhibitors have been also reported. Two mutant IDH inhibitors, enasidenib (AG-221) and ivosidenib (AG-120), have been approved in ongoing trials on Acute Myeloid Leukemia (AML), and their therapeutic benefits are now studied in other malignancies including CCA [34].

2. Transforming Growth Factor Beta (TGF β) Pathway

2.1. TGF β Signaling: From Receptor Activation to Transcription Of Target Genes

Members of the TGF β family are pleiotropic cytokines that exhibit important roles in tissue homeostasis, cell differentiation, and embryonic development. Extracellular TGF β ligands bind to transmembrane type I and type II TGF β receptors (T β RI and T β RII, respectively), thereby initiating a signaling cascade that ultimately leads to altered expression of protein-coding and noncoding target genes [35,36]. Initiation of the TGF β pathway involves binding of ligands to T β RII, formation of a heterotetrameric complex between T β RII and T β RI, and phosphorylation of T β RI by T β RII, the latter possessing serine/threonine kinase activity in its intracellular domain. Phosphorylation of T β RI turns on its serine/threonine kinase activity, resulting in the phosphorylation of the members of the SMAD family, SMAD2 and SMAD3, which interact, at their carboxy-terminal domain, with the common mediator SMAD4 and transduce the signal into the nucleus. The transport of the activated SMAD complex into the nucleus is mediated by proteins of the nuclear pore, such as importin- β 1 and CAN/Nup214, and requires the GTPase RAN [37]. In the nucleus, the SMAD complexes associate with various coactivator or corepressor factors to regulate gene transcription in a positive or negative manner, respectively (Figure 1).

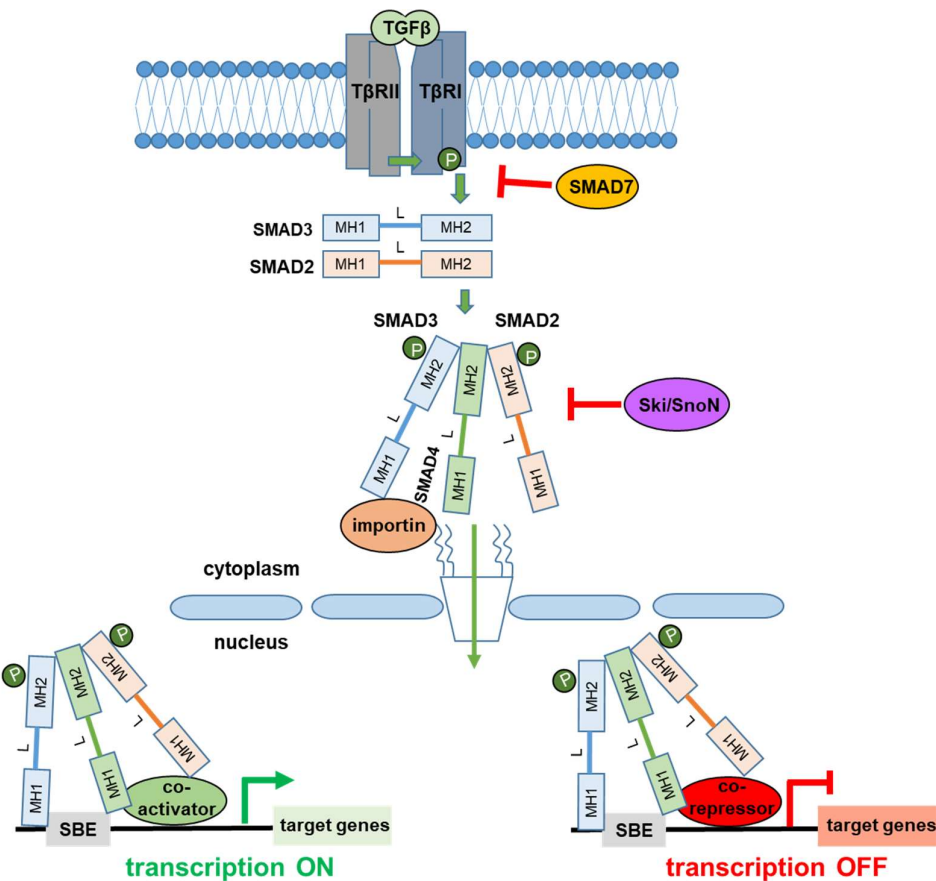


Figure 1. Basics of transforming growth factor β (TGF β) signaling. Binding of TGF β ligands to their receptors initiates the signal, through the phosphorylation of T β RI by T β RII. Then, T β RI transmits the signal to SMAD2 and SMAD3 by phosphorylating their MH2 domains. This phosphorylation enables SMAD2 and SMAD3 activation and the formation of a complex with SMAD4, which, in turn, enters into the nucleus through nuclear pores with the assistance of importins. In the nucleus, the activated SMAD complex regulates the expression of target genes, in a positive or negative manner, depending on its association with transcriptional coactivators or corepressors. The signaling is subjected to negative regulation by SMAD7, which prevents SMAD2/3 activation and induces degradation of T β RI, and by Ski/SnoN, which interferes with the formation of active SMAD2/3/4 complex. MH1: MAD homology 1 domain, L: Linker domain, MH2: MAD homology 2 domain, T β RI: type I TGF β receptor, T β RII: type II TGF β receptor.

Structurally, the transcription factors SMAD2, SMAD3, and SMAD4 consist of three main domains, an N-terminal (or MH1), a central (or linker), and a C-terminal (or MH2) domain [38]. The MH1 domain enables SMAD3 and SMAD4 to directly recognize and bind to specific SMAD-binding elements (SBEs) at promoter or enhancer DNA regions [39]. The linker domain is subjected to extensive post-translational modifications that influence stability and interactions with proteins from other signaling pathways. The MH2 domain is important for the interaction of SMAD2 and SMAD3 with T β RI, SMAD4, and other protein partners.

In parallel to TGF β -induced SMAD activation, TGF β receptors can also induce non-SMAD pathways, such as MAP kinase (MAPK), phosphatidylinositol-3' kinase (PI3K), and the cell polarity regulator PAR6, which can mediate its biological functions [40]. The various physiological actions of TGF β signaling require control mechanisms to ensure that the magnitude of the signal meets the temporal, spatial, or developmental needs of the cellular systems. Thus, several mechanisms prevent an overactivation of the TGF β signaling. Notably, SMAD7 and Sloan Kettering Institute proto-oncogene (SKI)/SKI-related novel gene (SnoN) proteins are the best described negative regulators of TGF β

signaling. SMAD7 is a member of the SMAD family and its inhibitory role on TGF β signaling is exerted in two ways. First, SMAD7 antagonizes with SMAD2/3 for binding to T β RI receptors, thereby limiting the phosphorylation of SMAD2/3. Second, SMAD7 promotes the ubiquitin-dependent proteosomal degradation of T β RI receptor by recruiting E3-ubiquitin ligases (SMURFs) to the receptor [41]. SKI and SnoN proteins interact with SMAD2/3 or SMAD4 and interfere with the formation of an active SMAD2/3-SMAD4 complex, thus also interfering with the propagation of the TGF β signal [41] (Figure 1).

2.2. Crosstalk of TGF β Signaling with Other Signaling Pathways

TGF β -mediated cellular responses are frequently achieved by the cooperation of TGF β with other signaling pathways. Thus, the crosstalk between TGF β and a plethora of pathways, such as WNT, HIPPO, NF- κ B, Notch, hedgehog, JAK/STAT, MAPK, and PI3K-AKT, is supported by the current literature [42]. Interestingly, most of these pathways are deregulated in CCAs [43]. As an example, TGF β and HIPPO pathways are functionally associated to regulate CCA progression. Hyperactivation of yes-associated protein-1 (Yap1) and transcriptional coactivator with PDZ-binding motif (Taz), due to a genetic depletion of Mps One Binder kinase activator (Mob)1a/1b in mouse liver, results in an increased incidence of combined HCC-CCA and iCCA. These effects are accompanied by increased levels of TGF β 2 and TGF β 3 ligands. Interestingly, a positive correlation between YAP1 and SMAD2 activation has been shown in patients with HCC-CCA and iCCA [44]. Interleukin-6 (IL-6) and TGF β pathways also converge to potentiate malignancy and resistance to chemotherapy in biliary tract cancer [45].

2.3. Physiological Responses in the Liver

TGF β signaling dictates transcriptional programs, which influence diverse physiological processes, such as cell cycle arrest, apoptosis, EMT, and immune surveillance. When the tight regulatory mechanisms of TGF β signaling activity are circumvented, pathological conditions, such as fibrotic diseases and tumorigenesis, may arise due to excessive and uncontrolled activity of the pathway. For example, the pathogenesis of liver fibrosis involves extravagant production and deposition of extracellular matrix (ECM) components i.e., collagen, produced at the liver tissue, in response to chronic liver damage [46]. At the onset of liver fibrosis, a combinatorial action of inflammatory responses, infiltrating immune cells, and cytokine signaling (e.g., TGF β) triggers the activation of hepatic stellate cells (HSC) and their transition towards myofibroblasts [47]. Failure of recovery, as a consequence of constant liver injury, favors a persistent pro-fibrotic microenvironment, ultimately leading to severe fibrotic disease. Both SMAD-dependent and non-SMAD signaling can mediate the effects of TGF β in HSC, such as the induction of connective tissue growth factor (CTGF) [48].

In epithelial cells, TGF β blocks cell proliferation, mainly through the induction of cyclin-dependent kinase (CDK) inhibitors, such as P21, P15, and P27, and the reduction of the oncogene MYC [49]. The pro-apoptotic effects of TGF β are well-described in normal liver or HCC cell lines. For example, TGF β induces apoptosis of Hep3B HCC cell line, a process that involves the activation of caspases. In the case of HUH7 cells, TGF β -mediated apoptosis engages the activation of caspases and the downregulation of anti-apoptotic proteins, such as BCL-XL and XIAP [50]. In other cellular models, the involvement of the c-Jun N-terminal kinase (JNK) pathway [51], the induction of the tumor suppressor TGF β inducible early growth response protein 1 (TIEG1) [52], or the death-associated protein kinase (DAPK) [53] have been reported as mediators of apoptosis downstream of TGF β .

TGF β also plays a central role in promoting EMT, a process whereby epithelial cells lose their well-defined morphology and adhesion and acquire mesenchymal traits, allowing them to migrate from their original site [54]. EMT is crucial for normal embryogenesis, however, during tumor progression, epithelial carcinomas can profit from hyperactivation of EMT-inducing signaling pathways, such as WNT and TGF β , to gain migratory properties, enabling them to metastasize. Many reports pinpoint the positive contribution of EMT to metastasis [55], although a few of them support that EMT is

not a determinant factor for pancreatic [56] or lung metastasis [57], but is associated with increased resistance of these cancers to chemotherapeutic agents. In fact, EMT has been also linked to the generation of cancer stem cells (CSCs), defined as undifferentiated cancer cells, which possess stem cell characteristics, contribute to tumor heterogeneity, and show low sensitivity to chemotherapy [58]. Therefore, by positively regulating EMT, TGF β is also capable of conferring stemness features to cancer cells, as described in HCC [59]. At the molecular level, TGF β restricts epithelial phenotypes either by downregulating components of tight junctions, such as E-cadherin, or by redistributing them away from the cell membrane, thereby leading to their disassembly. In addition, TGF β induces the expression of EMT transcription factors (SNAIL, SLUG, ZEB1, ZEB2, TWIST) and mesenchymal markers (fibronectin, vimentin, collagens) to achieve EMT [60].

Immune system responses are also controlled by the TGF β pathway, which, in general, exhibits immune-suppressive effects. Immune suppression by TGF β is a mechanism by which cancer cells may escape immune surveillance. TGF β disrupts the ability of immune cells to recognize and eliminate cancer cells by shifting the differentiation of naïve CD4⁺ cells towards T-regulatory cells, thereby restricting the production of effector T cells. In addition, it prevents natural killer cells from destroying tumor cells. A characteristic example for the role of TGF β in cancer progression, via modulation of the immune system, derives from a study using CCA cells. The findings of this study suggested that interference of the TGF β pathway by neutralizing antibodies against T β RII in dendritic cells (DC) caused increased activation of effector T cells and, in turn, enhanced targeting and lysis of co-cultured CCA cells by the immune system [61]. In addition, another cause of CCA progression is chronic inflammation, in response to injury or due to the presence of high levels of pro-inflammatory cytokines at the bile duct [62]. Interestingly, TGF β induces the expression of IL6 in iCCA cell lines, an effect that facilitates CCA growth [63]. Other major contributors of tumor progression are the tumor associated macrophages (TAM) [64]. These immune cells are recruited at the tumor site and secrete cytokines, such as TGF β , thereby creating a pro-inflammatory microenvironment that favors cancer progression. Also, TAM and other immune cells have been identified at tumor areas in patients with eCCA and are correlated with poor prognosis [65]. Interestingly, addition of supernatant from HuCCT1 CCA cells on TAM enforced the latter to express high levels of TGF β , IL10, and VEGF, suggesting that the interplay between TGF β and TAM is of high importance in CCA tumor microenvironment [66].

2.4. TGF β Functional Duality in Cancer

TGF β is a challenging target for cancer treatment, due to its ability to both inhibit and facilitate tumor progression. During tumor initiation, TGF β exhibits tumor-suppressing functions by halting proliferation and inducing programmed cell death. In contrast, in advanced malignancies, TGF β preferentially exerts tumor-promoting actions by affecting the behavior of the cancer cells themselves, or by creating a favorable microenvironment for tumor growth [67]. The unresponsiveness of advanced cancers to the tumor-restricting properties of TGF β is a consequence of either genetic mutations of downstream cytostatic genes that, otherwise, are induced by the pathway, or mutations in components of the core signaling pathway, such as *TGFBR2* and *SMAD4* [68]. For example, CCA cells do not respond to the growth inhibitory effects of TGF β , due to high expression levels of cyclin D1. On the other hand, normal biliary epithelial cells, expressing physiological levels of cyclin D1, undergo cell cycle arrest in response to TGF β [69]. In the case of liver cancer, TGF β induces cytostatic and pro-apoptotic factors at early stages of cancer, but later on it promotes EMT and also stimulates the generation of cancer associated fibroblasts (CAF) in the tumor stroma, which maintain active TGF β signaling and contribute to metastasis [70].

