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Aude Merdrignac

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HAL Id: tel-02421163

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THESE DE DOCTORAT DE

L'UNIVERSITE DE RENNES 1
COMUE UNIVERSITE BRETAGNE LOIRE

ECOLE DOCTORALE N° 605
Biologie Santé
Spécialité : Biochimie, Biologie moléculaire et cellulaire

Par **Aude MERDRIGNAC**

Etude de la voie TGF β dans le cholangiocarcinome intrahépatique Implication des ARN longs non-codants

Thèse présentée et soutenue à Rennes, le 14 juin 2019
Unité de recherche : Inserm UMR 1241 - Nutrition, métabolisme et Cancer - NuMeCan

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Remerciements

Je remercie en premier lieu Cédric Coulouarn, mon directeur de thèse, qui m'encadre depuis le Master 2 et sans qui ce travail n'aurait pu aboutir. Tu as eu la patience de partager tes connaissances et ton expertise. Je te remercie particulièrement de ta rigueur et de ta compréhension sur mon emploi du temps.

Je remercie chaleureusement Gaëlle Angenard qui m'a accompagnée dans mes expérimentations et soutenue quand les résultats n'étaient pas ceux escomptés. Ce travail n'aurait pu se faire sans ton aide technique ni celle de l'équipe : Kilian, Coralie, Camille...

Je remercie le Professeur Sulpice qui a été le premier à me proposer ce projet et qui m'a encouragée dans la réalisation de ce travail. J'espère que notre collaboration se poursuivra dans la même confiance.

Je remercie le Professeur Boudjema de la formation qu'il m'a apportée par son expertise chirurgicale, son expérience et sa rigueur.

Je remercie Madame le Professeur Paradis et Monsieur le Professeur Soubrane qui me font l'honneur d'accepter d'être les rapporteurs de ce travail.

Je remercie Monsieur le Professeur Thomas Decaens, Monsieur Thomas Derrien et Madame Laura Fouassier et qui me font l'honneur d'accepter d'être les examinateurs de ce travail.

Je remercie le Docteur Damien Bergeat mon collègue et soutien au cours du Master et le Docteur Véronique Desfourneaux pour la formation précieuse à ses côtés.

Je remercie enfin et surtout Anaël et Lizenn.

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Liste des abréviations

ABCC1: ATP binding cassette subfamily C member 1
ACE: antigène carcino-embryonnaire
ANRIL: antisense noncoding RNA in the INK4 locus
APC: adenomatous polyposis coli
ARID1A: AT-rich interaction domain 1A
a-SMA : actine-alpha de type musculaire lisse
ATB: lncRNA-activated by TGF β
BAP1: BRCA1 associated protein-1
CA19-9: carbohydrate antigene 19-9
CASC15: cancer susceptibility candidate 15
CBX7: chromobox 7
CCI: cholangiocarcinome intrahépatique
CD: cluster of differentiation
CDKN: cyclin-dependent kinase Inhibitor
CDKN2B-AS1: cyclin-dependent kinase inhibitor 2B - antisense RNA 1
CDS: coding DNA sequence
CHC: carcinome hépatocellulaire
CK: cytokératine
CPS1-IT1: carbamoyl-phosphate synthase - intronic transcript 1
CRNDE: colorectal neoplasia differentially expressed
CSP : cholangite sclérosante primitive
CPS1-IT1: CPS1 intronic transcript 1
CTGF: connective tissue growth factor
CYFRA 21-1: cytokeratin-21-fragment
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
EMT: epithelial-mesenchymal transition
EPCAM: epithelial cell adhesion molecule
EPIC1: epigenetically-induced lncRNA1
EZH2: enhancer of zeste 2 polycomb repressive complex 2 subunit
FGFR: fibroblast growth factor receptor
FXR: farnesoid X receptor
H3K27me3 : trimethylated lysine 27 of histone H3
H3K4deme : demethylated lysine 4 of histone H3
HGF/MET: hepatocyte growth factor
HOTAIR: HOX transcript antisense RNA
HOTTIP: HOXA distal transcript antisense RNA
HOXD10: homeobox D10
IDH1/2: isocitrate dehydrogenase 1/2
IL: interleukin
JAG-1: jagged1
JAK: janus kinase
JAM2 : junctional adhesion molecule 2
JNK: c-Jun NH2-terminal kinase
KRT: keratin
LGL : lethal giant larvae
LOXL2: lysyl oxidase like 2
LSD1: lysine-specific histone demethylase 1
MALAT1: metastasis associated lung adenocarcinoma transcript 1
MAPK: mitogen-activated protein kinase
MEG3: maternally expressed gene 3
MLL : mixed lineage leukemia

mPGES-1: microsomal prostaglandin E synthase-1
 mTOR: mechanistic target of rapamycin
 NBAT1: neuroblastoma associated transcript 1
 NEAT1: nuclear paraspeckle assembly transcript 1
 NF-Y: nuclear transcription factor Y
 PANDA: p21-associated ncRNA DNA damage-activated
 PATJ : PALS1-associated tight junction protein
 PCAT1: prostate cancer associated transcript 1
 PCDH10 : protocadherin 10
 PD1:programmed cell death 1
 PDGFR: platelet-derived growth factor receptor
 PD-L1:programmed cell death-ligand 1
 PGR : progesterone receptor
 PI3K: phosphatidylinositol 3-kinase
 PIP: prolactin-inducible protein
 PLK2: polo like kinase 2
 PPAR:peroxisome proliferator-activated receptor
 PTCH: patched
 PTEN : phosphatase and tensin homolog
 PTENP1: phosphatase and tensin homolog pseudogene 1
 PTHRP: parathyroid hormone-related protein
 PVT1: plasmacytoma variant translocation 1
 q-RT-PCR: quantitative-real-time polymerase chain reaction
 REST: RE1 silencing transcription factor
 RhoA: Ras homolog family member A
 ROR: regulator of reprogramming
 (S)HH: sonic hedgehog
 SMO: smoothed, frizzled class receptor
 SNHG1: small nucleolar RNA host gene 1
 SNO : ski-related novel gene
 SNP: single nucleotide polymorphism
 SOCS-3: suppressor of cytokine signaling 3
 SOX: Sry-related HMG box
 S1PR2: sphingosine-1-phosphate receptor 2
 SPP1: secreted phosphoprotein 1/osteopontin
 SPRY4-IT1: sprouty RTK signaling antagonist 4-intronic transcript 1
 STAT3: signal transducer and activator of transcription 3
 TAMs: tumour-associated macrophages
 TANs: tumour-associated neutrophils
 TCGA: the cancer genome atlas
 TEM: transition épithélio-mésenchymateuse
 TF: transcription factor
 TGF β : transforming growth factor β
 TH: transplantation hépatique
 TLINC: TGF β induced long intergenic non coding RNA
 TRAIL: TNF-related apoptosis inducing ligand
 TP53: tumor protein 53
 TUG1: taurine upregulated gene 1
 VEGF: vascular endothelial growth factor
 VEGFR: vascular endothelial growth factor receptor
 VIM: vimentine
 ZEB: zinc finger E-box binding
 ZEB2-AS : ZEB2 antisense RNA

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I. Introduction

1. Cholangiocarcinome intrahépatique

i. Epidémiologie et facteurs de risque

Le cholangiocarcinome est un cancer développé à partir des cellules de l'épithélium de l'arbre biliaire. Il est classé selon sa localisation anatomique par l'Organisation Mondiale de la Santé en intrahépatique (au-delà des divisions en canaux biliaires de 2nd ordre), périhilaire ou distal¹ (figure 1).

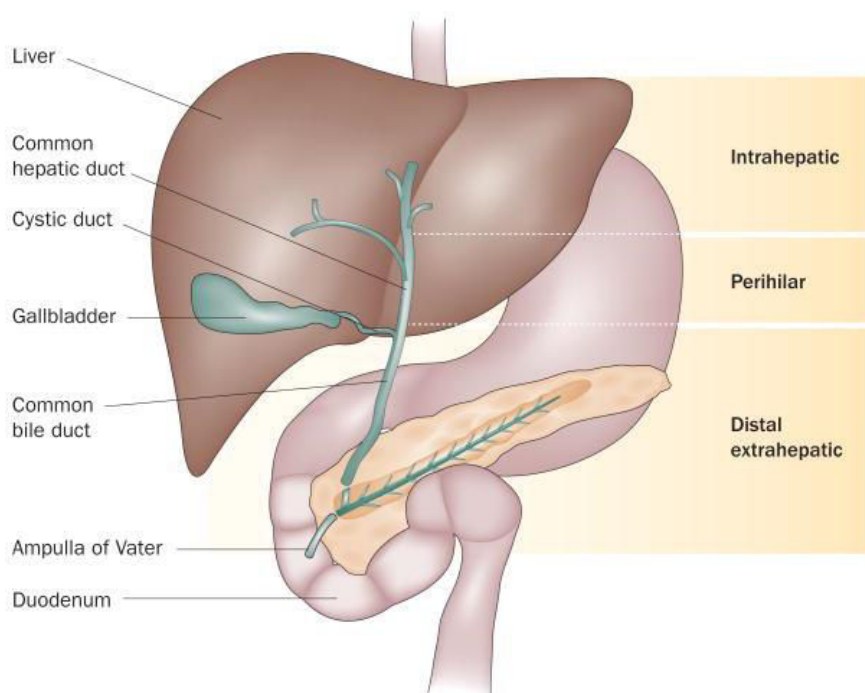


Figure 1: Classification anatomique des cholangiocarcinomes. *Blechacz, Nat Rev Gastroenterol Hepatol, 2011*².

Nous n'aborderons dans ce travail que la localisation intrahépatique (CCI). Le CCI est classiquement la 2^{ème} tumeur hépatique primitive après le carcinome hépatocellulaire (CHC) représentant 10 à 20% des tumeurs hépatiques primitives³. Cependant, son incidence est très variable dans le monde (figure 2). Elle est forte en Asie du Sud-Est (jusqu'à 85/100000 en Thaïlande) et à l'opposé faible dans certains pays d'Occident (0.3/100000 au Canada)⁴.

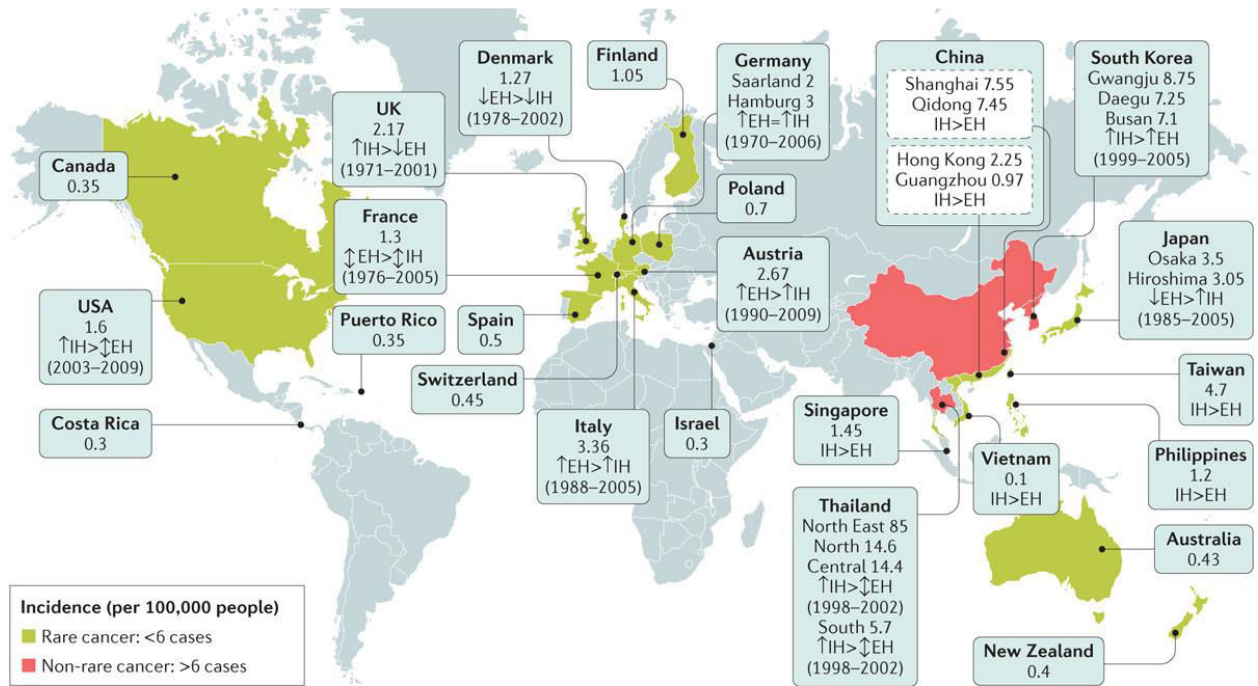


Figure 2: Incidence mondiale du cholangiocarcinome au cours de la période 1971-2009. Les pays avec la plus faible incidence sont en vert (<6/100000 cas, cancer rare), alors que les pays avec la plus forte incidence sont en rose (>6/100000 cas). La forme avec la plus forte incidence (intra-hépatique (IH) versus extra-hépatique (EH)) et la tendance de l'incidence (↑augmentation, ↓stabilité, ↓diminution) sont indiquées si les données sont disponibles. *Banales, Nat Rev Gastroenterol Hepatol, 2016*⁵.

L'incidence forte en Asie du Sud-Est est expliquée par des facteurs de risque spécifiques comme l'infection parasitaire par les douves *Opisthorchis viverrini* et *Clonorchis sinensis*. Les anomalies de l'arbre biliaire, comme les kystes du cholédoque, et les calculs biliaires intra-hépatiques fréquents dans cette région sont également impliqués.

En Occident, dans la majorité des cas, le CCI survient sur un foie sain comme l'a montré la série récente de 522 patients de l'Association Française de Chirurgie⁶. Cependant, la cholangite sclérosante primitive (CSP) est un facteur de risque bien connu qui augmente la prévalence du cholangiocarcinome à 5 à 10% au cours de la vie⁷. Plus récemment, les facteurs de risque classiques du CHC ont également été reconnus comme impliqués dans le CCI (hépatites virales chroniques B et C, cirrhose, obésité, diabète et alcool) suggérant des voies de carcinogénèse communes aux deux tumeurs⁸. L'ensemble des facteurs de risque connus du cholangiocarcinome sont présentés dans la table 1.

Table 1: Facteurs de risque du cholangiocarcinome

Généraux

Age >65 ans
Obésité
Diabète

Maladies inflammatoires

Cholangite sclérosante primitive
Lithiase hépatique (Cholangiohépatite orientale)
Calculs de l'arbre biliaire
Anastomose bilio-digestive
Cirrhose hépatique

Maladies infectieuses

Opisthorchis viverrini (douves hépatiques)
Clonorchis sinensis (douves hépatiques)
Hépatite C
Hépatite B
Infection VIH

Drogues, toxines et produits chimiques

Alcool
Tabac
Thorotraste
Dioxine
Vinyl chloride
Nitrosamines
Asbestose
Pilule contraceptive orale
Isoniazide

Congénitaux

Kystes du cholédoque (type I, solitaire, extrahépatique; type IV, extrahépatique et intrahépatique)
Maladie de Caroli
Fibrose hépatique congénitale

*Table adaptée de Banales, Nat Rev Gastroenterol Hepatol, 2016*⁵

Globalement, l'incidence du CCI est en augmentation même si l'évolution des classifications au cours du temps a pu modifier l'incidence des différentes localisations^{9,10}. Une des causes de cette augmentation pourrait être le syndrome métabolique¹¹.

ii. Histologie et microenvironnement tumoral

La forme basée sur l'aspect macroscopique de CCI la plus fréquente est la forme *mass-forming* trouvée chez 86 % des patients opérés. Le type *mass-forming* correspond à une masse infiltrant le parenchyme hépatique via le système porte et les vaisseaux lymphatiques

^{12,13}

Les 2 autres formes sont le type *periductal infiltrating* et le type *intraductal growth* (figure 3).

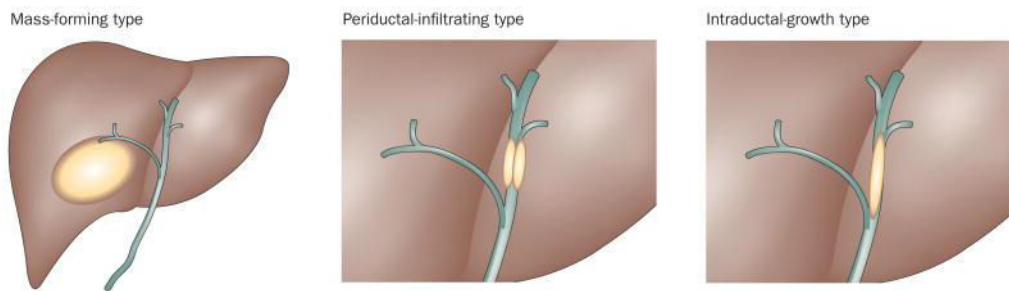


Figure 3: Types de CCI selon l'aspect macroscopique. Blehacz, *Nat Rev Gastroenterol Hepatol*, 2011².