3. TGF β Pathway in Cholangiocarcinoma

3.1. Genomic Alterations

In many cancers, genes encoding members of the TGF β pathway are frequently subject to mutations, reflecting the importance of this pathway in tumor progression [71]. Indeed, studies involving whole genome or exome sequencing from human CCA tissues have recorded genomic aberrations in main signaling pathways, and among them, TGF β has a special place [27,28]. Using a large cohort of 103 iCCA patients, Zou and coworkers identified RAS/PI3K, P53, and TGF β pathways to be influenced by alterations in the exome. *SMAD4* was described as one of the 25 significantly mutated genes, with a mutational rate of 4% ($P < 0.01$) [28]. In another study, *SMAD4* was mutated in 3.6% of patients with iCCA ($n = 55$) and in 25% of patients with eCCA ($n = 20$) [72]. Although, this difference in the frequency of *SMAD4* mutations between iCCA and eCCA was not statistically significant ($P = 0.333$), probably as a result of a limited number of eCCA cases, it suggests that eCCA exhibits a molecular phenotype that resembles pancreatic cancer rather than iCCA [29,72]. The expression of *SMAD4* was also evaluated by immunohistochemistry in normal liver and iCCA tissues with different differentiation status and clinical stages. *SMAD4* inactivation was found in 22 out of 49 iCCA specimens (44.9%, $P = 0.029$), whereas all normal liver tissues ($n = 9$) expressed *SMAD4*. A significant negative correlation between *SMAD4* expression and advanced clinical stages was highlighted [73]. Indeed, loss of *SMAD4* expression was associated with lymph node and intrahepatic metastasis ($P < 0.001$), poorly to moderately differentiated histological grade ($P = 0.013$), and advanced TNM stage ($P = 0.018$). In addition, the expression of *SMAD4* and cell cycle regulators (P53, P16, P27, cyclin D1, and Rb) was measured in 42 resected iCCAs by immunohistochemistry. Loss of expression of *SMAD4* was reported in 45.2% of cases, as well as genes encoding cell cycle inhibitors (e.g., 35.7% for P16) [74]. Loss of *SMAD4* was positively correlated with advanced pTNM stage ($P = 0.039$). Taken together, these studies suggest that different mechanisms contribute to inactivate *SMAD4* and possibly the tumor suppressive arm of the TGF β pathway, both in eCCA, involving *SMAD4* inactivating point mutations, and in iCCA, involving a transcriptional down-regulation of *SMAD4*.

3.2. TGF β Regulates CCA Tumor Progression

TGF β is one of the main signaling pathways that promotes CCA progression. Many studies support the TGF β -mediated induction of EMT in CCA cell lines [75]. Stimulation of human CCKS-1 and TFK-1 cells with TGF β led to a significant induction of SNAIL, VIM, and S100A4 and the reduction of E-cadherin and cytokeratin 19, thereby promoting migration and invasion [76]. TGF β also exhibits pro-EMT functions in iCCA cell lines KKKU-M213 and HuCCA-1. Enhanced expression of VIM and SLUG and secretion of the metalloproteinase MMP9, accompanied by a concomitant increase in cell migration and invasion, were observed after TGF β treatment [77]. TGF β stimulation activated both SMAD-dependent and SMAD-independent pathways, exemplified by the induced phosphorylation of SMAD2/3 and ERK1/2, respectively. Interestingly, inhibition of the kinase activity of MEK diminished the induction of EMT but, in contrast, potentiated the anti-proliferative effects of TGF β [77]. This observation raises the possibility that selective blockade of the noncanonical TGF β signaling may suppress the pro-tumorigenic, while preserving the anti-tumorigenic effects of TGF β in CCA. Disruption of TGF β pathway in human CCA primary cell cultures, using the T β RI kinase inhibitor LY2157299 (galunisertib), compromised cell migration. Notably, the same inhibitor did not influence cell cycle progression or apoptosis, indicating a selective inhibitory effect towards EMT [78]. Moreover, inhibition of the kinase CK2, which is linked to TGF β signaling, attenuated proliferation and increased apoptosis of the primary CCA cells [78]. The observed effects propose a combinatorial use of T β RI and CK2 kinase inhibitors for the treatment of CCAs. In another report, treatment of Huh28 and RBE CCA cell lines with the 3-hydroxy-3-methylglutaryl-coenzyme-CoA (HMG-CoA) reductase inhibitor, lovastatin, resulted in reduced TGF β 1 expression as well as reduced tumor cell proliferation and migration [79]. An interesting link between TGF β signaling, EMT, and the generation

of CCA cells with stemness properties was provided by Shuang and co-workers [80]. According to this study, exposure of TFK-1 cells to TGF β resulted in the acquisition of a mesenchymal phenotype and an increase in the population of cells positive for the cancer stem cell marker aldehyde dehydrogenase1 (ALDH1). Moreover, decreased cell death rates of TFK-1 cells in response to the DNA-damaging agent 5-fluorouracil (5-FU) was observed as a consequence of TGF β treatment, suggesting that TGF β confers chemoresistance of CCA cells to anti-cancer drugs [80]. It is worth noting that BMP7, a ligand that belongs to the TGF β family of proteins but signals through a different combination of receptors and effector SMADs, is capable of antagonizing the effects of TGF β -induced EMT in CCAs. Indeed, combinatorial treatment of M213 and M139 CCA cells with TGF β 1 and BMP7 attenuated migration and the increased expression of the EMT markers TWIST and N-cadherin, observed after TGF β 1 stimulation [81].

The effects of hepatitis B (HBV) and C (HCV) viruses on the progression of iCCA were evaluated in vivo using zebrafish as an experimental model. Livers from animals overexpressing both hepatitis B virus X (HBx) and hepatitis C virus core (HCP) proteins developed fibrosis and iCCA characterized by activated noncanonical TGF β signaling, exemplified by enhanced MAPK and SMAD linker phosphorylation. In addition, in vivo disruption of TGF β 1 expression by morpholinos attenuated fibrosis and iCCA progression, suggesting the importance of TGF β signaling during hepatitis-induced iCCA [82]. Moreover, experiments in transgenic zebrafish, whereby the expression of *tgfb1* was specifically induced in hepatocytes, showed increased incidence of HCC and CCA as a consequence of *tgfb1* chronic expression. Interestingly, HCC tumors were characterized by a switch from Smad-dependent to Erk-dependent TGF β pathway, whereas CCA tumors exhibited activation of both canonical and Erk pathways [83].

On the other hand, there are very few reports about a tumor-suppressing role of the TGF β pathway in CCAs. TGF β -treated RBE human CCA cells exhibited elevated apoptosis, a process that was SMAD-dependent and augmented by inhibition of the c-Jun N-terminal kinase (JNK), using the chemical inhibitor SP600125 [84]. Blocking JNK activity not only resulted in enhanced TGF β -induced apoptosis, but also in increased levels of C-terminal phosphorylated SMAD2 and SMAD3 and a general induction of TGF β -dependent transcriptional responses. Nevertheless, JNK inhibition did not influence SMAD linker phosphorylation, implying alternative molecular mechanisms for the inhibitory role of JNK on SMAD activation, in this context [84]. In a recent report, PTPN3, a protein tyrosine phosphatase which acts as a tumor suppressor, was shown to enhance T β RI stability, independently of its catalytic activity. PTPN3 was described as an antagonist of SMURF2 by overlapping with its binding domain to T β RI. SMURF2 is a well-known ubiquitin E3 ligase, recruited by SMAD7 and targeting T β RI for proteasomal degradation [85]. Interestingly, PTPN3 point mutations (L232R) may disrupt its interaction with T β RI, allowing SMAD7/ubiquitin E3 ligase complexes to exert their inhibitory role towards T β RI and, thus, abolishing TGF β pro-cytostatic effects [86]. Mutant PTPN3 L232R is frequently found in iCCA and results in loss of its anti-tumorigenic function. This finding could be of clinical importance for the subset of patients that carry PTPN3 L232R mutations, although a direct correlation between mutant PTPN3 and inactivation of TGF β signaling in iCCA patients was not addressed in this study.

3.3. Noncoding RNAs as Emerging Effectors of TGF β Signaling during CCA Progression

Noncoding RNA (ncRNA) are transcripts that lack protein-coding potential. Instead, they are transcribed from genes and perform regulatory or structural roles in cells. Noncoding RNA longer than 200 nucleotides are defined as long ncRNA (lncRNA). The rest are collectively designated as short ncRNA and, among many types, include the microRNA (miRNA), whose functional role is to target messenger RNA (mRNA) for degradation or to inhibit mRNA translation, thereby limiting gene expression [87]. The role of ncRNA in cancer progression, downstream of TGF β signaling, has been appreciated during the last years. For instance, in HCC, the lncRNA activated by TGF β (lncRNA-ATB) is a target gene of TGF β signaling and contributes to metastasis by sponging miR-200,

thereby stabilizing the EMT transcription factors ZEB1 and ZEB2, and by stabilizing IL11 mRNA, thus favoring tumor cell dissemination [88].

The crosstalk between TGF β signaling and miRNA in modulating EMT has been investigated in samples from patients with CCA. After measuring the expression levels of epithelial and mesenchymal markers in tumor and adjacent nontumor tissues from 20 patients, Zhang and co-workers observed low expression of the epithelial markers E-cadherin and miR-200b, and high expression of the mesenchymal markers fibronectin and α -smooth muscle actin (α -SMA) in the tumor tissues [89]. In addition, higher levels of TGF β 1 were detected in the tumor tissues, as compared to nontumor adjacent tissues. In vitro experiments using human HCCC and RBE cell lines stimulated with TGF β confirmed the downregulation of miR-200b and the establishment of an EMT transcriptional program. The mRNA encoding AP2 α and MAPK7 proteins were identified as direct targets of miR-200b. Enforced expression of miR-200b led to decreased tumor formation and EMT in an in vivo mouse model, highlighting its tumor-suppressing role and the importance of its repression by TGF β to elicit the EMT program [89]. In a rat model of iCCA, increased tumor size and enhanced intrahepatic metastasis were detected upon overexpression of TGF β 1 [90]. In addition, TGF β 1 promoted colony formation in a rat bile duct epithelial cell line BDE-Neu. The authors also used RNA interference to deplete TGF β 1 and chemical inhibition of T β RI kinase activity by LY2157299 and SB431542 to further confirm that both manipulations negatively affected cell growth and migration of RBE and SSP25 CCA cell lines. At the molecular level, miR-34a was identified as a central effector downstream of TGF β signaling. The expression of miR-34a was reduced in response to TGF β , which was followed by an increase in the expression of the direct targets of miR-34a, including CDK6, cyclin D1, and c-Met. Stabilization of CDK6 and cyclin D1 promoted cell growth, and elevated c-Met levels reinforced migration [90]. In another study, the anti-migratory role of miR-34a in CCA was also established, although an alternative mechanism for the miR-34a-mediated suppression of EMT has been described. According to Qiao and co-workers, miR-34a targeted SMAD4 and suppressed TGF β -induced EMT in vitro. Moreover, the levels of miR-34a and SMAD4 were inversely correlated in human eCCA tissues, with decreased miR-34a and increased SMAD4 expression being observed [91]. The miRNA miR-29a was suppressed by TGF β in the CCA cell lines FRH-0201 and CCLP-1. In addition, CCA tissues expressed lower miR-29a levels compared to adjacent nontumor tissues. Overexpression of miR-29a dampened the TGF β -induced cell migration and invasion in CCA cells. The histone deacetylase HDAC4 was identified as a direct target of miR-29a and rescue experiments. Using simultaneous overexpression of both HDAC4 and miR-29a showed a reversion of the EMT phenotype, which was abolished, due to miR-29a overexpression alone. Furthermore, HDAC4 promoted EMT in CCA cells, although the direct molecular targets of HDAC4 were not deeply investigated [92].

A recent report highlighted the importance of intercellular transfer of miRNA through extracellular vesicles (EVs) during CCA progression. As an example, TGF β represses the expression of miR-30e, a negative regulator of SNAIL in HuCCT1 cells. Enforced expression of miR-30e results in a decreased expression of SNAIL1 and of several EMT markers. In addition, miR-30e was packaged in EVs and transported to recipient HuCCT1 cells, where it exhibited its anti-EMT functions. Interestingly, TGF β treatment reduced the abundance of miR-30e in EVs of CCA cells, suggesting a negative role of miR-30e in regulating EMT under physiological conditions [93].

Although several studies have identified lncRNA associated with CCA progression [94], the knowledge concerning lncRNA acting as regulators of CCA progression in response to TGF β signaling is currently limited. Nevertheless, the lncRNA TGF β -induced long noncoding RNA (TLINC) was reported to be highly expressed in response to TGF β stimulation and to facilitate a pro-inflammatory microenvironment by enhancing cytokines, such as IL8 [95]. This finding strengthens the idea that additional lncRNAs could be effectors of TGF β -regulated responses in CCA.

More recently, circular RNA (circRNA) emerged in the literature as a new class of ncRNA that may play a critical role in cancer. Numerous circRNA are generated from alternative back-splicing of coding and ncRNA, forming continuous loop without 3' and 5' extremities. Recent data highlighted

the deregulation of circRNA in several cancers [96]. At the functional level, evidence indicates that circRNA play a key regulatory role at transcriptional and translational levels by possibly acting as miRNA sponges, or as scaffolds for RNA binding proteins (RBP) to form RNA-protein complexes [97]. The singular circular structure of circRNA renders them extremely resistant to exoribonucleases. Therefore, circRNA exhibit an expanded half-life, as compared to their linear counterpart RNA. Moreover, studies demonstrated that circRNA may also be found circulating in body fluids, freely or in exosomes [98]. Altogether, these characteristics are promising regarding the capability of circRNA as reliable diagnostic and predictive biomarkers in cancer. Although there is currently no report about circRNA deregulation in CCA, this new class of ncRNA could act as key regulator in the TGF β network and thereby in cancer. Notably, Goodal et al. demonstrated that TGF β could promote RNA circularization by inducing the binding of the RBP Quaking on introns flanking circular junctions, which could foster the pro-oncogenic feature of some circRNA [99]. These data reinforce the idea that circRNA could also be effectors of TGF β -mediated responses in cancer, including CCAs.

3.4. Therapeutic Targeting of the TGF β Signaling in CCA

The importance of the TGF β signaling in CCA renders this pathway a promising target for developing anti-tumor therapies. Thus, drugs that specifically target the TGF β pathway at different levels (i.e., maturation of latent TGF β to active TGF β dimers, ligand binding to its receptors, T β RI kinase activity) have been or are currently tested for possible anti-tumor effects in different cancers, including liver cancers [100–104]. In CCA, anti-TGF β -based therapeutic strategies have been mainly evaluated in preclinical models. Thus, *in vivo* experiments using a rat model of induced liver fibrosis showed decreased levels of fibrosis and CCA in animals treated with a neutralizing monoclonal antibody against TGF β . In contrast, control animals exhibited extensive liver fibrosis, which, eventually, led to the development of larger tumors [105]. Other mouse xenograft models showed increased tumor dissemination and larger tumors at the site of injection when mice were injected with CCKS-1 cells and simultaneously treated with TGF β 1 as compared to vehicle control-treated mice. Interestingly, the pro-tumorigenic effects of TGF β were abolished upon administration of a soluble form of T β RII that competes for binding to TGF β ligands with the membrane-bound T β RII [76]. In addition to these preclinical models, ongoing clinical trials make use of the chimeric antibody M7824, which is composed of the extracellular domain of human T β RII and the C-terminus of human anti-PD-L1 heavy chain [106,107]. M7824 has a double anti-tumor function as it serves as a trap for TGF β ligand binding at the tumor microenvironment and restricts the immune checkpoint factor programmed cell death ligand-1 (PD-L1), thereby restoring immune responses against the tumor [106,107]. Thus, a recently started multicenter phase II clinical trial is evaluating M7824 monotherapy in locally advanced or metastatic second line biliary tract cancer, including CCAs and gallbladder cancer (NCT03833661).

4. Conclusions

This overview of TGF β functions in liver homeostasis underlined the critical role of its dysregulation in cancer onset and progression. The fact that CCA evolves in a desmoplastic microenvironment, whereby TGF β is extremely abundant and frequently associated with a poor prognosis, emphasizes the clinical relevance of TGF β -targeted-therapies. Unfortunately, the literature described TGF β as an elusive target considering that, depending on the cell transcriptional context, it exhibits a dichotomous action. Thus, any therapeutic strategy aiming at modulating the TGF β pathway must consider its potential repercussion, either by repressing the pro-apoptotic and tumor suppressor arm, or by improving the pro-oncogenic and pro-metastatic action. A better insight into the molecular mechanisms regulating the functional duality of TGF β could improve the efficiency of targeted therapies for a better management of patients with CCA (Figure 2).

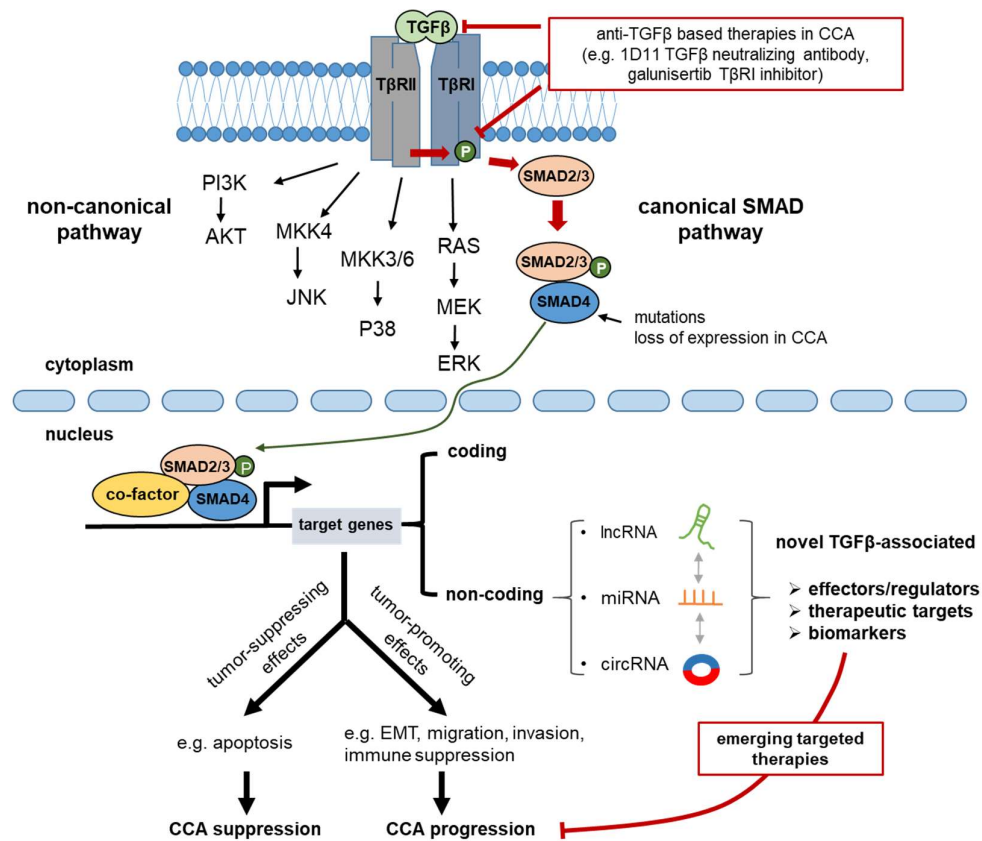


Figure 2. TGFβ signaling in cholangiocarcinoma (CCA) progression. TGFβ activates SMAD-dependent (canonical) and SMAD-independent (non-canonical) pathways in order to evoke transcriptional programs that, ultimately, regulate physiological responses in CCA. Consistent with its dual role in cancer, TGFβ can either prevent CCA progression, by inducing apoptosis, or enhance CCA progression, by promoting EMT, migration, invasion, and suppression of the immune system. Many coding and noncoding TGFβ-target genes can mediate the effects of the pathway during CCA progression and a number of them could potentially serve as drugable targets and biomarkers of CCA. Current therapeutic approaches aim at targeting components of the core pathway, such as the TGFβ ligands and the type I TGFβ receptor (TβRI).

The latest evidence promises a bright future for ncRNAs including miRNA, lncRNA, and circRNA as innovative downstream TGFβ effectors, or as clinically relevant biomarkers in CCA (Figure 2). A better understanding of the intricate and coordinated network of coding and ncRNAs will certainly allow us to elucidate how the functional duality of TGFβ in CCA is regulated. Some of these ncRNAs are also found circulating in body fluids and could represent specific biomarkers for a better management of patients with CCA.

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One stone, two birds: circACTN4, a nexus for a coordinated activation of Hippo and Wnt/ β -catenin pathways in cholangiocarcinoma

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Cholangiocarcinomas (CCAs) are a group of tumors arising along the hepatobiliary tree. CCA is a rare cancer, but its incidence has been increasing over the last decade. CCA is associated with a poor prognosis and limited effective therapeutic options.¹ Innovative strategies are therefore urgently needed to identify novel therapeutic targets and clinically relevant biomarkers to ultimately improve the survival of patients with CCA.¹ Circular RNAs (circRNAs) are splicing products that form a continuous loop by joining the 3' extremity of a downstream exon with the 5' extremity of an upstream exon. Although circRNAs are mainly outlined for their ability to sponge microRNAs (miRNAs) and RNA binding proteins (RBPs), their modes of action remain largely unknown.² Recently, circRNAs have emerged as new regulatory molecules that contribute to human carcinogenesis and hold promise as biomarkers.² However, very few studies have reported on the role of deregulated circRNAs in CCA so far.³⁻⁷ Notably, an increased expression of circ-CCAC1 has been identified by circRNA expression profiling in tumor tissues and bile-resident extracellular vesicles (EVs) from patients with CCA.⁷ CCA-delivered exosomal circ-CCAC1 was reported to be transferred to endothelial cells to disrupt endothelial barriers and induce angiogenesis.⁷ Mechanistically, circ-CCAC1 increased cell permeability by sequestering enhancer of zeste homolog 2 (EZH2) in the cytoplasm, thereby increasing SH3GL2 expression to suppress intercellular junction proteins.⁷ Besides this well-designed functional study, most of the published reports highlighting circRNAs in CCA solely focused on the clinical relevance of their expression. Thus, a comprehensive analysis of their functional role in the gene regulatory networks contributing to CCA onset and progression has barely been addressed. In addition, 80-90% of reports focused on the sponging capacity of circRNAs as a mechanism of action. We would like to point out that circRNAs are more than simple sponges for miRNAs. Indeed, emerging data from the literature demonstrate that circRNAs take an active role in complex transcriptional and post-transcriptional regulatory systems.

In this issue of *Journal of Hepatology*, Chen and colleagues illustrate this concept by investigating the function and the clinical relevance of circular RNA ACTN4 (circACTN4) in intra-hepatic CCA (iCCA). They demonstrate that circACTN4 is induced in iCCA and promotes proliferation and invasion of tumor cells *in vitro* and *in vivo*. Importantly, the study implies circACTN4 as a signaling nexus that enables the coordinated activation of Hippo and Wnt/ β -catenin pathways, notably by acting on different targets in the cytoplasm and the nucleus (Fig. 1). Indeed, circACTN4 is reported i) in the cytoplasm, to sponge miR-424-5p, which could target the oncogenic transcriptional coactivator Yes-associated protein 1 (YAP1) from the Hippo pathway and ii) in the nucleus, to recruit the Y-box binding protein 1 (YBX1) transcription factor to initiate the transcription of *Frizzled-7* (*FZD7*), a positive regulator of the Wnt/ β -catenin signaling pathway.⁸ CircACTN4 is also shown to favor the interaction between YAP1 and β -catenin and their nuclear accumulation, which ultimately leads to CCA cell growth and metastasis (Fig. 1). Thus, this study demonstrates how circRNAs can act as sponges for miRNAs and as scaffolds to locate transcription factors to the nucleus. The data suggest that the specific location of circRNAs within the cells could play a significant role in controlling gene expression. Overall, the study by Chen and colleagues unraveled circRNAs as intracellular nodes acting simultaneously in the cytoplasm and in the nucleus through specific regulatory processes. However, this study raises unanswered questions. Notably, the dynamic regulation of circRNAs shuttling between the nucleus and the cytoplasm, in relationship to their mode of action, is an important feature which remains to be elucidated. In addition, whether or not circACTN4 acts as a scaffold by interacting with DNA and/or other co-factors to recruit YBP1 to the promoter of *FZD7* remains to be determined.

The mechanisms regulating the spatial localization of circRNAs are poorly understood, although this parameter is probably of special importance in determining their activity. The reason why intron-containing circRNAs are restricted to the nucleus (to act as transcriptional regulators) while exonic circRNAs are enriched in the cytoplasm (to operate as post-transcriptional regulators by sponging miRNAs and/or RBPs) is unclear.⁹ Several reports explored possible mechanisms whereby circRNAs could shuttle from the nucleus to the cytoplasm. One of them described an evolutionarily conserved pathway that controls the nuclear export of circRNAs depending on the length of the transcripts. Hence, long (>800 nt) or short circRNAs could be

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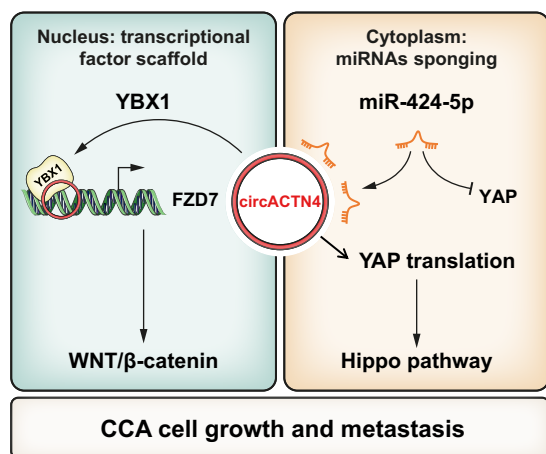


Fig. 1. Squaring the circle: emerging roles of circRNAs in CCA. In this issue of *Journal of Hepatology*, Chen and colleagues report the role and the clinical relevance of circACTN4 in CCA.⁸ The study demonstrates that circACTN4 is induced in CCA and exhibits specific but complementary functions depending on its subcellular localization. Thus, in the cytoplasm circACTN4 acts as a sponge sequestering miR-424-5p and therefore preventing the degradation of YAP1, from the Hippo pathway. In the nucleus, circACTN4 recruits YBX1 to initiate *FZD7* transcription and contributes to activate the Wnt/β-catenin pathway. Altogether, the coordinated activation of Wnt/β-catenin and Hippo signaling pathways by circACTN4 contributes to CCA cell growth and metastasis.

translocated to the cytoplasm with the assistance of UAP56 or URH49, respectively.¹⁰ Wang and colleagues described a nuclear export process specific for a circular repeat containing intron. They identified a NXF1:NXT1-related active process regulating the level of intronic circRNAs in the cytoplasm.¹¹ Another study pinpointed N⁶-methyladenosine (m⁶A) epitranscriptomic modifications favoring the export of circNSUN2 to the cytoplasm.¹² These studies highlight the importance of better characterizing the mechanisms regulating circRNA spatial localization to better understand the underlying molecular mechanisms involved in CCA progression. In the future, the modulation of such transport mechanisms could be considered as an option to modulate circRNA availability and activity.

When it comes to circRNAs, most studies describe a cytoplasmic circRNA sequestering a couple of miRNAs, which are therefore not available to inhibit their predicted target genes. However, it is becoming obvious from emerging functional studies that investigating the molecular mode of action of circRNAs should not be considered through a unique axis. Indeed, several reports, including the one from Chen and colleagues in this issue of *Journal of Hepatology*,⁸ established that circRNAs could operate in a multi-layered coordinated fashion, by acting at different levels on several gene regulatory networks. The ability of a single circRNA to concomitantly sponge miRNAs in the cytoplasm and to recruit RBPs in the nucleus to mediate RNA Pol II transcription has been also reported in gastric cancer.¹³ Hence, circFAT1 exerts tumor suppressive actions by regulating the miR-548g/RUNX1 axis in the cytoplasm and by targeting YBX1 in the nucleus to inhibit its activity.¹³ However, the process regulating circFAT1 spatial localization is unknown.¹³ Factors regulating RNA circularization, such as QKI5 RBP, could also act at a post-transcriptional level to competitively regulate the level of canonical and back-splicing. That competition could

be explained by a shared spliceosome machinery for intron and exon definition and back-splicing.^{14,15} Although initially viewed as non-coding RNAs, growing evidence indicates that circRNAs could also be translated, notably by cap-independent mechanisms that rely on the internal ribosome entry site and m⁶A modifications.¹⁶ Characterizing the function of circRNA-derived peptides is still in its infancy, but several reports described a role as protein baits.¹⁷ An original mode of action of circβ-catenin (a circRNA derived from β-catenin) has recently been reported.¹⁸ Circβ-catenin differs from its cognate mRNA by a new stop codon generated by the circularization junction. Thus, circβ-catenin is translated into a 370-amino acid β-catenin isoform which acts as a decoy. Indeed, this shorter isoform can stabilize full-length β-catenin by antagonizing GSK3β-induced β-catenin phosphorylation and degradation, leading to Wnt/β-catenin pathway activation and promotion of tumor cell growth.¹⁸ In breast cancer, circSMARCA5 was shown to combine several of the functions described above. Indeed, circSMARCA5 binds its parental gene locus forming an R-loop with DNA and regulates concomitantly the splicing machinery and transcriptional pausing, leading to the production of a non-functional truncated SMARCA5 protein.¹⁹ Eventually, growing evidence suggests that circRNAs act as important cellular regulators with various modes of action. However, how many functions a single circRNA can fulfill needs to be determined. This is a fundamental point to address before thinking of the clinical translation of circRNAs as therapeutic targets, as suggested by Chen and colleagues for circACTN4.

Chen and colleagues reported in this issue of *Journal of Hepatology* that increased expression of circACTN4 in iCCA tissues was associated with worse survival.⁸ Although, the prognostic potential of numerous circRNAs has already been described, none have been validated for clinical use. Nonetheless, circRNAs have attracted substantial interest owing to their presence in body fluids (e.g. serum, plasma, saliva), circulating either freely or encapsulated in EVs, particularly in exosomes (100-200 nm EVs).²⁰ Moreover, circRNAs display an expanded half-life compared to linear RNAs, suggesting they could challenge the sensitivity and specificity of standard biomarkers. Thus, measuring the level of circulating circRNAs in liquid biopsies represents an appealing alternative to tissue biopsies.²⁰

Taken collectively, the study by Chen and colleagues provides an elegant example of how circRNAs could contribute to a coordinated activation of signaling pathways involved in CCA progression. Along with other pioneering studies in the field, it highlights the promising potential of circRNAs as therapeutic targets and clinically relevant biomarkers. Nevertheless, the field is still in its infancy, with many unanswered questions, notably regarding the exact functions and modes of action of circRNAs in the gene regulatory network, and how they contribute to tumor progression. Integrating coding and non-coding RNA profiles could lead to a comprehensive picture of circRNA/miRNA/mRNA networks and to a better understanding of the underlying molecular mechanisms involved in CCA tumorigenesis.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

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Authors' contributions

All authors contributed equally to the intellectual content of the manuscript. All authors wrote the initial draft and contributed significantly to the final version.