Histologiquement, le CCI peut être classé en 2 sous types selon son origine anatomique sur l'arbre biliaire intrahépatique : mixte issu des petits canaux biliaires et mucineux issu des larges canaux biliaires. Le type mixte, correspondant presque exclusivement au type *mass-forming*, est fréquemment associé aux hépatopathies chroniques (cirrhose ou hépatites virales) et n'est pas précédé de lésion prénéoplasique. Le type mucineux, qui peut avoir toutes les apparences macroscopiques, est plus fréquemment associé à la CSP. Il peut être précédé de lésions néoplasiques et partage des caractères avec les cholangiocarcinomes distaux et les cancers pancréatiques⁵. La différenciation du CCI avec des métastases hépatiques d'autres cancers du tractus digestif est aidée par des marqueurs immunohistochimiques de l'origine biliaire (e.g. positivité de la cytokératine 7, CK7)^{14,15}.

Le CCI correspond à un adénocarcinome plus ou moins différencié avec un riche stroma fibreux desmoplastique^{5,16}. Ce stroma contient principalement du collagène de type 1 et des myofibroblastes caractérisés par l'expression de novo d'actine-alpha de type musculaire lisse (α -SMA)¹⁷. De nombreuses cellules inflammatoires (macrophages) et vasculaires sont également présentes dans le microenvironnement du CCI. L'analyse moléculaire du stroma tumoral après microdissection laser réalisée dans notre équipe avait mis en évidence une surexpression du *Transforming Growth Factor β* (TGF β)¹⁸. Ces résultats étaient concordants avec l'action profibrotique connue du TGF β dans le foie. Les macrophages produisent des médiateurs profibrotiques dont le TGF β qui est responsable de l'activation de cellules hépatiques étoilées quiescentes en myofibroblastes¹⁹. Les myofibroblastes jouent un rôle

majeur dans la progression du CCI. Ils agissent sur la prolifération, la migration, l'invasion, la résistance à l'apoptose et la TEM des cholangiocytes tumoraux par la régulation autocrine et paracrine de voies de signalisation (e.g. voie Hedgehog) ¹⁷. Leur abondance comme celle de protéines de la matrice extra cellulaire (e.g. périostine), est corrélée à un mauvais pronostic chez les patients opérés ^{16,17}.

iii. Cancérogénèse et altérations moléculaires

Le CCI résulte d'un processus complexe de cancérogénèse impliquant la transformation de cholangiocytes et dans certains cas de cellules souches. Des marqueurs de cellules souches cancéreuses (e.g. CD13, CD44) sont présents dans au moins 30% des cellules de cholangiocarcinome ²⁰. L'injection de cellules souches cancéreuses a permis de générer des CCI humains dans des foies cirrhotiques murins ²⁰. L'hypothèse de la transformation en CCI à partir d'hépatocytes a été également proposée. En effet, des modèles murins ont permis de convertir des hépatocytes en cholangiocytes via l'activation des voies de signalisation Notch et Akt ^{21,22}.

Parmi les altérations moléculaires rencontrées dans le CCI, les mutations génétiques les plus fréquemment mises en évidence sont *KRAS* (19%), *TP53* (16%) et *IDH1/2* (15%) ²³. Les mutations *KRAS* ont été associées à un phénotype plus agressif ²⁴. D'autres mutations sont moins fréquentes : *ARID1A*, *BAP1*, *BRAF* et *EGFR* ²³. Des fusions de gènes codant pour des protéines tyrosine kinase, comme *FGFR2* qui agit comme récepteur membranaire aux facteurs de croissance des fibroblastes, sont décrites dans 20% des cas ²³. Elles aboutissent à la formation de protéines de fusion qui ont une action oncogénique en activant des voies de signalisation e.g. la voie MAPK ²⁵. De rares altérations chromosomiques correspondant à des gains ou des pertes sur l'un des bras chromosomiques ont été décrites e.g. gain sur 7p et perte sur 4q ²⁶. Des altérations épigénétiques par hyperméthylation de promoteur (i.e. inactivation) ont également été décrites, comme par exemple au niveau du promoteur du gène codant p16INK4A, un régulateur négatif du cycle cellulaire ²⁶.

Les voies de signalisation principalement dérégulées dans le CCI concernent l'inflammation comme IL6/STAT3, les facteurs de croissance comme EGFR et la voie KRAS/MAPK (figure 4)²⁶. Des dérégulations des voies HGF/MET, VEGF et WNT/ β -caténine ont également été décrites.

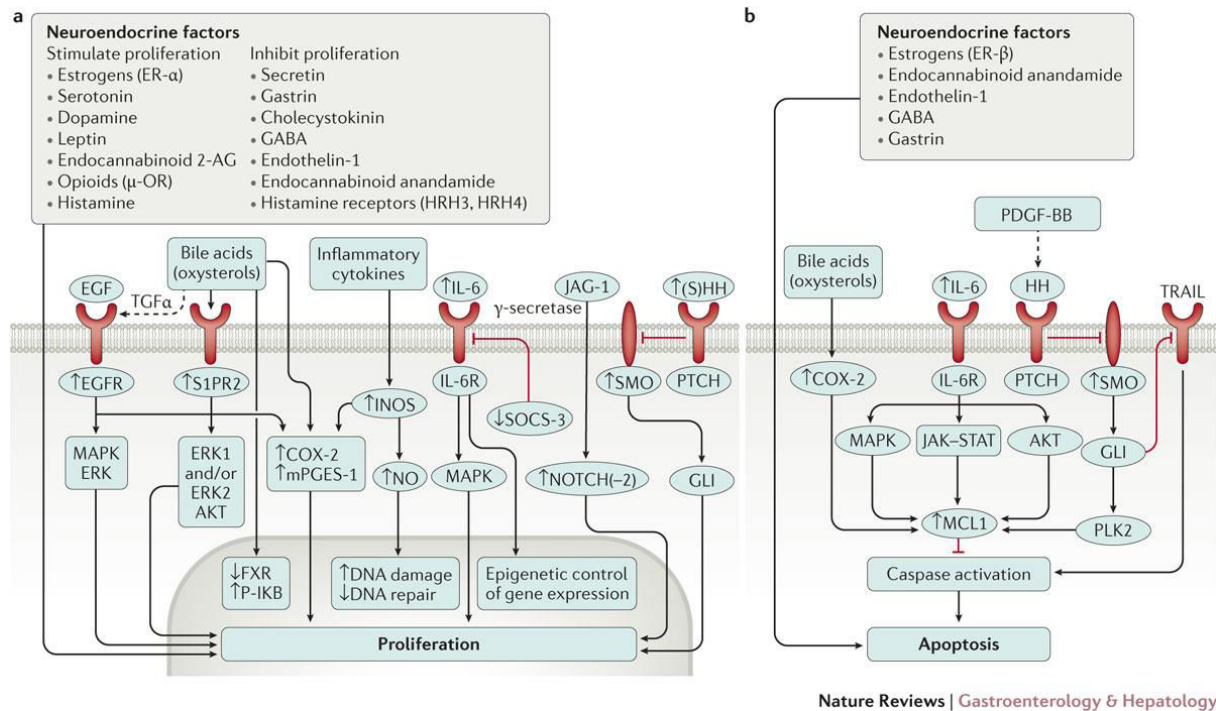


Figure 4: Voies de signalisation intracellulaires impliquées dans la prolifération et l'apoptose dans le cholangiocarcinome. *Banales, Nat Rev Gastroenterol Hepatol, 2016*⁵.
 2-AG: 2-arachidonylglycerol. COX-2: cyclooxygénase 2. EGF: épidermal growth factor. EGFR: épidermal growth factor receptor. ER: œstrogène récepteur. GABA: γ -aminobutyric acid. IL-6R: IL-6 récepteur. NO: nitric oxide. OR: opioïde récepteur. TGF: transforming growth factor. iNOS: inducible nitric oxide synthase. JAG-1: Jagged1. S1PR2: Sphingosine-1-Phosphate Receptor 2. mPGES-1: microsomal prostaglandin E synthase-1. FXR: farnésioïde X récepteur. P-IKB: Phosphatidylinositol kinase. SOCS-3: suppressor of cytokine signaling 3. SMO: smoothened, frizzled class récepteur. PTCH: patched. (S)HH: sonic hedgehog. JAK: Janus kinase. PLK2: polo like kinase 2. TRAIL: TNF-related apoptosis inducing ligand.