Supplementary data

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Commentary

Exosomal circRNAs: new players in the field of cholangiocarcinoma

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Cholangiocarcinoma (CCA) is a deadly cancer worldwide associated with limited therapeutic options. A recent study published in *Clinical Science* by Wang and colleagues [*Clin. Sci.* (2019) **133**(18), 1935–1953] brought new perspectives to CCA management and therapy by focusing on circular RNAs (circRNAs). CircRNAs belong to an emerging class of functional non-coding RNAs (ncRNAs) regulating numerous biological processes. Notably, circRNAs have been associated with cancer onset and progression, although reports in CCA are very limited so far. In this work, the expression of circular RNA circ-0000284 (aka circHIPK3) was specifically elevated in CCA cell lines, human tumor tissues and plasma exosomes. Gain and loss of function approaches were performed to better understand the molecular mechanisms regulated by circ-0000284. Notably, the authors evaluated the role of circ-0000284 as a microRNA (miRNA) sponge. By prediction analysis and functional tests, a direct interaction was demonstrated with miR-637 that targets lymphocyte antigen-6 E (LY6E). Increased expression of circ-0000284 was associated with enhanced migration, invasion and proliferation of CCA cell lines. Interestingly, exosomal-mediated circ-0000284 was reported to exhibit pro-oncogenic effects on surrounding normal cells. Altogether, these data highlight circRNAs not only as new players in CCA pathogenesis but also as promising molecules for innovative non-invasive biomarkers, as circRNAs are enriched and stable in exosomes. Further investigations on extracellular vesicles should provide the necessary tools to improve CCA diagnosis, and move toward targeted-therapies.

Introduction

Cholangiocarcinoma (CCA) refer to a heterogeneous group of aggressive tumors that arise within both intrahepatic and extrahepatic bile ducts. CCA represent the second most common cancer in the liver after hepatocellular carcinoma (HCC). CCA incidence has been steadily increasing during the last decades and the absence of characteristic symptoms restrains early diagnosis [1]. In addition, resistance of CCA to conventional chemotherapies and radiotherapies approaches limits therapeutic options [2]. Surgical approaches remain the best possibly curative alternative for a total recovery. However, even total resections with safety margins are associated with a high rate of recurrence and a 5-year survival ranging from 15% to 40% [3]. Hence, fresh ideas aiming at identifying novel therapeutic strategies and biomarkers are urgently needed to improve the survival of patients with CCA.

circRNAs: an emerging class of functional ncRNAs

Recently, circular RNAs (circRNAs) emerged in the literature as a puzzling class of non-coding RNAs (ncRNAs). For a long time, circRNAs were viewed as splicing noise or byproducts of splicing errors. Eventually, they drew increasing interest after being found dysregulated in several types of cancer, including CCA [4]. Subsequently, growing evidences indicated that circRNAs play a key role in cancer onset and progression, by regulating notably cell proliferation, migration or invasion [5]. It is now recognized that

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circRNAs, unlike linear RNAs, are generated through back-splicing and form a covalently closed continuous loop without 3' and 5' extremities. This specific structure renders circRNAs more stable and resistant to exonuclease degradation. Hence, circRNAs could be of a valuable assistance for clinical diagnosis, possibly serving as potent predictive non-invasive biomarkers, notably in CCA [6]. The origin, biogenesis and functions of circRNAs remained unclear for many years. Nevertheless, points of consensus are starting to emerge in the literature. For instance, one of the latest studies in the field strengthened the established process governing circRNA biogenesis [7]. Thus, it was confirmed that circRNAs are natural byproducts of spliceosome-mediated splicing. Notably, a model whereby intron definition, exon definition and back-splicing require the same spliceosome machinery has been highlighted, suggesting that alternative back-splicing may compete with canonical splicing and thus regulate cell function at a post-transcriptional level. Interestingly, canonical and alternative back-splicing may be regulated by *cis* or *trans* factors [7]. Accordingly, Conn et al. identified the TGF β -induced RNA-binding protein Quaking (QKI) as a regulator of circRNA biogenesis binding on introns flanking circRNA-forming exons [8].

Almost 30 years after the discovery of the first spliced circular products [9], active research in the field improved our understanding of circRNA function. Thus, circRNAs were mainly described as post-transcriptional regulators due to their capability to sponge microRNAs (miRNAs). However, additional functions have been attributed to circRNAs over the last years, including sequestration of proteins, competition with canonical mRNA splicing and transcriptional regulation of their host gene [10]. In addition, a new school of thought is reconsidering the non-coding nature of some circRNAs. This assertion is supported by recent evidences identifying translation initiation systems in circRNAs [11].

circRNAs: a wide-open field for research in CCA

To date, only few recent studies investigated the role of circRNAs in CCA pathogenesis. One of them focused on CDRIAS, a circRNA already largely described for its implication in cancer onset and progression. Thus, CDRIAS expression was significantly higher in CCA tissue samples and correlated with clinical features such as lymph node invasion, advanced TNM stage and a poor overall survival [12]. Another study reported the expression of circRNAs in intrahepatic cholangiocarcinoma (iCCA). Gene expression profiling using pangenomic microarrays identified TGF β -induced long ncRNA TLINC (formerly known as CASC15) in iCCA cell lines. Subsequently, this work revealed the presence of circular isoforms of TLINC in iCCA tissue samples [13]. Although the function of TLINC circRNA in CCA carcinogenesis was not explicitly explored, a putative role in cancer stem cell biology is supported by an elevated expression of TLINC circRNA in human embryonic stem cells. [14]. A couple of studies performed by Xu et al. pinpointed two aberrantly expressed circRNAs in both CCA cell lines and human tumors. The involvement of these circRNAs in CCA progression has been determined by both *in vitro* and *in vivo* experiments. Thus, circ-0001649 and circ-0005230 were respectively down- and up-regulated in CCA cell lines and human tumors and their role as putative miRNA sponge was evaluated. In both cases, these circRNAs were associated with CCA progression and deserve further investigations as therapeutic targets for CCA [4,15]. In fact, most of the studies reported in CCA so far highlighted circRNA deregulations and their clinical relevance rather than performing a deep and comprehensive functional analysis.

A comprehensive analysis of circ-0000284 (circHIPK3) in CCA

The recent study published in *Clinical Science* by Wang and colleagues identified for the first time in CCA, circ-0000284, an isoform of the well-known circHIPK3 circRNA involved in tumor progression [16]. Prediction data reported 21 isoforms of circHIPK3 deriving from the same pre-mRNA [17]. Although all these isoforms were extensively described in cancer, circ-0000284 is definitely the most investigated one [18–24]. However, opposite actions for circ-0000284 were reported depending on the type of cancer (Table 1). Thus, circ-0000284 can exhibit either an oncogenic or a tumor suppressor role. As it stands, it is impossible to conclude firmly on a general function of circ-0000284 in cancer. However, it seems increasingly clear that circ-0000284 acts mainly as a miRNA sponge (Table 1). To this extent, there are strong evidences showing that circ-0000284 rather exhibits pro-oncogenic features. Further investigations are needed to determine whether specific circHIPK3 isoforms carry either oncogenic or tumor-suppressive features. As far as CCA is concerned, the recent work by Wang and colleagues demonstrated that circ-0000284 was able to sponge miR-637 and to promote CCA progression. Hence, the authors demonstrated that circ-0000284 acts as an oncogene by promoting CCA cell migration, invasion and proliferation. Further mechanistic experiments established the ability of circ-0000284 to sponge miR-637, leading to increased expression of lymphocyte antigen-6 E (LY6E) through a competitive binding to miR-637 (Figure 1). LY6 complex is a group of alloantigens that was first discovered on lymphocytes. Interestingly, increased expression of LY6 family was reported in multiple types of cancer,

Table 1 Examples of cancer-specific role and regulatory functions of circ-000284

Cancer type	Function	Oncogene or tumor suppressor	Reference
Bladder cancer	miRNA sponge (miR-558)	Tumor suppressor	[23]
Colorectal cancer	miRNA sponge (miR-7)	Oncogene	[24]
Hepatocellular carcinoma	miRNA sponge (miR-124)	Oncogene	[20]
Glioma	miRNA sponge (miR-654)	Oncogene	[21]
Nasopharyngeal carcinoma	miRNA sponge (miR-4288)	Oncogene	[22]
Cholangiocarcinoma	miRNA sponge (miR-637)	Oncogene	[16]

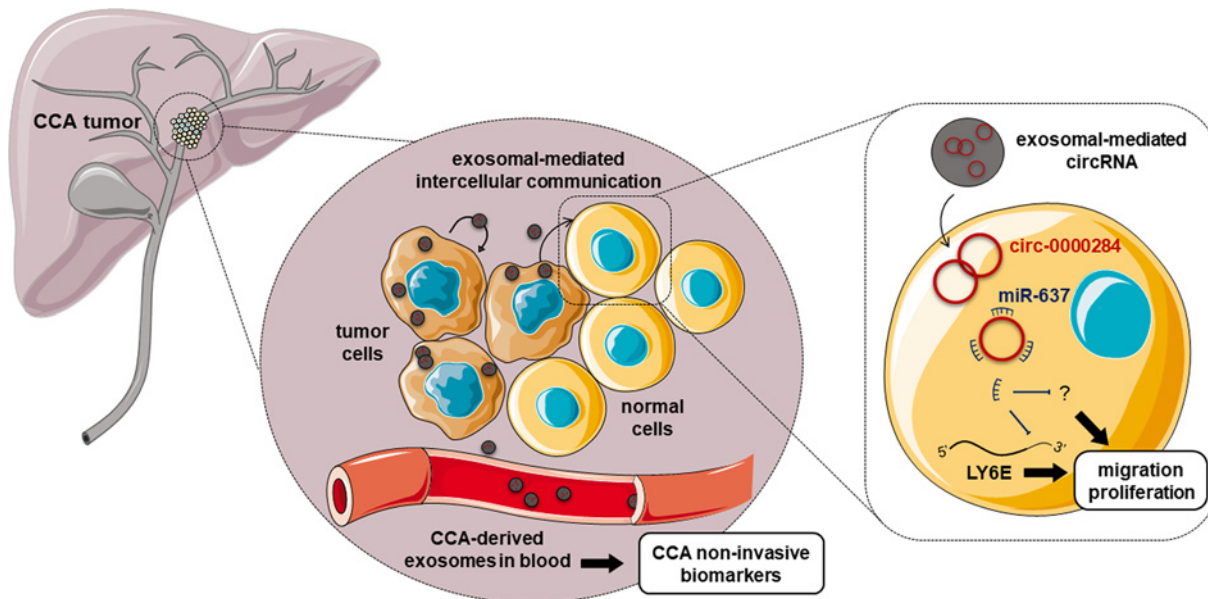


Figure 1. Circ-000284 promotes tumor progression in CCA cells acting as competitive endogenous RNA by sponging miR-637

Circ-000284 holds sway over surrounding cells biological functions through exosomal communication. Exosomes released in blood represent a potential biomarker for CCA (modified from Servier Medical images: <https://smart.servier.com>).

Table 2 Examples of disease-specific targets and regulatory functions of miR-637

miR-637 target	Endogenous miR-637 competitor	Disease	Reference
CDK6	LINC00473	Glioma	[26]
KLK4	circCEACAM5	Ovarian cancer	[27]
RING1	C5orf66-AS1 lncRNA	Cervical cancer	[28]
STAT3	circHIPK3	Colorectal cancer	[29]
NUPR1	FAL1 lncRNA	Colorectal cancer	[30]
NUPR1	LINC01234	Oral squamous cell carcinoma	[31]
APELIN	circNOTCH1	Gastric cancer	[32]
MMP19	circERBB2	Gastric cancer	[33]
AKT1	FAL1 lncRNA	Hirschsprung's disease	[34]

and was subsequently associated with poor overall and disease-free survival. Thus, high LY6E expression was significantly correlated with poor clinical outcome in colorectal cancer, glioma, ovarian cancer, gastric cancer, breast cancer and lung cancer. Altogether, these data suggest that LY6 family members play a crucial role in tumor progression and represent clinically relevant target [25]. To date, these data are the first to support a correlation between a high LY6E expression and a pro-oncogenic action in CCA, and providing a noteworthy mechanistic explanation [16]. However, LY6E is not the only target of miR-637 as reported in the literature (Table 2). Thus, miR-637 could affect CCA progression by targeting additional transcripts encoding proteins involved in cell proliferation and migration (e.g. CDK6,

STAT3, AKT1) (Table 2). In addition, miR-637 could be itself regulated by non-coding endogenous competitors other than solely circHIPK3, as described in other diseases [26–34] (Table 2).

Exosomal circRNAs as non-invasive functional biomarkers in CCA

An important aspect of the study by Wang and colleagues relies on evaluating for the first time in CCA the influence of exosomal-transmitted circRNAs on their immediate cellular microenvironment. Hence, autocrine and paracrine pro-tumorigenic actions mediated by exosome-carried circRNAs (exo-circRNAs) were demonstrated (Figure 1). Although the elevated expression of exo-circ-0000284 has been measured in the host surrounding cells, no further information is provided as regard to the mechanisms that promote cell migration and cell proliferation in these host cells. It was assumed that transformation of host cells was mediated by the same mechanism than the one described in tumor cells where exo-circ-0000284 was originating from. Thus, a mechanism involving miR-637 sponging needs to be more clearly defined. In addition, the possibility that exo-circ-0000284 involves different miRNA regulatory networks in host cells should not be excluded. Interestingly, an increased expression of circ-0000284 was also shown in plasma-derived exosomes from patients with CCA. To our knowledge, this is the first report of circulating exo-circRNAs in CCA. Thus, these findings provided a promising alternative for existing CCA biomarkers. Similarly, a recent paper highlighted the clinical relevance of protein biomarkers conveyed in body fluids via extracellular vesicles (EVs) in CCA [35]. Thus, CCA-derived EVs from patient serum might serve as cargo for oncogenic proteins [35]. Similar to circRNAs, EV-derived biomarkers are about to progressively challenge the poor established biomarkers in CCA. For instance, serum level of carbohydrate antigen 19-9 (CA19-9) or carcinoembryonic antigen (CEA) are two possible biomarkers to suspect CCA diagnosis. However, several studies reported the poor specificity and sensitivity of these biomarkers, highlighting the need for more efficient substitutes for CCA diagnosis and prognosis [36–38]. For that purpose, it would be of great interest to identify clinically relevant circRNA signatures in CCA to improve patient outcome, as circRNAs are easily detectable and stable in body fluids.

Conclusion

In conclusion, recent data provide evidence of circRNAs potential as robust biomarkers with diagnostic and prognostic values in CCA. Remarkably, Wang and colleagues identified, for the first time in CCA, an exosome-mediated circRNA that was able to display autocrine and paracrine actions promoting CCA progression. Interestingly, this circRNA was also found in plasma-derived-exosomes, opening new perspectives for the use of exo-circRNAs as non-invasive biomarkers. Further investigations should provide the necessary tools to improve CCA diagnosis, and move toward targeted-therapies.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

CCA, cholangiocarcinoma; circRNA, circular RNA; EV, extracellular vesicle; exo-circRNA, exosomes circRNA; HCC, hepatocellular carcinoma; LY6E, lymphocyte antigen-6 E; miRNA, microRNAs; ncRNA, non-coding RNA.

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Article

A Minimal Subset of Seven Genes Associated with Tumor Hepatocyte Differentiation Predicts a Poor Prognosis in Human Hepatocellular Carcinoma

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Simple Summary: Liver cancer is one of the most commonly diagnosed cancers worldwide and the fourth leading cause of cancer-related deaths. Hepatocellular carcinoma (HCC) accounts for at least 80% of all malignant liver primary tumors. A better characterization of molecular mechanisms underlying HCC onset and progression may lead to discover new therapeutic targets and biomarkers. In this study, we performed an integrative transcriptomics analysis to evaluate the clinical relevance of genes associated with hepatocyte differentiation in human HCC. The HepaRG cell line model was used to define a gene expression signature reflecting the status of tumor hepatocyte differentiation. This signature was able to stratify HCC patients into clinically relevant molecular subtypes. Then, a minimal subset of seven differentiation-associated genes was identified to predict a poor prognosis in several cancer datasets.