Différentes classifications moléculaires ont été proposées comme par exemple Sia et al. en 2013 (figure 5)²⁷. La classe « proliférative » est plus agressive et de plus mauvais pronostic. Elle présente un enrichissement de voies de signalisation oncogéniques comme RAS, EGF, HGF/MET qui sont des altérations semblables au CHC de mauvais pronostic. La classe « inflammatoire » est de meilleur pronostic avec un enrichissement des voies de signalisation de la réponse immunitaire comme STAT3 menant à une surexpression de cytokines (e.g. IL4, IL10).

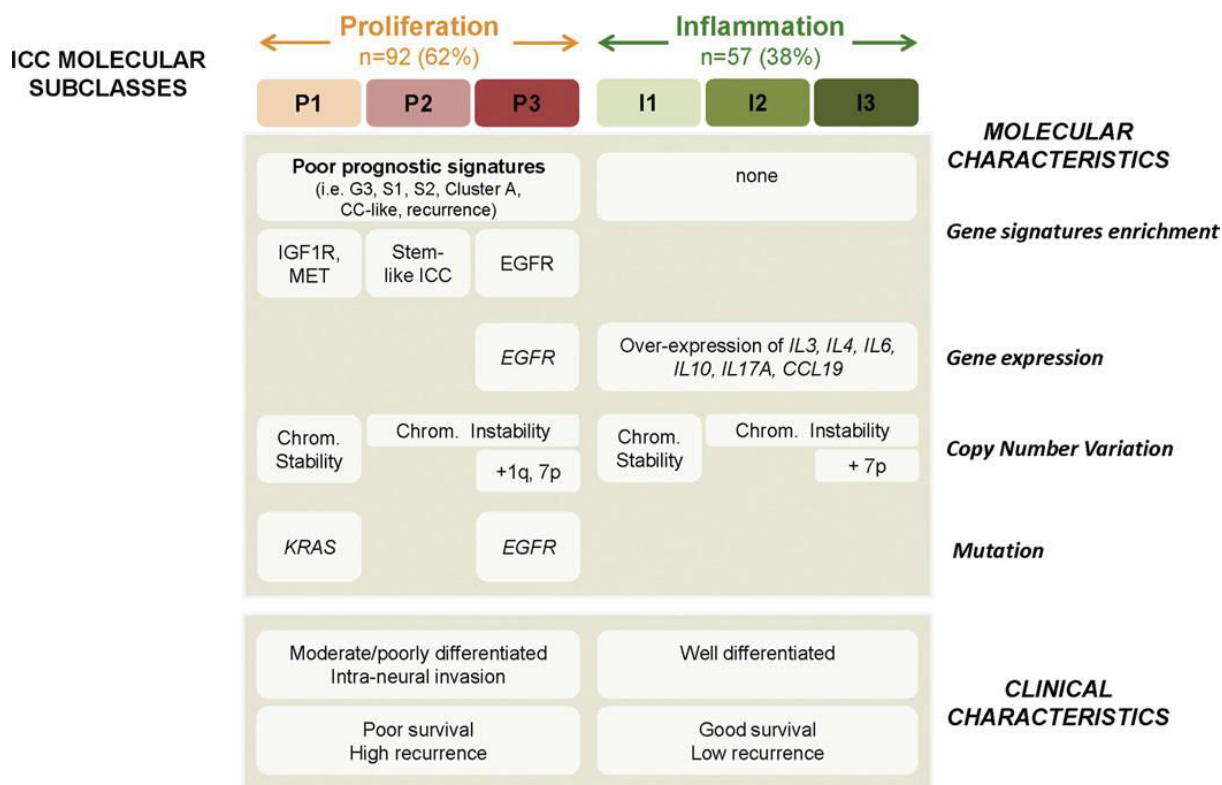


Figure 5: Résumé des classes de CCI. Les caractéristiques moléculaires et cliniques diffèrent entre les 2 classes. *Sia, Gastroenterol, 2013*²⁷.

iv. Diagnostic

Le diagnostic du CCI est souvent tardif car au stade précoce les patients sont asymptomatiques. A un stade avancé, les signes sont aspécifiques : perte de poids, douleurs abdominales, hépatomégalie, plus rarement ictère. Les marqueurs tumoraux sériques actuels, CA 19-9 et ACE, manquent de spécificité pour le CCI. La sensibilité et la spécificité du CA 19-9 ne sont que de 62% et 63% respectivement². L'obstruction biliaire peut faussement augmenter le taux de CA 19-9 mais un taux élevé pourrait être prédictif de non résecabilité et d'une moins bonne survie sans récurrence après chirurgie^{28,29}. De nombreux autres biomarqueurs sériques potentiels (e.g. CYFRA 21-1, SPP1) ont été identifiés mais sans résultats assez satisfaisants pour une application clinique actuellement^{30,31}. Des travaux dans l'équipe avaient notamment identifié LOXL2, EPCAM et SPP1 comme biomarqueurs pronostiques indépendants au niveau tissulaire^{18,32,33}. Dans le but d'identifier de nouveaux biomarqueurs non invasifs, Banales et al. ont isolé des vésicules extracellulaires dont la majorité étaient des exosomes³⁴. L'analyse protéomique des

exosomes a montré des signatures potentiellement utiles à visée diagnostique pour différencier CCI, CHC et CSP.

La tomодensitométrie abdominale et l'imagerie par résonance magnétique permettent de suspecter le diagnostic. L'aspect typique est une lésion hépatique prenant le contraste au temps artériel avec une augmentation du rehaussement au temps veineux en raison d'une prise de contraste lente du stroma fibreux ³⁵. La différenciation avec un CHC est parfois difficile particulièrement pour les tumeurs de petite taille <2cm. Seule la preuve histologique fait donc le diagnostic formel.

v. Traitement et pronostic

Le pronostic du CCI est mauvais avec 50% de récurrence à 1 an et une médiane de survie globale de 33 mois après chirurgie ³⁶. La chirurgie reste cependant le seul traitement potentiellement curatif pour les 30 à 40% de patients avec une tumeur résectable ³⁷. La résection ne doit pas laisser de résidu tumoral (R0) ce qui implique pour 80% des patients une hépatectomie majeure et un curage ganglionnaire dans 70% des cas ¹³. Les facteurs de risque de récurrence après résection sont l'envahissement ganglionnaire, l'invasion péri-neurale et la présence de nodules satellites intrahépatiques ³⁶. En l'absence de chirurgie, la médiane de survie est de 12 à 15 mois ³⁷. La transplantation hépatique (TH) pour CCI reste actuellement contre indiquée dans la plupart des équipes. La survie à 5 ans est inférieure à 50% dans les séries de patients chez lesquels l'indication pour la TH était initialement une suspicion de CHC ³⁸. Cependant, des études rétrospectives récentes analysant des groupes homogènes de patients avec des CCI uniques <2cm montrent des résultats encourageants avec des survies de 65 à 73% à 5 ans ³⁸.

Pour les patients non résectables, la chimiothérapie systémique utilisée actuellement combine gemcitabine et cisplatine. Les résultats de l'essai ABC-02 publiés en 2010 avaient montré une amélioration de la médiane de survie globale de 8.1 à 11.7 mois grâce à cette combinaison ³⁹. Un essai de seconde ligne de chimiothérapie intra artérielle (gemcitabine et

oxaliplatine) est en cours en France (GEMOXIA, FFCG 1606, clinicaltrials.gov : NCT03364530).

En situation adjuvante, les résultats récents de l'essai de phase III BILCAP ont montré une augmentation de la survie globale avec la capécitabine ⁴⁰. L'essai français PRODIGE12-ACCORD18 n'avait par contre pas montré d'intérêt à un traitement adjuvant par gemcitabine et oxaliplatine ⁴¹.

Parmi les autres possibilités thérapeutiques en cours de développement, la radioembolisation à l'yttrium 90 combinée à la chimiothérapie permettrait un allongement de la survie globale ⁴². Plusieurs essais de ce schéma pour les CCI non résécables sont en cours aux Etats-Unis et en Europe (clinicaltrials.gov : NCT02807181, NCT02512692).

L'essai de thérapies ciblées suite à l'identification des mutations présentes dans le CCI est en plein développement ⁴³. Pour l'instant, les différentes études de phase II et III ciblant VEGFR, EGFR, PDGFR, BRAF n'ont pas permis d'améliorer de façon significative la survie des patients ²⁶. Des inhibiteurs IDH1, IDH2 et FGFR sont en cours d'évaluation ⁴⁴.

Des essais d'immunothérapie sont également en cours avec le nivolumab et le pembrolizumab (clinicaltrials.gov : NCT02834013, NCT02628067), des anticorps antiPD1, qui ont déjà montré leur efficacité notamment dans le traitement du mélanome ⁴⁵.

2. Voie du « Transforming Growth Factor β » (TGF β)

i. Voie de signalisation / Mécanisme de transduction du signal

La voie de signalisation du TGF β joue un rôle majeur dans le développement des mammifères via la modulation de nombreux processus cellulaires (e.g. prolifération, différenciation) qui vont influencer sur l'homéostasie et la régénération tissulaire. Les dérégulations de cette voie peuvent donc être à l'origine de nombreuses pathologies dont les cancers ⁴⁶. La famille du TGF β humain, qui est une cytokine pléiotropique, comprend plus de 30 membres dont les TGF β s (isoformes 1, 2 et 3), les activines et les protéines morphogénétiques de l'os. Le ligand se fixe à son récepteur (du type sérine/thréonine kinase) à la surface cellulaire qui forme un complexe de récepteurs bi-dimérique: TGF β RI et

TGFβRII. L'activation de la voie canonique dite SMAD-dépendante fait suite à une cascade de phosphorylations activant les protéines SMAD dans le cytoplasme. Le complexe activé formé par SMAD2/3 phosphorylé et SMAD4 (co-SMAD) va migrer dans le noyau cellulaire pour réguler la transcription de gènes cibles de la voie avec l'aide de co-activateurs et de co-répresseurs (figure 6) ⁴⁷. La voie de signalisation TGFβ régule également des modifications post-transcriptionnelles via notamment la biogenèse des microARNs (figure 6).

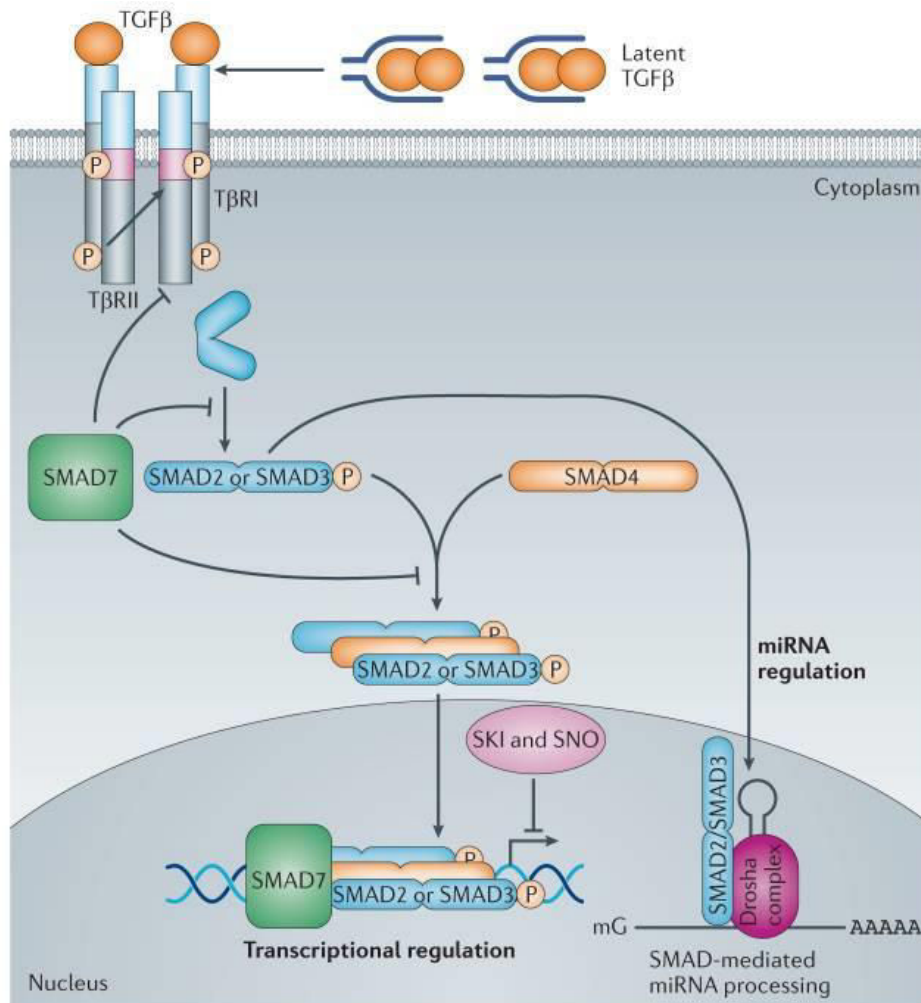


Figure 6: Représentation schématique de la voie de signalisation canonique TGFβ dépendante des SMADs. Akhurst, *Nat Rev Drug Discov*, 2012 ⁴⁸. SNO : ski-related novel gene.

L'activation de la voie SMAD indépendante (non canonique) peut se faire via le *crossstalk* avec de nombreuses autres voies dont p38 MAPK, m-TOR, RAS, RhoA, PI3K/AKT, JNK ⁴⁹. Les mécanismes de régulation de la voie TGFβ sont complexes. Par exemple, SMAD7 est un inhibiteur de la famille SMAD qui exerce un rétrocontrôle négatif sur la voie et agit comme

médiateur des interactions avec les autres voies de signalisation e.g. voie Wnt/ β -caténine⁵⁰. Sa surexpression est fréquente quand la voie TGF β est perturbée⁴⁹.

ii. Rôle du TGF β dans la cancérogénèse

Le TGF β a un rôle paradoxal dans la cancérogénèse. L'activation de la voie donne des effets opposés (suppresseur de tumeur ou tumorigénique) selon le contexte cellulaire (figure 7). Les mécanismes orientant vers l'un ou l'autre des effets du TGF β ne sont pas clairement identifiés⁵¹.