Abstract: Hepatocellular carcinoma (HCC) is a deadly cancer worldwide as a result of a frequent late diagnosis which limits the therapeutic options. Tumor progression in HCC is closely correlated with the dedifferentiation of hepatocytes, the main parenchymal cells in the liver. Here, we hypothesized that the expression level of genes reflecting the differentiation status of tumor hepatocytes could be clinically relevant in defining subsets of patients with different clinical outcomes. To test this hypothesis, an integrative transcriptomics approach was used to stratify a cohort of 139 HCC patients based on a gene expression signature established in vitro in the HepaRG cell line using well-controlled culture conditions recapitulating tumor hepatocyte differentiation. The HepaRG model was first validated by identifying a robust gene expression signature associated with hepatocyte differentiation and liver metabolism. In addition, the signature was able to distinguish specific developmental stages in mice. More importantly, the signature identified a subset of human HCC associated with a poor prognosis and cancer stem cell features. By using an independent HCC dataset (TCGA consortium), a minimal subset of seven differentiation-related genes was shown to predict a reduced overall survival, not only in patients with HCC but also in other types of cancers (e.g., kidney, pancreas, skin). In conclusion, the study identified a minimal subset of seven genes reflecting the differentiation status of tumor hepatocytes and clinically relevant for predicting the prognosis of HCC patients.

Keywords: hepatocellular carcinoma; prognosis; signature; differentiation; overall survival; integrative transcriptomics



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1. Introduction

Liver cancer is one of the most commonly diagnosed cancers worldwide (841,000 cases) and the fourth leading cause of cancer-related deaths (782,000 cases) [1]. Both the incidence

and the mortality of liver cancer have increased over the last two decades. Hepatocellular carcinoma (HCC) accounts for at least 80% of all malignant liver primary tumors. HCC frequently occurs in a background of fibrotic and/or cirrhotic liver and is usually asymptomatic during the early phases of carcinogenesis. Thus, most of the patients are diagnosed with advanced stage HCC, which limits the therapeutic options and their efficacy [2]. Tumor heterogeneity in advanced HCC also impedes the development of effective treatments. Genome-scale studies at the DNA level (e.g., mutation analysis, epigenetic profiling) established the landscape of molecular alterations occurring in HCC. Mutations in the *TERT* promoter and in *TP53*, and *CTNNB1* genes were identified as the most frequent driver mutations in HCC [3].

Gene expression profiling studies at the RNA level defined key genes and pathways commonly altered in HCC [4] and identified clinically relevant tumor subtypes [5–7]. By integrating these studies, a general consensus on the molecular classification of human HCC has been achieved [5]. Thus, HCC are classified into two main molecular classes: a proliferation class and a non-proliferation class. The proliferation class includes aggressive and poorly differentiated HCC associated with frequent signs of vascular invasion. These HCC are more frequently mutated for *TP53* [8]. This proliferation class is further subdivided into a “WNT-TGF β ” subclass defined by the activation of WNT and TGF β pathways and an exhausted immune response, and a “progenitor” subclass defined by the expression of progenitor markers [6,9,10]. In contrast, the non-proliferation class includes more differentiated HCC with hepatocyte-like features, the up-regulation of liver-specific genes, and a better prognosis [5,8]. A gene regulatory network linked with β -catenin dysfunction and dedifferentiation was found to be negatively correlated with HCC prognosis [11]. It was also reported that the overexpression of hepatocyte nuclear factor-1 alpha (HNF1A), a key transcription factor driving hepatocyte differentiation, inhibited HCC growth in mice [12].

Altogether, unsupervised gene expression profiling and supervised functional studies established a functional link between hepatocyte dedifferentiation, HCC progression and a poor clinical outcome, suggesting that dedifferentiation markers could be clinically relevant to predict HCC prognosis [6–8,13]. However, no experimentally validated signature including an exhaustive list of genes linked with differentiation has been reported so far. Most of the prognostic signatures derived from transcriptomics studies consist of hundreds of genes, some of them related to differentiation, rendering a routine clinical implementation challenging [14]. Here, we used the HepaRG cell line model cultured under well-controlled conditions recapitulating tumor hepatocyte differentiation [15–17] combined with an integrative transcriptomics approach [18,19] to identify a minimal subset of seven genes reflecting the differentiation status of tumor hepatocytes and clinically relevant for predicting overall survival in independent cohorts of patients with HCC.

2. Materials and Methods

2.1. Culture of HepaRG Cells

HepaRG cells were cultured as previously described [18]. Briefly, cells were grown in William’s E medium supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 5 mg/mL insulin, and 50 mmol/L hydrocortisone hemisuccinate. Differentiation of HepaRG from progenitors to mature well-differentiated hepatocytes was achieved within 4 weeks by culturing the cells in the supplemented medium in presence of 2% dimethyl sulfoxide (DMSO) for the last 2 weeks. All experiments, hereinafter referred to as progenitor HepaRG, were carried out using cells isolated 3 days after plating, while experiments referred to as differentiated HepaRG were carried out using hepatocytes selectively isolated by mild trypsinization from DMSO-treated cultures 4 weeks after the initial plating. All cell cultures were conducted at 37 °C in a 5% CO₂ atmosphere. Independent culture experiments were carried out in triplicate.

2.2. Gene Expression Profiling

Total RNA was purified from cells at 80% confluence with a RNeasy kit (Qiagen). Genome-wide expression profiling was conducted using the low-input Quick Amp Labeling Kit and human SurePrint G3 8 60 K pangenomic microarrays (Agilent Technologies) as previously described [4]. Starting from 150 ng total RNA, amplification yield was $9.7 \pm 0.6 \mu\text{g}$ cRNA and specific activity was $20.3 \pm 1.3 \text{ pmol Cy3 per } \mu\text{g cRNA}$. Gene expression data were processed using the Feature Extraction and GeneSpring software (Agilent Technologies) and further analyzed using R-based ArrayTools. Clustering analysis was carried out using Cluster 3.0 and TreeView 1.6 using uncentered correlation and average linkage options. MIAME compliant microarray data have been deposited into the Gene Expression Omnibus (GEO) database (GSE181963).

2.3. Data Mining and Integrative Transcriptomics

Gene annotation was based on Gene Ontology terms and enrichment for specific biologic functions or canonical pathways was evaluated using FuncAssociate 2.0 [20]. Gene set enrichment analysis (GSEA) was conducted by using the Java tool developed at the Broad Institute (Cambridge, MA, USA) as previously described [21]. Unsupervised GSEA was carried out with the whole C2 collection of curated gene sets from the molecular signatures database (MSigDB). Enrichment score was determined after 1000 permutations. Integration of genomic data was conducted as previously described [19] using publicly available gene expression data sets downloaded from GEO. Survival analysis was conducted using the TCGA datasets (<https://portal.gdc.cancer.gov>, accessed on July 2021).

2.4. Real-Time Reverse Transcriptase PCR

Expressions of relevant genes were measured by quantitative real-time reverse transcriptase PCR (qRT-PCR), as previously described [22]. Quantitative analysis of PCR data was conducted with the $2\Delta\Delta\text{Ct}$ method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ct values for normalization. Melting analysis was conducted to validate the specificity of PCR products. PCR and gene expression profiling experiments were conducted using RNA extracted from independent cultures ($n = 3$).

2.5. Statistical Analysis

Data are presented as the mean \pm standard deviation (SD). Statistical analyses were performed using R-3.5.1 and GraphPad Prism 7.0. For gene expression profiling, differentially expressed genes were identified by a 2-sample univariate t test and a random variance model ($p < 0.01$; false discovery rate $< 1\%$) as described [23]. Permutation p values for significant genes were computed on the basis of 10,000 random permutations. For group comparison of quantitative variables, a Mann–Whitney test was applied. Categorical data were analyzed by chi-squared testing. The cumulative survival rate was estimated by the Kaplan–Meier method and the survival curves were compared with the log-rank test. p values < 0.05 were considered as significant statistical differences.

3. Results

3.1. Flowchart of the Study Design

Gene expression profiling was performed to identify genes significantly differentially expressed between differentiated and progenitor HepaRG cells (Figure 1). Then, an integrative transcriptomics analysis was conducted using publicly available human HCC gene expression datasets downloaded from GEO to evaluate the clinical relevance of the hepatocyte differentiation signature derived from HepaRG, as previously described [18]. The so-called NCI dataset (GSE1898 and GSE4024) was used as a training set ($n = 139$ HCC) and the TCGA RNA-seq dataset (<https://portal.gdc.cancer.gov>, accessed on July 2021) was used as a validating dataset ($n = 372$ HCC), as well as the GSE14520 dataset ($n = 245$ HCC). Kaplan–Meier plots and log-rank statistics were used to identify genes associated with overall survival (OS). Merging the OS-associated genes from the training and validat-

ing datasets was performed to identify a minimal subset of differentiation-related genes predicting OS in patients with HCC (Figure 1).

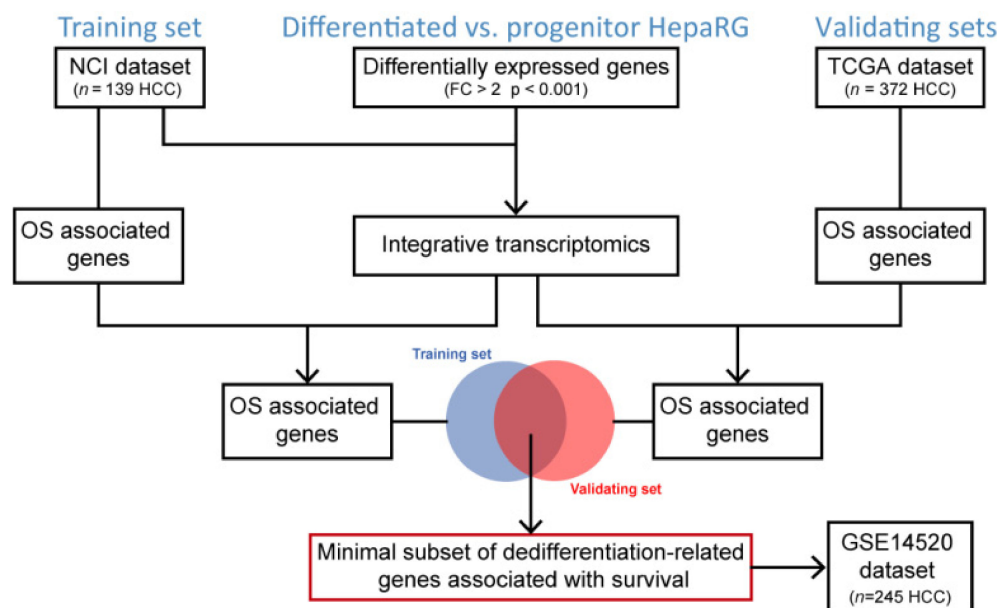


Figure 1. Flowchart of the study design. OS, overall survival.

3.2. Differentiation-Associated Genes in HepaRG Cells

By using stringent statistical criteria ($p < 0.001$ and fold change $FC > 2$), the volcano plot analysis identified 197 probes, corresponding to 174 non-redundant and well-annotated genes differentially expressed between differentiated and progenitor HepaRG cells. This signature included 105 up- and 69 down-regulated genes in differentiated HepaRG cells as compared to HepaRG progenitors (Figure 2A). The clustering analysis highlighted genes related to liver-specific metabolisms (Figure 2B and Table S1). Thus, genes associated with the complement and coagulation pathways (e.g., *C2*, *C4B*, *CFH*, *CR2*, *PROC*) or encoding secreted proteins involved in the response of the liver to acute systemic inflammation (e.g., *A2M*, *CRP*, *FGA*, *ORM1*, *SAA2*, *SERPINA1*) [24,25] were significantly induced in differentiated HepaRG cells. Similarly, genes encoding members of the cytochrome P450 superfamily (e.g., *CYP1A1*, *CYP8B1*) involved in drug metabolism and lipid synthesis were more expressed in differentiated HepaRG, as well as genes encoding alcohol-metabolizing enzymes (e.g., *ADH1A*, *ALDH2*) and nuclear receptors regulating xenobiotic metabolism (e.g., *NR1I3*). Other genes more expressed in differentiated HepaRG cells were involved in glutathione (e.g., *GPX3*) and amino acid (e.g., *PAH*, *PIPOX*) metabolisms, ketogenesis (e.g., *HMGCS2*) or encoding cell junctions (e.g., *CDH1*, *TJP3*), aquaporins (e.g., *AQP9*, *AQP11*) and transmembrane proteins (e.g., *SLC17A1*, *SLC22A23*), all of them reflecting well-differentiated functional hepatocytes. Conversely, the genes more expressed in HepaRG progenitors were associated with cell cycle, proliferation and apoptosis (e.g., *CDK20*, *NUAK1*, *BMF*, *CASP7*), hypoxia (e.g., *CITED2*), chemoattraction (e.g., *CXCL1*, *CXCR7*) and angiogenesis (e.g., *EDIL3*, *NRP1*). Interestingly, several genes were known to be regulated by the TGF β pathway (e.g., *BMF*, *COL4A4*) and/or expressed in several cancers (e.g., *HOTAIR*, *MRC2*, *POSTN*, *SPARC*). Next, the expression of eight representative genes were evaluated by qRT-PCR (Figure 2C), including four genes more expressed in differentiated HepaRG cells (*FGA*, *CDH1*, *CR2* and *CFH*) and four genes more expressed in HepaRG progenitors (*COL4A4*, *CXCR7*, *HOTAIR* and *POSTN*). The expression of all genes was validated (Figure 2C). Further supporting the gene selection, GSEA demonstrated that well-curated liver-related gene signatures were significantly enriched in the gene expression profiles of differentiated HepaRG cells (Figure 2D), including liver-specific genes [26] as well as gene sets associated with liver development [27] and well-differentiated HCC tumors (e.g., the

so-called HCC molecular subclass S3 defined by Hoshida and colleagues [6]). Altogether, these data validate the gene expression signature established in HepaRG cells and suggest that it could be relevant in vivo, both in mouse liver development and in human liver carcinogenesis.

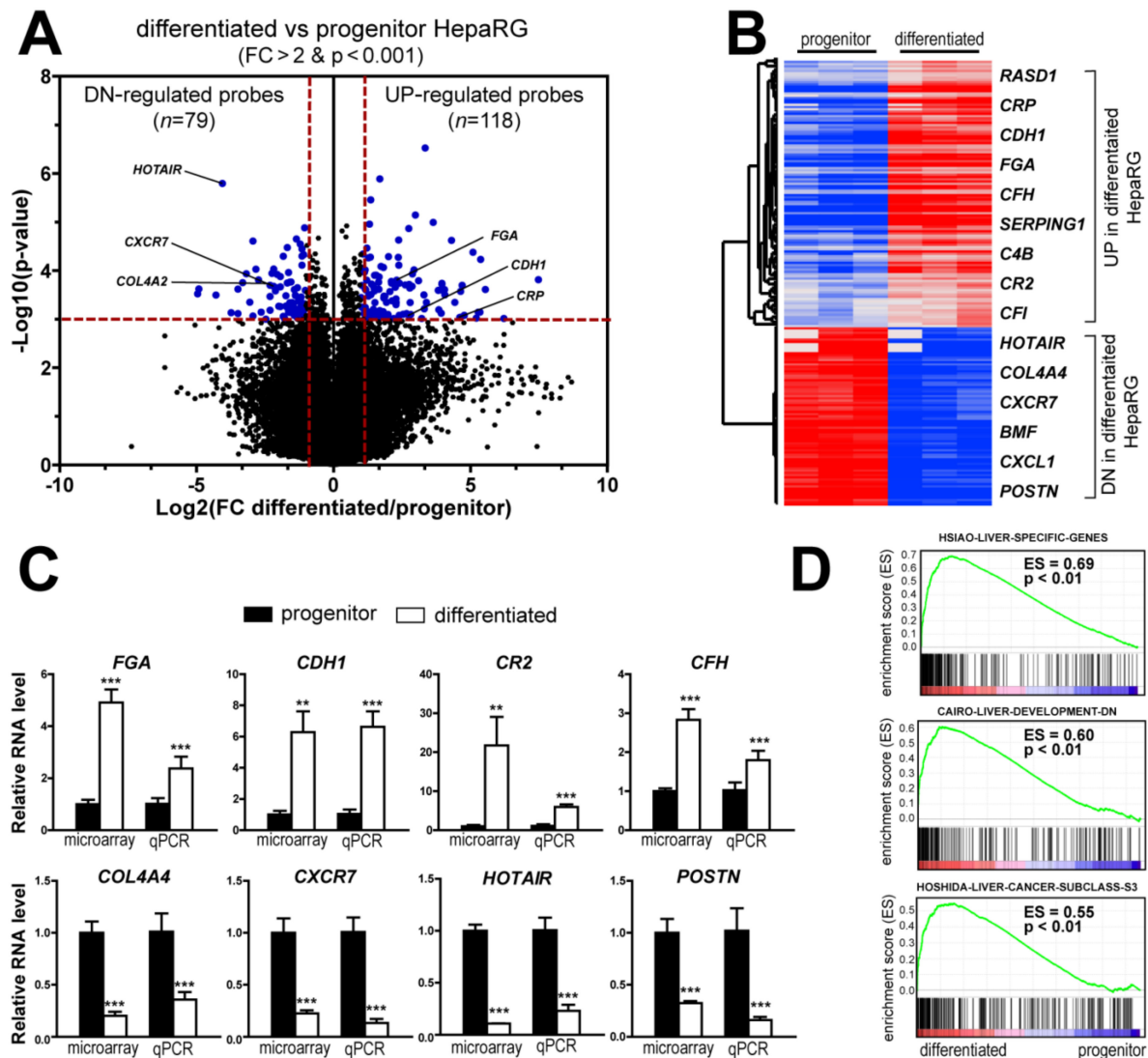


Figure 2. Differentiated and progenitor HepaRG cells exhibit distinct gene expression profiles related to hepatocyte differentiation. **(A)** Volcano plot of genes differentially expressed between differentiated and progenitor HepaRG cells. Differentiated HepaRG cells display a specific signature of 118 probes (105 genes) up-regulated and 79 probes (69 genes) down-regulated. Horizontal dashed red line: $p < 0.001$. Vertical dashed red lines: FC > 2. **(B)** Hierarchical clustering analysis and examples of genes up- and down-regulated in differentiated and progenitor HepaRG cells. The complete list of probes and genes is provided in the Supplementary Table S1. **(C)** Gene expression analysis of representative genes differentially expressed in differentiated and progenitor HepaRG cells, as determined by microarray and qRT-PCR. Statistical analysis was performed by a Mann–Whitney test (** $p < 0.01$, *** $p < 0.001$, $n \geq 3$). **(D)** GSEA analysis using the gene expression profiles of differentiated (left side) and progenitor (right side) HepaRG cells. GSEA highlights a significant enrichment ($p < 0.01$) of several liver-related signatures, including the so-called HSAIO_LIVER_SPECIFIC_GENES [26], CAIRO_LIVER_DEVELOPMENT_DN [27] and HOSHIDA_LIVER_CANCER_SUBCLASS_S3 [6] signatures.

3.3. HepaRG-Derived Signature Recapitulates Relevant Stages of Mouse Liver Development

To determine the in vivo relevance of the gene expression profiles derived from HepaRG cells (differentiated versus progenitor), we integrated them with the multi-stage gene expression profiles established in the course of mouse liver development [28]. The

GSE13149 dataset reports transcriptomic profiles at 14 time points across the C57/B6 mouse liver development, which include E11.5 (embryonic day 11.5), E12.5, E13.5, E14.5, E15.5, E16.5, E17.5, E18.5, Day0 (the day of birth), Day3, Day7, Day14, Day21, and normal adult liver. Orthologous genes were retrieved from both species, as previously described [19]. Clustering analysis based on mouse and human orthologous genes demonstrated that the gene expression profiles of differentiated HepaRG cells clustered with post-natal mouse samples, i.e., from Day0 to Day21 and adult liver samples (Figure 3A). Conversely, the gene expression profiles of HepaRG progenitor cells clustered with those of early embryonic livers, particularly between E11.5 and E14.5 (Figure 3A). Examples of the expression of developmentally regulated genes differentially expressed in differentiated and progenitor HepaRG cells (e.g., *PAH*, *HAO1*, *CXCR7*) are shown in Figure 3B.

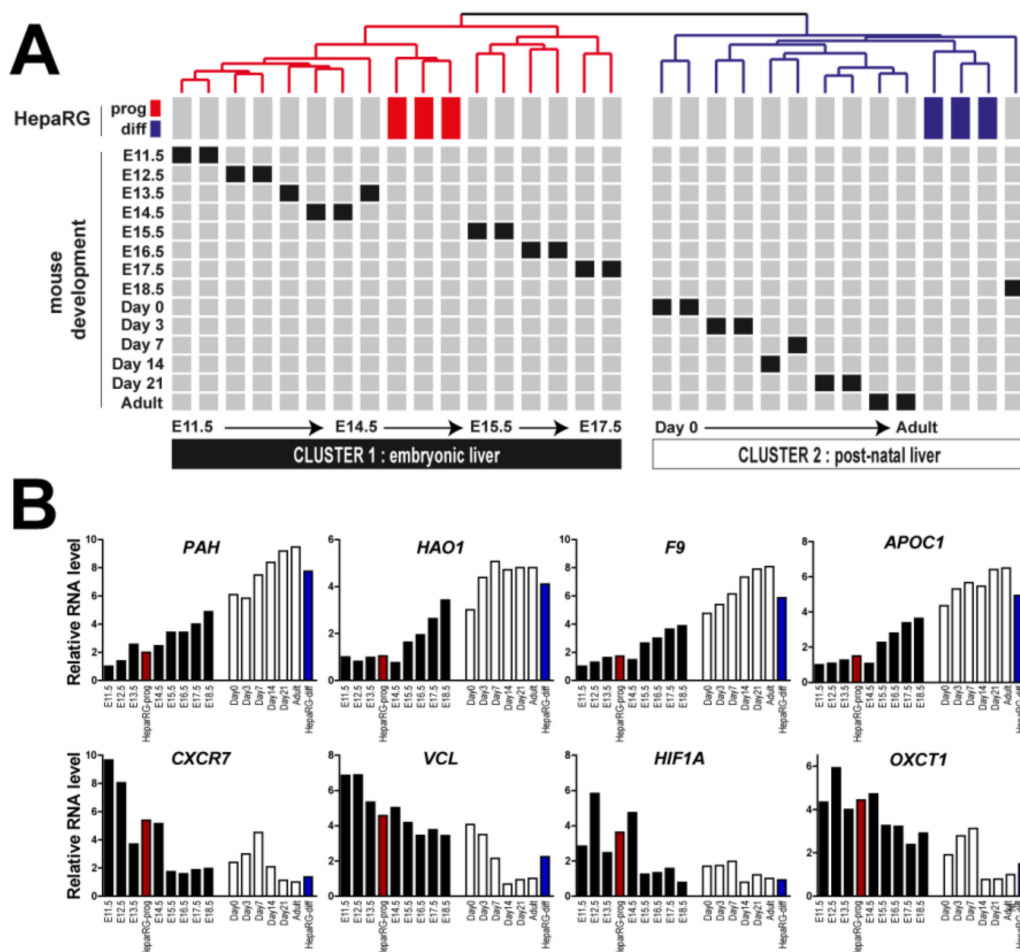


Figure 3. Differentiated and progenitor HepaRG gene profiles recapitulate relevant stages of mouse liver development. (A) Clustering of mouse and human samples based on the expression of orthologous genes identified from the signature of genes differentially expressed between differentiated and progenitor HepaRG cells. Data are presented in a matrix format in which rows and columns represent the position of each sample within the dendrogram. Mouse gene expression profiling was performed between E11.5 to adult stage. Clustering analysis identified two major clusters: cluster 1, which includes progenitor HepaRG cells (red boxes), is associated with embryonic mouse liver samples and cluster 2, which includes differentiated HepaRG cells (blue boxes), is associated with post-natal mouse liver samples. (B) Examples of the expression of developmentally regulated genes. Red bars: progenitor HepaRG cells; blue bars: differentiated HepaRG cells; black bars: embryonic stages in mice (from E11.5 to E18.5, left to right); white bars: post-natal stages in mice (from Day0 to adult stage, left to right).

3.4. HepaRG-Derived Signature Distinguishes Relevant Human HCC Subtypes

Based on the GSEA results demonstrating a significant enrichment of a signature featuring a well-differentiated human HCC subtype (Figure 2D), we hypothesized that the differentiation-associated signature established in HepaRG could be clinically relevant. To test this hypothesis, we integrated the HepaRG signature (Table S1) with the gene expression profiles of 139 cases of human HCC extensively characterized (NCI training dataset, Figure 1) [7,10,19,29]. Hierarchical clustering analysis of the integrated dataset (91 genes detected in both datasets) identified two major clusters (Figure 4A). Clusters 1 and 2 included HCC associated with differentiated and progenitor HepaRG, respectively. The biological and clinical parameters were not randomly distributed between the clusters (Figure 4A,B). Strikingly, cluster 1 included significantly more tumors, which were previously assigned to a better prognosis group, than cluster 2 (Figure 4A,B). Conversely, cluster 2 (progenitor HepaRG), was significantly enriched in HCC previously defined by poor prognosis signatures, including poor survival genes [7], hepatoblast-associated genes [10] and genes associated with the activation of a pro-oncogenic MET/HGF pathway [29] and a so-called late pro-metastatic TGF β signature [19] (Figure 4A,B). The serum level of alpha-fetoprotein (AFP), a well-established HCC biomarker, was significantly higher in these aggressive HCC (Figure 4A,B). Accordingly, HCC linked to HepaRG progenitors exhibited a reduced OS (Figure 4C). Altogether, the data demonstrate that the progenitor HepaRG signature predicts a poor prognosis in human HCC.

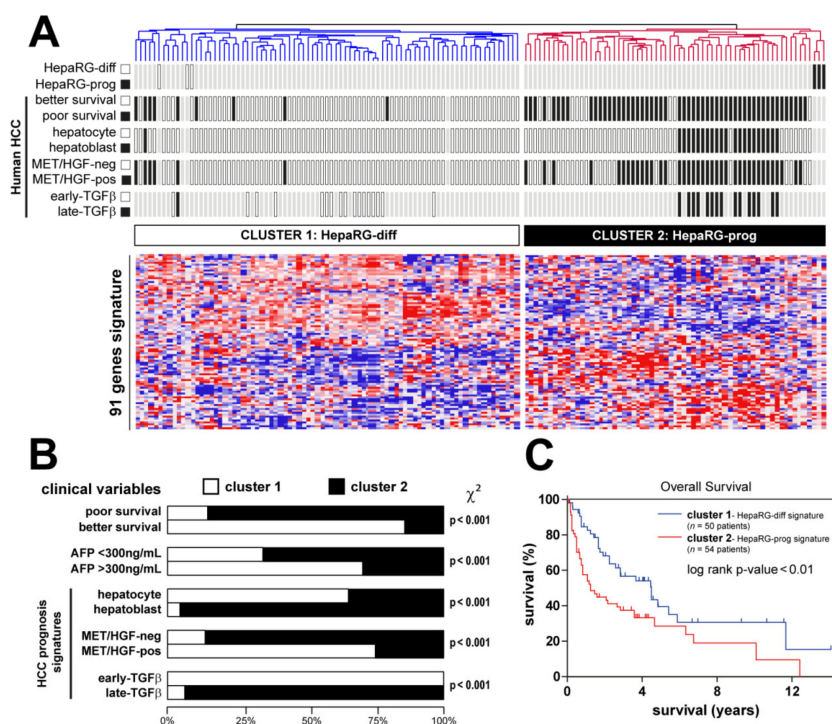


Figure 4. Clinical relevance of HepaRG signature in human HCC. (A) Dendrogram overview of in vitro experiments using differentiated and progenitor HepaRG cells (Figure 2) integrated with 139 cases of human HCC (NCI training set, Figure 1). Clustering analysis was based on the expression of genes differentially expressed between differentiated and progenitor HepaRG (Table S1). Two main clusters were identified: cluster 1 and cluster 2, associated with differentiated and progenitor HepaRG, respectively. Distribution of HCC samples between previously described subgroups with respect to survival (better vs. poor prognosis) [7], cell origin (hepatocyte vs. hepatoblast) [10], activation of MET/HGF (negative vs. positive) [29] and TGF β signaling (early vs. late) [19] is indicated on the left side. (B) Statistical analysis of HCC distribution between clusters 1 and 2 based on previous gene expression signatures and clinical parameters. Cluster 2 (defined by a high expression of genes reflecting the HepaRG progenitor state) shows a significant enrichment in human HCC with the following features: poor survival, hepatoblast traits, activation of MET/HGF, late TGF β pathways and higher serum AFP levels. (C) Kaplan–Meier plot and log-rank statistics analysis revealed a significant decrease in overall survival for patients included in cluster 2.

3.5. A Minimal Subset of Seven Differentiation-Associated Genes Predicts a Poor Prognosis in Several Human Cancers

Next, from the clinically relevant differentiation-associated human gene expression signature, we selected a minimal subset of genes able to predict survival in independent cohorts of patients with HCC (Figure 1). From the NCI training dataset, 15 genes were associated with OS and 21 genes from the TCGA validating dataset, including seven common genes (Figure 5A). The expression of these seven genes (*HMGCS2*, *BDH1*, *ALDH2*, *PIPOX*, *HAO1*, *AQP9* and *PAH*) was negatively correlated with the survival of patients with HCC in two independent datasets (Figure 5A). In agreement with the selection process, data mining demonstrated that this minimal subset of genes was significantly enriched in metabolism (ketone bodies, butanoate, bile acid and glyoxylate metabolism, as well as lysine degradation, peroxisome and Phe, Tyr and Trp biosynthesis) (Figure 5B).