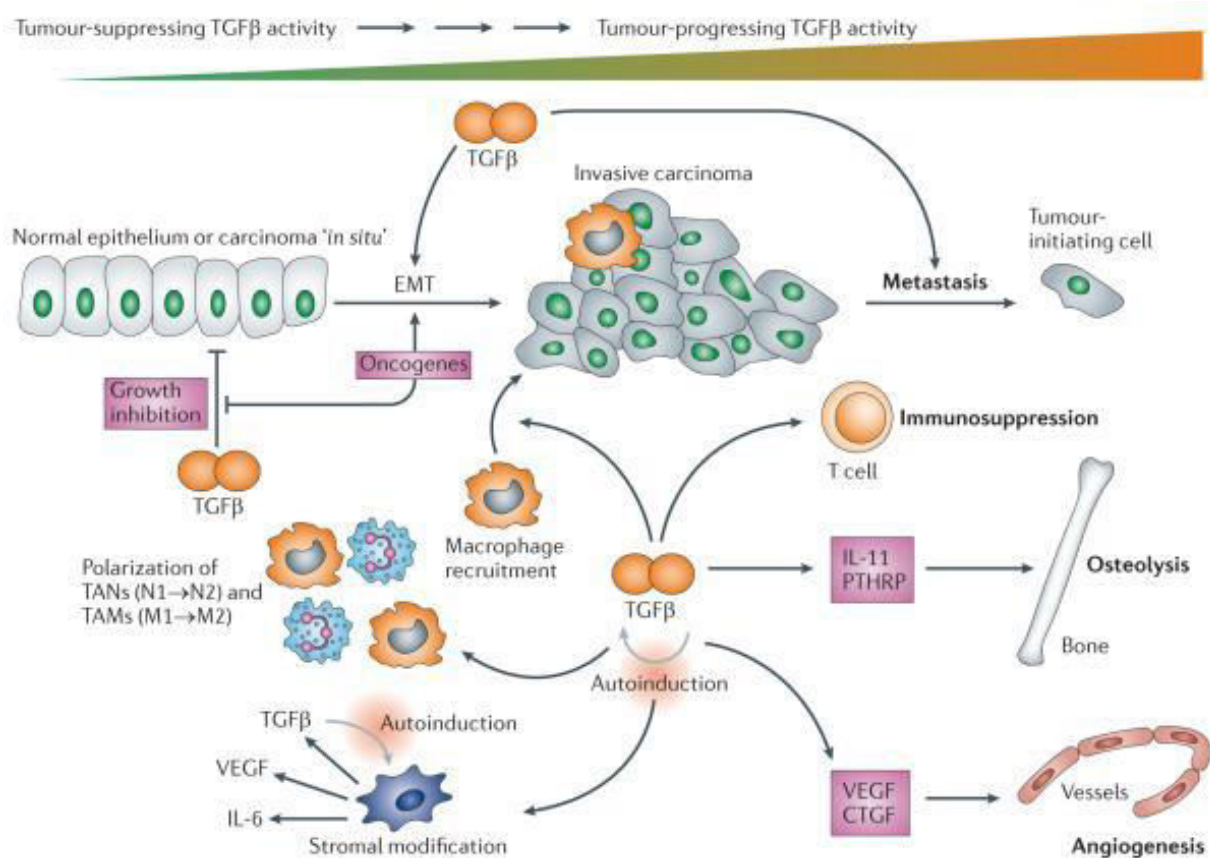


Figure 7: Schéma de l'activité biphasique de la voie TGF β pendant la cancérogénèse: de la suppression à la promotion de la tumeur. Akhurst, *Nat Rev Drug Discov*, 2012⁴⁸
 Les flèches gris clair indiquent la boucle d'activation conduisant à des niveaux plus élevés de TGF β dans les pathologies néoplasiques et non néoplasiques.
 CTGF, connective tissue growth factor; EMT, epithelial-mesenchymal transition; IL, interleukin; PTHRP, parathyroid hormone-related protein; TAMs, tumour-associated macrophages; TANs, tumour-associated neutrophils; VEGF, vascular endothelial growth factor.

A des stades précoces (prénéoplasiques ou cancer *in situ*), le TGF β va plutôt exercer un rôle cytotatique, par exemple en contrôlant le cycle cellulaire via c-MYC, p21 et cycline D⁴⁹. Après activation de signaux oncogéniques, le TGF β va favoriser l'apoptose et la sénescence pour limiter la prolifération cellulaire et l'apparition de néoplasie⁵¹. Cependant, nous avons

vu également précédemment le rôle profibrotique du TGF β dans les hépatopathies chroniques. Aux stades avancés de cancer, le TGF β va augmenter la croissance tumorale et l'invasion. Les effets tumorigéniques du TGF β passent par l'activation de la transition épithélio-mésenchymateuse (TEM), la mobilisation de myofibroblastes et l'immunosuppression ⁵¹.

La TEM est un processus dynamique réversible pendant lequel les cellules épithéliales adoptent progressivement les caractéristiques structurelles et fonctionnelles des cellules mésenchymateuses (figure 8) ⁵². Elle est mise en évidence au niveau membranaire par la perte d'E-cadhérine et l'expression de protéines mésenchymateuses comme Vimentine, N-cadhérine et fibronectine qui vont favoriser l'invasion ⁴⁹. Le processus de perte d'E-cadhérine peut être régulé par les voies SMAD dépendante ou SMAD indépendante du TGF β et des interactions entre les deux ont été montrés ⁴⁹. Au niveau transcriptionnel, les facteurs qui régulent la TEM sont SNAIL, TWIST et ZEB ⁵³.

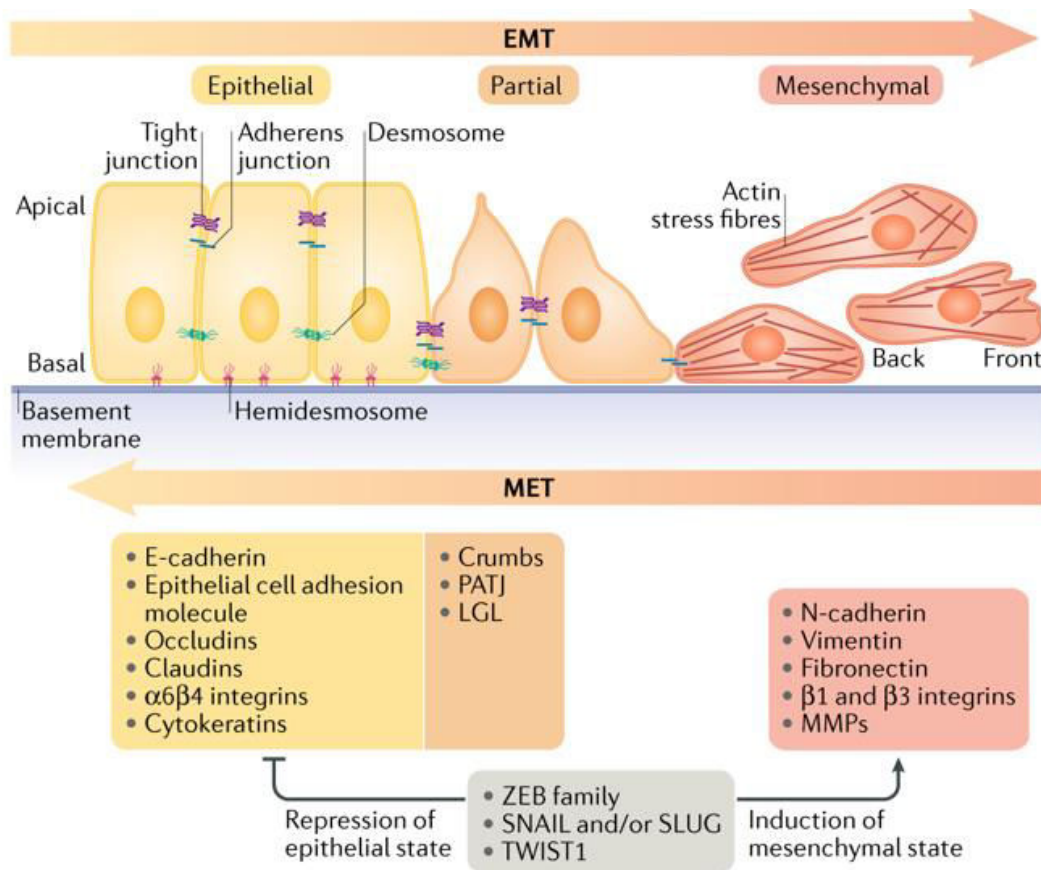


Figure 8: Schéma d'une transition épithélio-mésenchymateuse typique. *Dongre, Nat Rev Mol Cell Biol, 2019*⁵⁴.
 PATJ : PALS1-associated tight junction protein. LGL : lethal giant larvae. ZEB : zinc finger E-box binding.

La régulation de l'expression de marqueur de cellules souches cancéreuses comme CD133 par le TGF β a été également été montré. Des modèles de xénogreffes CD133+ induites par le TGF β ont montré des tumeurs plus agressives avec des cellules résistantes à la chimiothérapie et à l'apoptose^{55,56}. A un stade tumoral avancé, le TGF β joue aussi un rôle crucial dans la répression du système immunitaire notamment via l'inactivation des cellules T_H17⁴⁹.

Outre son rôle dans la TEM, le TGF β exerce aussi une action prométastatique en favorisant l'angiogénèse à un stade tumoral avancé. Le TGF β régule l'expression du VEGF ainsi que la prolifération et la migration des cellules endothéliales (figure 7)^{57,58}.

Cependant, selon l'organe siège du cancer et le sous type tumoral, la réponse au TGF β peut être différente. Le type de réponse des tumeurs (évolution métastatique et pronostic) au TGF β permet de les classer en différents groupes. Par exemple dans le cancer du sein, une

signature spécifique de la réponse au TGF β n'est pas associée au même sous type de tumeur (récepteur œstrogène + ou -) ni au même site de rechute métastatique (poumon ou os) ⁵⁹.

De même, des mutations des récepteurs TGF β et de SMAD4 ont été identifiées fréquemment dans le cancer du pancréas et du colon. Cependant, ces mutations seules ne vont pas être responsables du développement tumoral. Elles s'ajoutent à des lésions déjà induites par l'oncogène KRAS ou l'inactivation du gène APC ⁵¹

iii. TGF β et cholangiocarcinome intrahépatique

Le TGF β joue un rôle majeur dans la TEM et donc le développement du microenvironnement tumoral (stroma). La richesse du stroma fibreux dans le CCI a donc fait supposer l'implication du TGF β dans son développement. L'induction de la TEM par le TGF β dans le CCI a été montré avec une augmentation des marqueurs mésenchymateux (VIM, S100A4, SNAIL) et une diminution des marqueurs épithéliaux (E cadhérine, CK19) ^{60,61}. La surexpression des facteurs de transcription SNAIL, TWIST et ZEB est associée à des CCI plus agressifs et de plus mauvais pronostic ⁵³. Dans les CCI présentant des caractéristiques de cellules souches cancéreuses, le profil transcriptomique a révélé un lien entre l'expression du microARN miR-200c avec la TEM. L'expression de miR-200c est inversement corrélée avec les gènes cibles de la voie TGF β . La surexpression de miR-200c dans la lignée HuH28 induit une inhibition de la TEM avec suppression des marqueurs mésenchymateux et augmentation des marqueurs épithéliaux ⁶². Plus récemment, des études de co-cultures chez le rat, de cellules de cholangiocarcinome avec des myofibroblates, ont montré que la formation d'un stroma fibreux (réaction desmoplastique) était dépendante du TGF β ⁶³. Les mécanismes de l'induction de la prolifération cellulaire par le TGF β sont multiples. L'IL6 et la cycline D1 via miR-34a seraient impliqués ⁶⁴⁻⁶⁶. Le TGF β 1 induit également l'angiogénèse via VEGF dans le CCI ⁶⁷. L'expression de TGF β 1 est corrélée à l'envahissement ganglionnaire, les métastases à distance et la récurrence dans le CCI ⁶⁸.