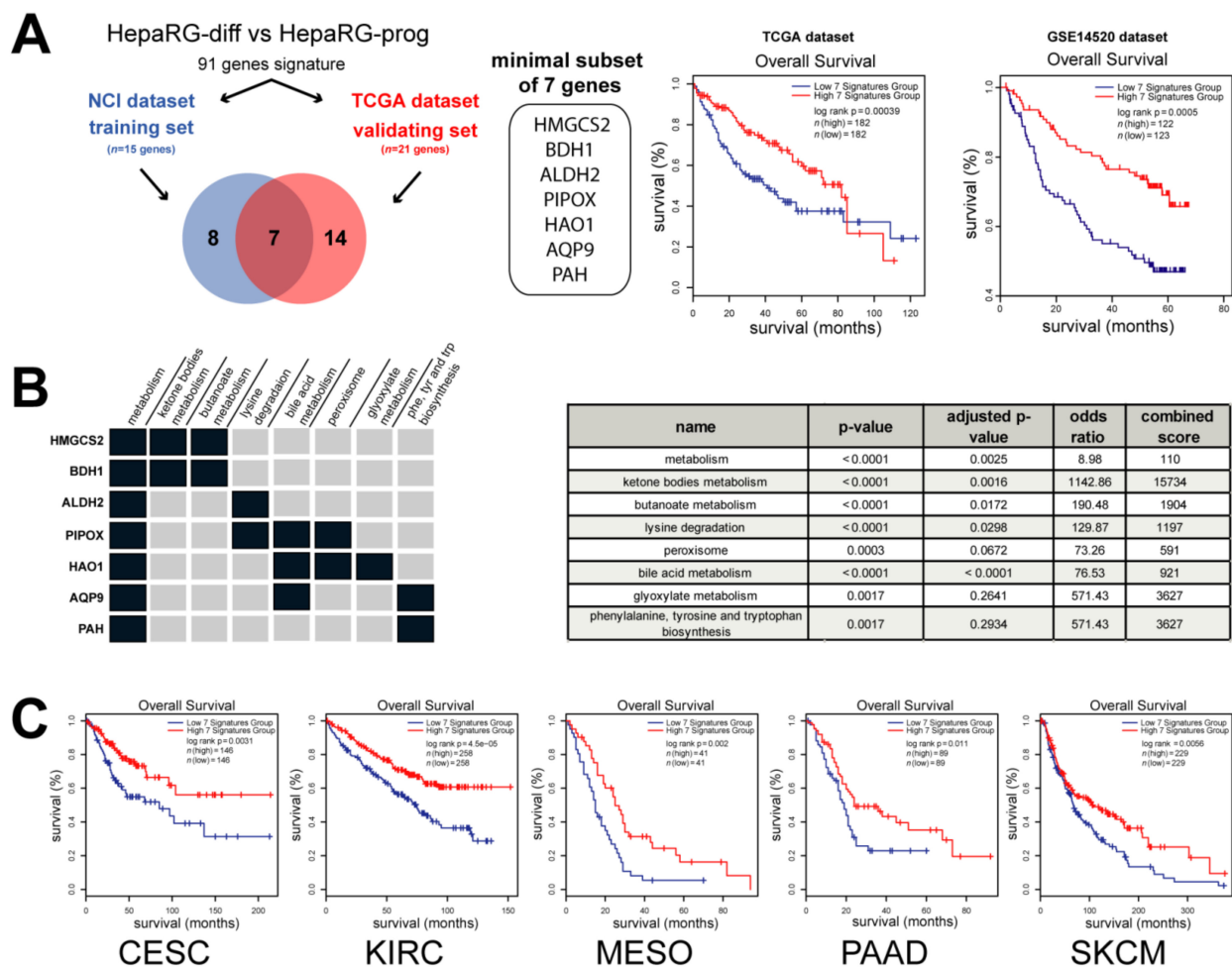


Figure 5. A minimal subset of 7 metabolic genes predicts a poor prognosis in cancer. (A) Analysis of genes from the HepaRG signature described in Figure 4 associated with the overall survival (OS) in two independent datasets: NCI (n = 15 genes) and TCGA (n = 21 genes). Survival analysis unraveled a minimal subset of 7 genes negatively correlated with patient outcome in two distinct datasets (i.e., TCGA, n = 364 HCC and GSE14520, n = 245 HCC). (B) Data mining analysis using Enrichr [30] demonstrated that the 7 genes were significantly associated with metabolism, such as ketone bodies, butanoate, bile acid and glyoxylate metabolisms, as well as lysine degradation, peroxisome and Phe, Tyr and Trp biosynthesis (p value < 0.01) (C) Analysis of overall survival by Kaplan–Meier plot and log-rank statistics in several cancers from TCGA, including cervical squamous cell carcinoma (CESC), kidney renal clear cell carcinoma (KIRC), mesothelioma (MESO), pancreatic adenocarcinoma (PAAD) and skin cutaneous melanoma (SKCM). Patients were divided into two groups based on the expression of the 7 genes taken as a signature (blue curve: low expression; red curve: high expression).

More interestingly, Kaplan–Meier plots and log-rank statistics demonstrated that a low expression of these seven metabolic genes (taken as a unique signature) predicted a decreased OS, not only in patients with HCC (Figure 5A), but also in patients with cervical squamous cell carcinoma, kidney renal clear cell carcinoma, mesothelioma, pancreatic adenocarcinoma and skin cutaneous melanoma (Figure 5C). Altogether, the data demonstrate that a minimal subset of seven metabolic genes associated with hepatocyte differentiation predicts a poor prognosis in several human cancers.

4. Discussion

In this study, by applying a functional and integrative transcriptomics approach based on the well-characterized HepaRG model of tumor hepatocyte differentiation, we identified a minimal subset of seven genes (*HMGCS2*, *BDH1*, *ALDH2*, *PIPOX*, *HAO1*, *AQP9* and *PAH*) predicting the survival of patients with cancer. These genes were mainly associated with metabolism, in agreement with metabolic reprogramming as a key hallmark of cancer cells [31]. All these genes were down-regulated in poor-prognosis HCC.

HMGCS2 encodes the 3-hydroxy-3-methylglutaryl-CoA synthase 2, a mitochondrial enzyme that catalyzes the second and rate-limiting reaction of ketogenesis, a metabolic pathway that provides lipid-derived energy. In agreement with our findings, *HMGCS2* was previously reported to be significantly down-regulated in HCC patients with high alpha-fetoprotein (AFP), tumor size >5 cm, multinodular, advanced tumor staging including BCLC, TNM and CLIP [32]. The Kaplan–Meier analysis also demonstrated that *HMGCS2* down-regulation contributed to an unfavorable OS in liver [32] and prostate [33] cancer. *HMGCS2* may function as a tumor suppressor. Indeed, its knockdown induces the proliferation and metastasis ability of HCC cells by enhancing c-Myc/cyclinD1 and epithelial-to-mesenchymal transition (EMT) signaling and alters lipid metabolism, resulting in increases in fatty acid, triglyceride, and cholesterol syntheses [34,35].

BDH1 (3-hydroxybutyrate dehydrogenase 1) is also involved in the metabolism of ketones. *BDH1* was reported to be down-regulated in glioblastoma [36] and acute myeloid leukemia (AML) [37]. The down-regulation of *BDH1* in AML was notably associated with a worse prognosis [37]. In addition, *BDH1* knock-down promotes the viability and the proliferation of AML cells [37]. The down-regulation of both *HMGCS2* and *BDH1* suggests that the deregulation of ketone metabolism is a hallmark of poor prognosis tumors. Thus, impaired ketogenesis has been shown to contribute to abnormal glucose metabolism and to provoke steatohepatitis [38,39]. Accordingly, therapeutic ketogenic diets have the potential to lower glucose availability to cancer cells and have been associated with better outcomes [39]. It was also reported that ketone bodies inhibited the proliferation of pancreatic cancer cells and that a ketogenic diet reduced tumor growth in mice [40].

ALDH2 (aldehyde dehydrogenase 2) is one of the major mitochondrial enzymes protecting cells from acetaldehyde toxicity. *ALDH2* down-regulation has been widely reported in HCC and associated with an unfavorable outcome [41,42]. The gain and loss of function experiments demonstrated that *ALDH2* inhibited HCC cell migration and invasion both in vitro and in vivo by modulating the redox status of cells and by activating the AMP-activated protein kinase (AMPK) signaling pathway [41]. Recently, it was reported that *ALDH2* deficiency promotes alcohol-associated liver cancer by activating oncogenic pathways via oxidized DNA-enriched extracellular vesicles [43].

PIPOX (pipecolic acid and sarcosine oxidase) encodes an enzyme participating in the pipecolate pathway (i.e., L-lysine degradation). *PIPOX* is a sarcosine-metabolizing enzyme that produces glycine from sarcosine, a potential oncometabolite. *PIPOX* has been poorly investigated in cancer, but its expression was particularly low in triple-negative breast tumors [44]. *PIPOX* was shown to reduce the oncogenic potential of prostate cells by metabolizing sarcosine and to be down-regulated in prostate cancer [45].

HAO1 (hydroxy acid oxidase 1) is a liver-specific peroxisomal enzyme that oxidizes glycolate to glyoxylate. *HAO1* has been linked to primary hyperoxaluria type 1. Supporting

our gene signature, a recent study demonstrated that *HAO1* is down-regulated in HCC and that a low expression of *HAO1* predicts a reduced OS [46].

AQP9 (aquaporin 9) has been extensively reported in cancer but with contrasting observations. Thus, a high expression of *AQP9* was significantly correlated with a worse prognosis in breast, colon and lung cancers, while predicted a better prognosis in gastric cancer [47]. In agreement with our results, *AQP9* was reported to be down-regulated in HCC tissues and human hepatoma cell lines in several studies and associated with a worse prognosis [48,49]. *AQP9* over-expression was shown to suppress the invasion of hepatoma cell by inhibiting EMT [48] and to inhibit the growth of subcutaneously xenografted liver tumors in nude mice [50].

PAH (phenylalanine hydroxylase, an enzyme which catalyzes the conversion of phenylalanine to tyrosine) has been associated with phenylketonuria, an autosomal recessive metabolic disorder. In HCC, a low expression of *PAH* has been reported as a prognostic marker for a poor prognosis [51].

Altogether, the data from the literature reporting the function of our minimal subset of seven genes predicting the survival of patients with HCC fully support a down-regulation in poor prognosis tumors. However, it has to be noted that the study exhibits some limitations. Notably, it is based on the gene expression profiling of a single HCC cell line. Due to the complexity of the liver structure, simplified in vitro models do not adequately reflect in vivo situations, especially the spatial heterogeneity and the metabolic zonation. Therefore, one can expect that further investigations using in vivo models would also be relevant to identify biomarkers related to hepatocyte differentiation. In addition, most of the current biomarkers are detected at the protein level. Thus, the detection of seven proteins at the same time by immunohistochemistry could be challenging. However, the detection of the gene signature at the protein level is not mandatory since alternative approaches already exist to assess complex gene expression signatures at the mRNA level in tissue biopsies. For instance, a six-gene signature has been recently established to accurately predict microvascular invasion in human HCC. These genes were identified by screening for the expression of 200 genes using a NanoString system in more than 300 frozen and/or FFPE samples [52]. The authors concluded that the approach could be applied in clinical practice with a routine tumor biopsy and integrated into therapeutic strategies [52].

5. Conclusions

In conclusion, our study identified a subset of seven genes reflecting the differentiation status of tumor hepatocytes. This minimal signature is clinically relevant for predicting the prognosis of HCC patients and can be easily implemented. Although these genes do not encode secreted proteins, it would be interesting to determine whether the products of the corresponding impaired metabolisms could be detected in the serum of the patients using poorly invasive methods and predictive of the clinical outcome.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cancers13225624/s1>, Table S1: List of genes differentially expressed between differentiated and progenitor HepaRG cells.

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Data Availability Statement: The data presented in this study are openly available from the gene expression omnibus database (<https://www.ncbi.nlm.nih.gov/geo>, accessed on July 2021).

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Distal bile duct cancer and pancreatic ductal adenocarcinoma: Call for a new definition of periampullary tumors

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ABSTRACT

Background: Distal cholangiocarcinoma (dCCA) and pancreatic ductal adenocarcinoma (PDAC) have always been considered as two distinct entities in terms of diagnosis and treatment. However, clinical and pathological similarities strongly suggest that distal biliary tumors are closer to pancreatic adenocarcinoma than to the rest of biliary tumors. This study aims at comparing the gene expression profiles of dCCA, PDAC and intrahepatic cholangiocarcinomas (iCCA) to stratify these tumors at the molecular level and to define their degree of similarity.

Methods: Three cohorts of patients with primary resected PDAC (n=10), iCCA (n=10) and dCCA (n=10) were studied at a transcriptomic level by gene expression profiling. Data mining by gene set enrichment analysis (GSEA) was performed to investigate specific enrichments for biological functions or canonical pathways.

Results: Hierarchical clustering analysis of PDAC, iCCA and dCCA tumors based on the expression on the 5,000 most variant genes identified two clusters : cluster 1 included all iCCA, and cluster 2 included both dCCA and PDAC. GSEA further demonstrated a significant enrichment of genes induced in PDAC, including key genes such as *MUC1*, *MUC4*, *CDX2* and *AGR2* in the gene expression profiles of dCCA. Accordingly, data mining highlighted a significant enrichment of common functional pathways in the dCCA and PDAC cluster distinct from the iCCA cluster.

Conclusion: The data demonstrate that i) dCCA exhibit distinct gene expression profiles from iCCA and could therefore be considered as a distinct tumor clinically, and ii) dCCA signature overlaps consistently with the PDAC signature.

INTRODUCTION

According to the WHO tumor classifications, distal cholangiocarcinoma (dCCA) have always been distinguished from pancreatic ductal adenocarcinomas (PDAC) in terms of diagnostic and therapeutic process (1). Therefore, dCCA remains associated to cancers of the biliary tract and does not benefit from the advances in the management of PDAC, particularly the benefit of mFOLFIRINOX regimen (2).

However, clinical and pathological similarities strongly suggest that distal biliary tumors are closer to pancreatic adenocarcinoma than to the rest of biliary tumors. Our team demonstrated in 2016 using a propensity score that dCCA and PDAC exhibit the same prognosis regarding overall survival and disease free survival (3). Since the publication of this article, many other papers have continued to explore the prognostic and histologic similarities between the two lesions without supporting it with tumor biology (4–6).

Gene expression profiling allowed considerable progress during recent years in defining and stratifying hepatobiliopancreatic tumors at the molecular level (7–9). In the present work, we profiled dCCA, PDAC and intrahepatic cholangiocarcinoma (iCCA) at the RNA level to determine whether the clinical and phenotypical similarities observed between dCCA and PDAC translated at the molecular level.

METHODS

Details are described in Supplementary Appendix 1. Briefly, three cohorts of patients with primary resected PDAC (n=10), iCCA (n=10) and dCCA (n=10) were studied at a transcriptomic level. A genome-wide expression profiling was performed using Agilent pangenomic microarrays. Differentially expressed genes were identified by a 2-sample univariate t test and a random variance model ($P < 0.01$; false discovery rate $< 1\%$). Several tools dedicated to the discovery of specific enrichments for biological functions or canonical pathways were used, including Enrichr algorithm (<http://amp.pharm.mssm.edu/Enrichr>) and gene set enrichment analysis (GSEA).

RESULTS

RNA was extracted from 30 freshly frozen resected PDAC, dCCA and iCCA tumors (10 in each group). As a first validation of our CCA datasets, GSEA identified a significant enrichment of gene signatures previously shown to be upregulated in iCCA (ES=0.64; $P < 0.05$) and extrahepatic CCA (dCCA) (10) in iCCA and dCCA gene expression profiles. In addition, GSEA using an integrated transcriptome meta-analysis comprised of several cohorts of PDAC matched with adjacent pancreatic tissues (GSE28735)(11) validated that dCCA gene expression profiles display a significant enrichment of genes induced in PDAC (ES=-0.69 and ES=-0.62; $P < 0.05$) (**Figure 1B**).

Following hybridization on microarrays and processing of the gene expression dataset 29,568 genes were retained for statistical analysis. Based on the expression of the 5,000 most variant genes, two clusters were identified by hierarchical clustering analysis. Cluster 1 included exclusively iCCA tumors while cluster 2 included all PDAC and dCCA tumors (**Figure 1A**).