Les travaux réalisés dans l'unité Inserm 1241 sur le CCI au cours des 10 dernières années ont cherché à identifier des altérations génomiques dans le stroma tumoral du CCI. Après séparation du stroma tumoral et des cholangiocytes tumoraux par microdissection laser de coupes de tumeurs humaines, une analyse transcriptomique a comparé le stroma non tumoral au stroma tumoral. L'analyse a montré une surexpression de l'ostéopontine (SPP1) mais aussi du TGF β dans le stroma tumoral qui était corrélée à un plus mauvais pronostic ¹⁸.

Ces données confirmaient l'importance du microenvironnement tumoral.

Andersen et al. en 2012 avaient également montré des signatures spécifiques des compartiments épithélial et stromal du CCI avec une augmentation du TGF β et de l'IL6 dans le stroma ²⁴.

Des mutations de composants de la voie TGF β ont déjà été observées dans le CCI : *SMAD4*, *SMAD6*, *SMAD7* et des mutations non-sens de *TGFBR1* ^{69,70}.

Cependant, la dualité fonctionnelle du TGF β fait qu'un traitement inhibant la voie TGF β pour traiter une fibrose pourrait augmenter le risque de développement d'un cholangiocarcinome. Dans un modèle murin, Mu et al. ont montré que le signal TGF β épithélial n'induisait pas la fibrose et protégerait du développement du cholangiocarcinome ⁷¹.

La régulation de la voie TGF β est un ensemble de processus complexes qui sont encore loin d'être tous identifiés. Les ARN non-codants pourraient être des acteurs de cette régulation.

3. ARN longs non-codants (ARNlnc)

Le génome codant ne représente que 2% des gènes. Depuis les années 90, le nombre d'ARN non-codants identifiés est en constante augmentation et dépasse le nombre des transcrits codants connus ⁷². Afin de simplifier leur classification, ils ont été classés arbitrairement selon leur taille. Les ARN courts ont moins de 200 nucléotides dont les microARN qui ont en moyenne 21 nucléotides et répriment la production de protéines. Les ARN circulaires (ARNcirc) constituent un groupe distinct d'ARN non-codants issus de la biogénèse et de l'épissage de transcrits linéaires, codants ou non-codants. Ils sont stables car résistants aux exonucléases en l'absence d'extrémité libre 5' ou 3' ⁷³.

i. Caractéristiques des ARNlnc

Les ARNlnc ont plus de 200 nucléotides et ont été peu étudiés jusqu'aux années 2000 contrairement aux microARNs. Ils appartiennent à une classe hétérogène d'ARN non-codants régulateurs qui influencent une large variété de processus biologiques et de voies de signalisation cellulaires. Après leur découverte dans les années 80, les ARNlnc ont initialement été peu étudiés car considérés comme du « bruit » transcriptionnel et donc non fonctionnels. Le projet *Encyclopedia of DNA elements* (ENCODE) a été initié en 2003 afin de faciliter l'identification et l'analyse de l'ensemble des éléments fonctionnels du génome. Le consortium GENCODE issu de ce projet a permis d'annoter environ 15000 transcrits correspondants à des ARNlnc. L'étude des ARNlnc a montré qu'ils sont généralement moins exprimés que les gènes codants pour des protéines et qu'ils ont des caractéristiques spécifiques d'organes ⁷⁴. Même si de nombreux ARNlnc ont été identifiés, leur fonction demeure inconnue pour la plupart.

Comme pour les gènes codants, plusieurs gènes donnant naissance aux ARNlnc sont conservés dans l'évolution ⁷⁵. Ils ont des similarités avec les ARNm: ils sont transcrits par la polymérase II, ont une extrémité 5', peuvent être épissés et polyadénylés, mais non codants. La classification courante des ARNlnc différencie 5 catégories selon leur organisation génomique : sens, antisens, bidirectionnel, intronique, et intergénique (figure 9) ⁷⁶.

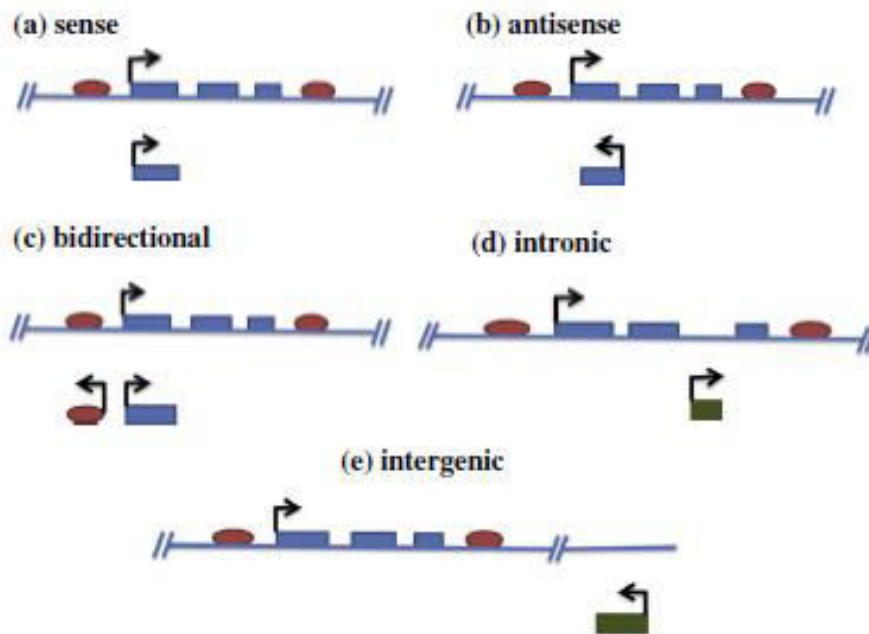


Figure 9: Classification des ARNlnc selon leur organisation génomique. *He, Cancer Lett, 2014*⁷⁶.

Au niveau fonctionnel, les ARNlnc peuvent s'associer à de l'ADN, à d'autres ARN ou à des protéines ce qui conduira à de multiples fonctions possibles (figure 10).

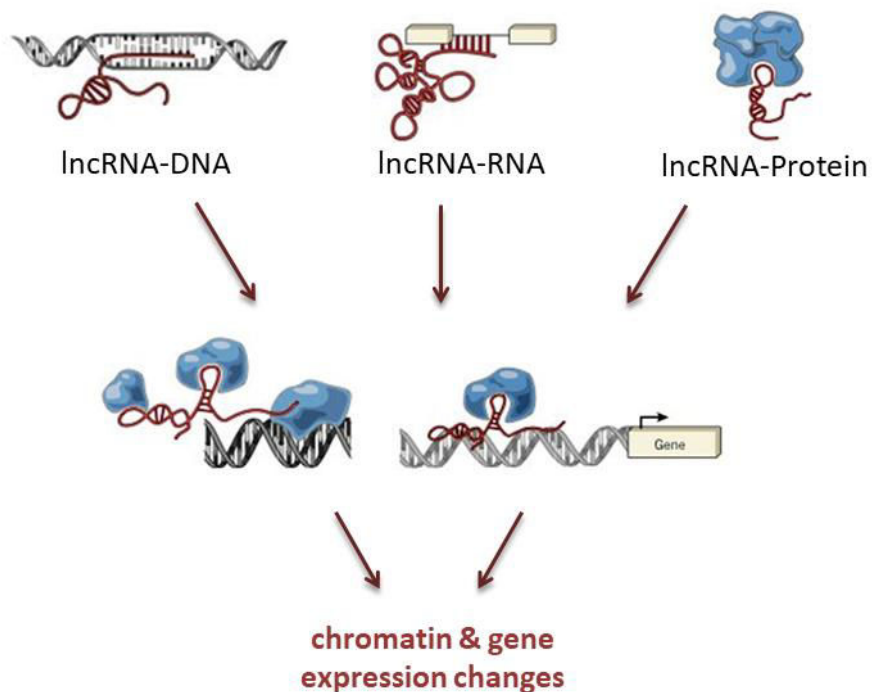


Figure 10: Associations moléculaires possibles des ARNlnc aboutissant à de multiples fonctions.