Key genes differentially expressed between cluster 1 (iCCA) and cluster 2 (dCCA and PDAC) were highlighted on the basis of their fold-change. Notably, *IGFBP1* (Insulin-like growth factor-binding protein 1), *MCL1* (Myeloid Cell Leukemia Sequence 1), *FOXP4* (Forkhead Box P4) and *FGF12* (Fibroblast Growth Factor 12) display an enhanced expression in cluster 1 when compared to cluster 2. Tumors included in cluster 2 exhibit a higher expression of *MUC1* (Mucin 1), *MUC4* (Mucin 4), *CDX2* (*Caudal Type Homeobox 2*) and *AGR2* (Anterior Gradient 2) (**Figure 1C**). Data mining using enrichR tool highlighted that genes highly expressed in cluster 1 are significantly associated with stemness features ($P < 0.001$). These genes are notably involved in

SMAD2/3, Hedgehog and HIF-1-alpha signaling. Genes highly expressed in cluster 2 are significantly associated with mucinous adenocarcinomas and pancreatic neoplasms phenotypes ($P < 0.001$) (**Figure 1D**).

DISCUSSION

This work is the first one to directly compare the gene expression profiles of iCCA, dCCA and PDAC at a pan-genomic level. We report that the clinical, prognostic and histological similarities of these tumors are translated at the transcriptomic level. Despite belonging to the biliary tumor family, dCCA exhibits a molecular profile more similar to PDAC than to iCCA. This observation challenges the paradigm of biliary tract cancers. Until now, cholangiocarcinoma was considered the same tumor throughout the biliary tree. The present work supports the idea that it is now necessary to consider the development environment: hepatocyte or pancreatic.

Although the samples used for gene expression profiling were taken in the middle of the tumor, contamination of parenchyma could occur. To rule out this possibility, we validated first that the histological origin of tumors was robust using well-defined gene signatures of iCCA and dCCA (10). Through GSEA, we validated that the gene expression profiles of our iCCA and dCCA tumors were enriched respectively in iCCA and dCCA specific signatures. In addition, hierarchical clustering analysis focused on the most variant genes (15%), bringing robustness to the result. The most variant genes were selected in order to establish well defined clusters gathering tumors with common transcriptomic features. Therefore, we identified a first cluster containing exclusively iCCA. These tumors display a high expression of genes related to the TGF-beta signaling pathway such as *FGF12* (Fibroblast Growth Factor 12), *FOXP4* (Forkhead Box P4) and *SMAD2* (SMAD Family Member 2). In addition, the increased expression of *BCL-1* (Cyclin D1) and *TFF1* (Trefoil Factor 1) could be a result of the enhanced activity of the TGF-beta signaling pathway, affecting cell migration, tumor local invasion and EMT (epithelial-mesenchymal transition) (12,13). This minimal signature could be used to differentiate the CCA subtype depicted in cluster 1.

We also highlighted a second group of tumors (dCCA and PDAC) showing a high expression for several mucin-related genes such as *MUC1* (Mucin 1), *MUC4* (Mucin 4), *CDX2* (*Caudal Type Homeobox 2*) and *AGR2* (Anterior Gradient 2). This core transcriptomic signature is characteristic of mucinous neoplasms. Interestingly, *AGR2* was described as a *SMAD4*-suppressible gene that modulates *MUC1* and *MUC4* levels and promotes the initiation and progression of pancreatic intraepithelial neoplasia (14). *AGR2* was also detected in hilar, dCCA, and a subset of iCCA showing mucinous features (15). Altogether, these data suggest that PDAC and dCCA share a common mucinous phenotype at a molecular level. This statement is notably supported by similarities in their mutational landscape. Notably, PDAC and eCCA share a high frequency for genomic alterations such as *KRAS* (respectively 62% and 28%) and *SMAD4* (respectively 18% and 11.3%) (PMID: 32314446 ; 31925805).

Although the hypothesis of the common entity between dCCA and PDAC is not a recent idea, it had not yet been firmly demonstrated at the molecular level. At the clinical level, several retrospective studies, including two with analysis using a propensity score, demonstrated that both periampullary cancers managed by pancreaticoduodenectomy (PD) presented an identical short and long term prognosis (3,16,17). This theory is however debated and sometimes contradicted by the grounds of a histological confusion between the two tumors (18). In the present work, the diagnosis was made by pathologists of an expert center, specialized in hepatobiliary disease. A large cohort published in 2017 contradicted the hypothesis developed here, reporting better survival of dCCA after PD. However, the 2 groups had different criticisms, notably on known prognostic factors such as lymph node involvement or positive margins (5). DCCA, is characterized by an endoluminal development leading

to a clinical expression by jaundice at an often-earlier stage than PDAC. This is why they can sometimes be attributed, without matching, better prognoses (19).

Histologically, the two tumors share similarities, starting from the pre-neoplastic stage. Pancreatic intraepithelial neoplasms have their pancreatic counterpart with BillIN and similarly intraductal papillary mucinous neoplasms in the pancreatic ducts have their counterpart in the biliary ducts with intraductal papillary neoplasm of the biliary tract (20,21). These similarities are confirmed by the expression of exclusive markers in both tumors such as CK7, MUC1, MUC4, S100P and absent in iCCA (4). The categorization of periampullary tumors by histological phenotype, rather than anatomical location, was studied by Williams et al in a large cohort of 510 patients (6). They demonstrated that periampullary adenocarcinomas of biliary phenotype (ampullary, duodenal, or distal CBD location) have survival similar to those with PDAC. The present study completes the comparative analysis of the two tumors by associating their features at the transcriptomic level.

These similarities can be explained by a common embryological origin. Although biliary cells line the biliary tree from the liver to the pancreas, their origins at these two levels are different. Hepatic biliary cells originate from periportal hepatoblasts in the hepatic diverticulum. In contrast, the pancreatic head and the distal bile duct originates from common endodermal layers (22). This common origin induces the development of an environment where the periductular and peribiliary glands merge and sometimes share the same enzyme secreting function (23).

This work is a novel finding and requires confirmation in a larger cohort. However, it offers a new perspective on the definition of periampullary tumors. Currently, the treatment of dCCA is limited to surgical resection, and for lack of anything better,

capecitabine is the gold standard of adjuvant therapy (1). The idea of grouping the two tumors into a single entity would open the door to modifying their management, and perhaps take advantage of the critical progress that the PDAC (2). The ongoing PRODIGE 38 (NCT02591030) trial comparing modified folfinox to cisplatin and gemcitabine in patients with metastatic biliary cancer may provide the first step towards an answer.

Conclusion

First, these data suggest that only dCCA has distinct transcriptomic features from iCCA and could be considered clinically as two different entities in their future diagnosis and management. Second, the transcriptomic signature of dCCA overlaps consistently with the signature of PDAC, supporting the hypothesis of a common entity.

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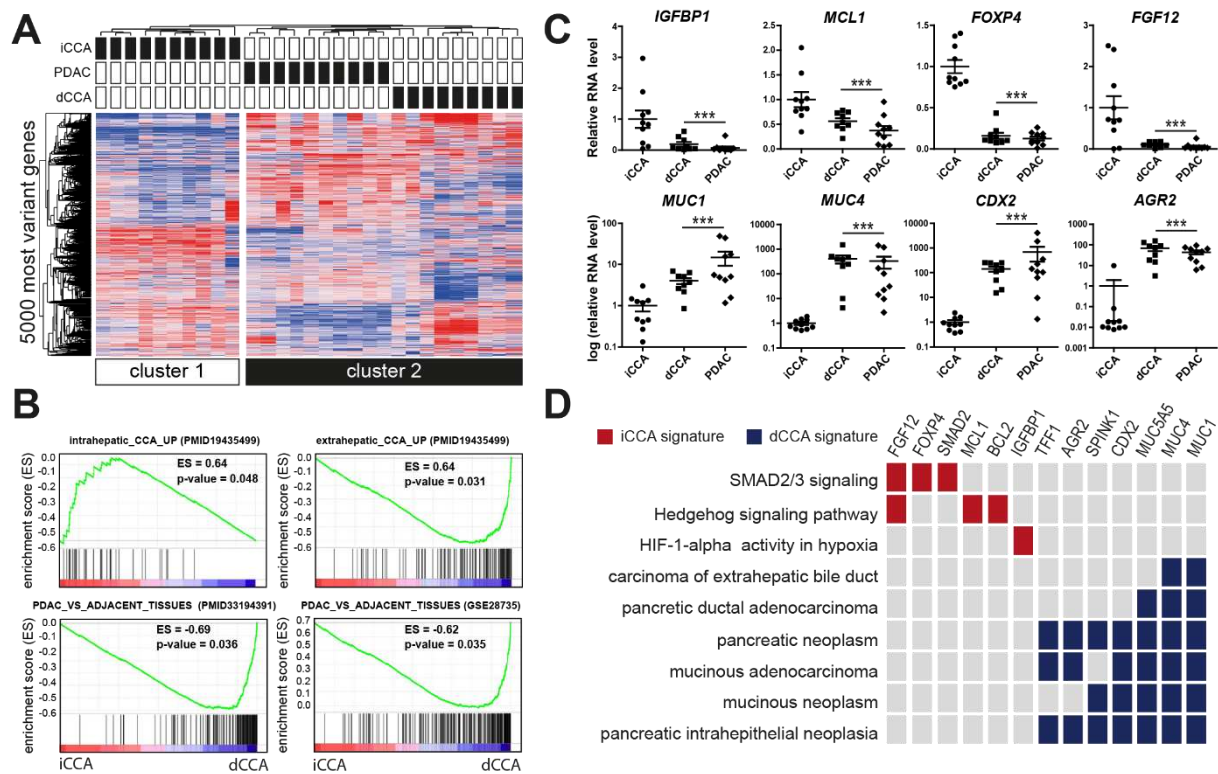


Figure 1. Gene expression profiling of biliary tract tumors recognizes distal bile duct cancer and intrahepatic cholangiocarcinoma as two different entities. (A) Clustering analysis based on the expression of the 5000 genes most differentially expressed. Two major clusters are identified: cluster 1 includes only iCCA while cluster 2 gathers all dCCA and PDAC. **(B)** GSEA using the gene expression profiles of iCCA and dCCA tumors. GSEA highlights a significant (p -value < 0.05) enrichment of iCCA (upper 2 panels) and PDAC (lower 2 panels) signatures respectively in iCCA and dCCA gene expression profiles. GSEA using an integrated transcriptome meta-analysis of PDAC and matched adjacent pancreatic tissues reveals that upregulated genes in PDAC are enriched in the gene expression profiles of dCCA. **(C)** Examples of key genes differentially expressed between cluster 1 and cluster 2 (p -value < 0.001 and $FC > 2$). **(D)** Data mining analysis using enrichR software (<http://amp.pharm.mssm.edu/Enrichr>) demonstrates that iCCA gene expression

profiles are significantly enriched in stemness associated signatures (e.g., HIF-1-alpha, Hedgehog signaling pathway, SMAD2/3 signaling pathway) while dCCA gene expression profiles are significantly enriched in signatures associated with mucinous adenocarcinomas and pancreatic neoplasms.

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Supplementary Appendix 1

Patients

Three cohorts of patients with primary resected PDAC (n=10), iCCA (n=10) and dCCA (n=10) were studied at a transcriptomic level. Freshly frozen and formalin fixed paraffin embedded (FFPE) tissues were provided by the biobank of the Rennes university hospital (PDAC, iCCA) and Beaujon university hospital (dCCA). Written informed consent was obtained from all patients. The study protocol fulfilled national laws and regulations and was approved by the local Ethics Committee. Histological and clinical features including those recorded upon follow-up examinations were obtained from hospital charts.

Gene expression profiling

Total RNA was purified with a miRNAeasy kit (Qiagen). Genome-wide expression profiling was performed using the low-input QuickAmp labeling kit and human SurePrint G3 8x60K probes pangenomic microarrays (Agilent Technologies, Santa Clara, CA). Differentially expressed genes were identified by a 2-sample univariate t test and a random variance model ($P < 0.01$; false discovery rate $< 1\%$).

Data mining

Clustering analysis was done using Cluster 3.0 and TreeView 1.6 using uncentered correlation and average linkage options. Gene expression data were processed using Feature Extraction and GeneSpring software (Agilent Technologies). Gene set enrichment analysis (GSEA) was performed using the Java tool developed at the Broad Institute (Cambridge, MA). The *Enrichr* algorithm (<http://amp.pharm.mssm.edu/Enrichr>) was also used to identify key genes associated with relevant signaling pathways.

Titre : Les ARN circulaires régulés par le TGF β dans le cholangiocarcinome intrahépatique

Mots clés : cholangiocarcinome intrahépatique ; TGF β ; ARN circulaires ; biomarqueurs ; vésicules extracellulaires

Résumé : Le cholangiocarcinome intrahépatique (CCAi) est un cancer de très mauvais pronostic qui présente des solutions thérapeutiques limitées. La combinaison de première ligne de référence qui combine le cisplatine à la gemcitabine ne présente que des bénéfices modérés pour le traitement du CCAi. Il est donc urgent d'identifier de nouveaux biomarqueurs et des cibles thérapeutiques pour améliorer le pronostic des patients atteints de CCAi. L'étude des ARN circulaires (ARNcirc), une nouvelle classe d'ARN impliquée dans la progression du cancer, représente une piste prometteuse dans ce but. Les ARNcirc impliqués dans la voie du TGF β , connue pour jouer un rôle important dans la progression du CCAi, pourraient notamment représenter des cibles intéressantes. Ainsi l'objectif de ce projet était d'identifier et de

caractériser les ARNcirc régulés par le TGF β qui agissent en tant qu'éponges à miARN, et d'évaluer leur potentiel en tant que biomarqueurs dans le CCAi. Nos résultats montrent que l'ARNcirc induit par le TGF β circLTBP2 agit comme ARN compétitif en séquestrant miR-338-3p, altérant ainsi son activité antitumorale. L'expression de circLTBP2 a été associée à une résistance accrue des cellules de CCAi à la gemcitabine et à un pronostic défavorable pour les patients atteints de CCAi. L'ARN circLTBP2 a également été mesuré dans le sérum de patients atteints de CCAi, ce qui suggère également un potentiel en tant que biomarqueur non invasif.

Title : TGF β -regulated circular RNA in intrahepatic cholangiocarcinoma

Keywords : intrahepatic cholangiocarcinoma; TGF β ; circular RNA; biomarkers; extracellular vesicles

Abstract: Intrahepatic cholangiocarcinoma (iCCA) is a deadly cancer worldwide with an increasing incidence and limited therapeutic options. Combined cisplatin-gemcitabine chemotherapy used as a first-line treatment for advanced iCCA shows only modest benefits. Thus, there is an urgent need to identify new biomarkers and therapeutic targets to improve the outcome of iCCA patients. One potential avenue for discovery is the study of circular RNA (circRNA), a new class of RNA that play a role in cancer progression. CircRNA that are involved in the TGF β pathway, which is known to be important in the development of iCCA, may represent promising targets for further

study. Therefore, the goal of this research was to identify and characterize TGF β -regulated circRNA that act as miRNA sponges and evaluate their potential as biomarkers in human iCCA. Our findings demonstrate that the TGF β -induced circLTBP2 acts as a competitive RNA that interferes with the activity of the tumor suppressor miR-338-3p. CircLTBP2 expression was associated with an increased gemcitabine resistance of iCCA cells and a poor prognosis for patients with iCCA. The identification of circLTBP2 in serum form patients also suggests a potential utility as a minimally invasive biomarker for iCCA.