Les ARNlnc sont localisés dans des compartiments cellulaires spécifiques selon leur fonction biologique ⁷⁷. L'identification croissante d'ARNlnc a conduit à découvrir de nombreux modes d'action (figure 11). Le rôle d'ARNlnc a été identifié dans de nombreuses pathologies et organes différents (cœur, cerveau foie, poumon) mais surtout dans les cancers. Dans la cancérogénèse hépatique, les premières altérations d'expression d'ARNlnc ont été identifiées dans le CHC dont des exemples sont cités ci-dessous.

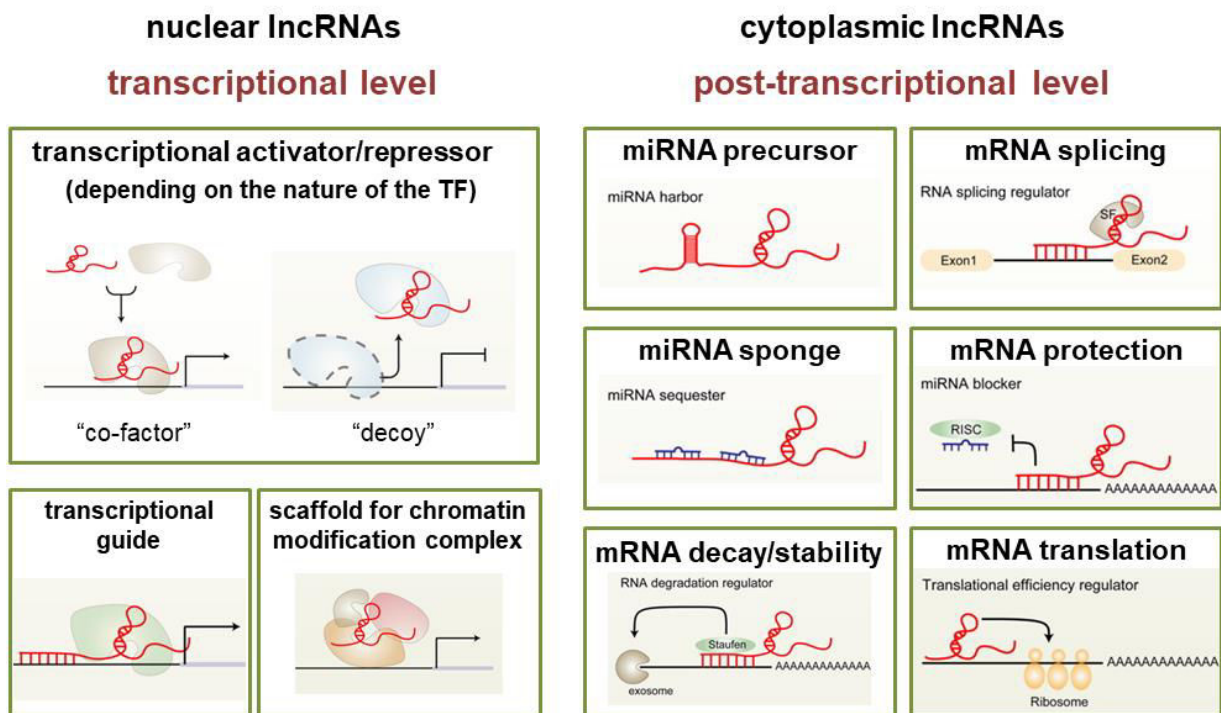


Figure 11: Exemples de description de modes d'actions des ARNlnc selon leur localisation nucléaire ou cytoplasmique.

Dans le noyau, les ARNlnc peuvent agir sur la chromatine et influencer la transcription :

- Guide : ces ARNlnc se lient à des protéines afin de former des complexes capables d'agir comme facteurs de transcription. Exemple de l'ARNlnc HOXA Distal Transcript Antisense RNA (HOTTIP) dans le CHC : HOTTIP interagit avec le complexe mixed lineage leukemia (MLL) qui conduit à la formation de H3K4me3 (triméthylation de la lysine 4 de la protéine d'histone H3) et à la transcription de gènes à proximité (figure 12) ⁷⁸.

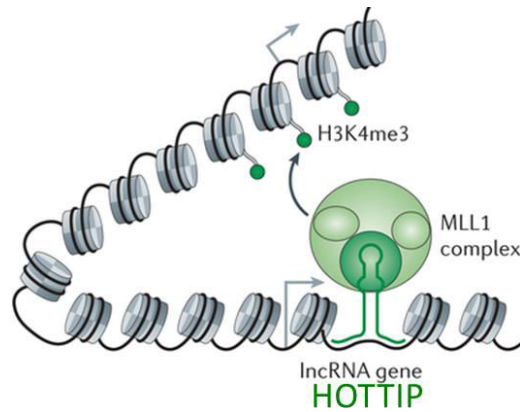


Figure 12: Schéma du mode d'action de HOTTIP. Wang, *Nature*, 2011⁷⁸.
MLL : mixed lineage leukemia.

- Echafaudage (*scaffold*) : ces ARNinc servent de plateformes sur lesquelles différents composants moléculaires vont s'assembler. Les complexes ainsi formés vont réguler au niveau épigénétique des régions génomiques spécifiques. L'ARNinc HOX Transcript Antisense RNA (HOTAIR) a ainsi un rôle oncogénique en modulant les complexes d'histones comme Polycomb Repressive Complex 2 (PRC2) impliqués dans la répression de gènes suppresseurs de tumeur (figure 13)⁷⁹.

PRC2 (Polycomb Repressive Complex 2): histone methyl-transferase complex
« writer » of the repressive mark H3K27 trimethylation

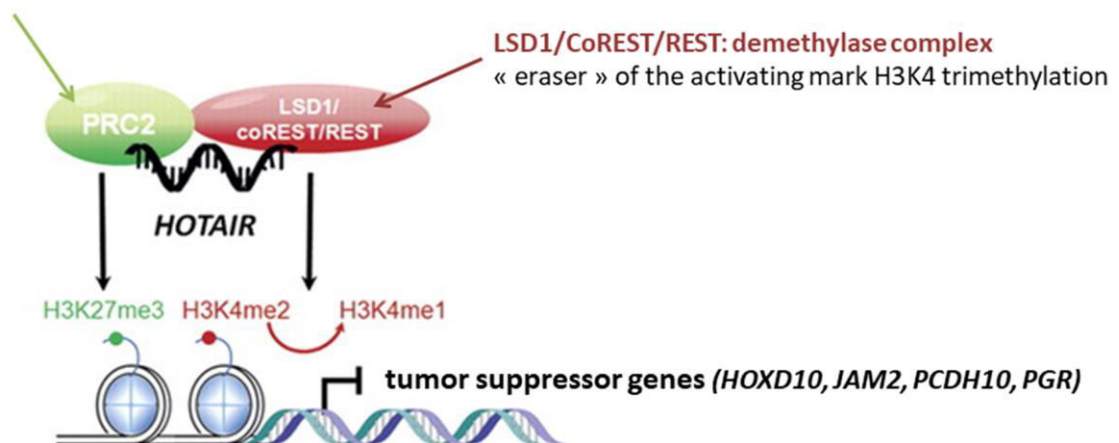


Figure 13: Schéma du mode d'action de HOTAIR sur les complexes d'histone PRC2 et LSD1/coREST/REST. D'après Tsai, *Science*, 2010⁷⁹.

PRC2 : Polycomb Repressive Complex 2. LSD1 : lysine-specific histone demethylase. REST : RE1-silencing transcription factor; 1. H3K27me3 : trimethylated lysine 27 of histone H3. H3K4me : methylated lysine 4 of histone H3. HOXD10: homeobox D10. JAM2 : junctional adhesion molecule 2. PCDH10 : protocadherin 10. PGR : progesterone receptor

- Régulateur transcriptionnel: ces ARNlnc vont être co-facteur de facteur de transcription ou répresseur en agissant comme leurre (*decoy*) pour les facteurs de transcription. L'ARNlnc p21-associated ncRNA DNA damage-activated (PANDA) limite ainsi l'apoptose en séquestrant le facteur de transcription nuclear transcription factor Y (NF-Y) (figure 14) ^{80,81}.

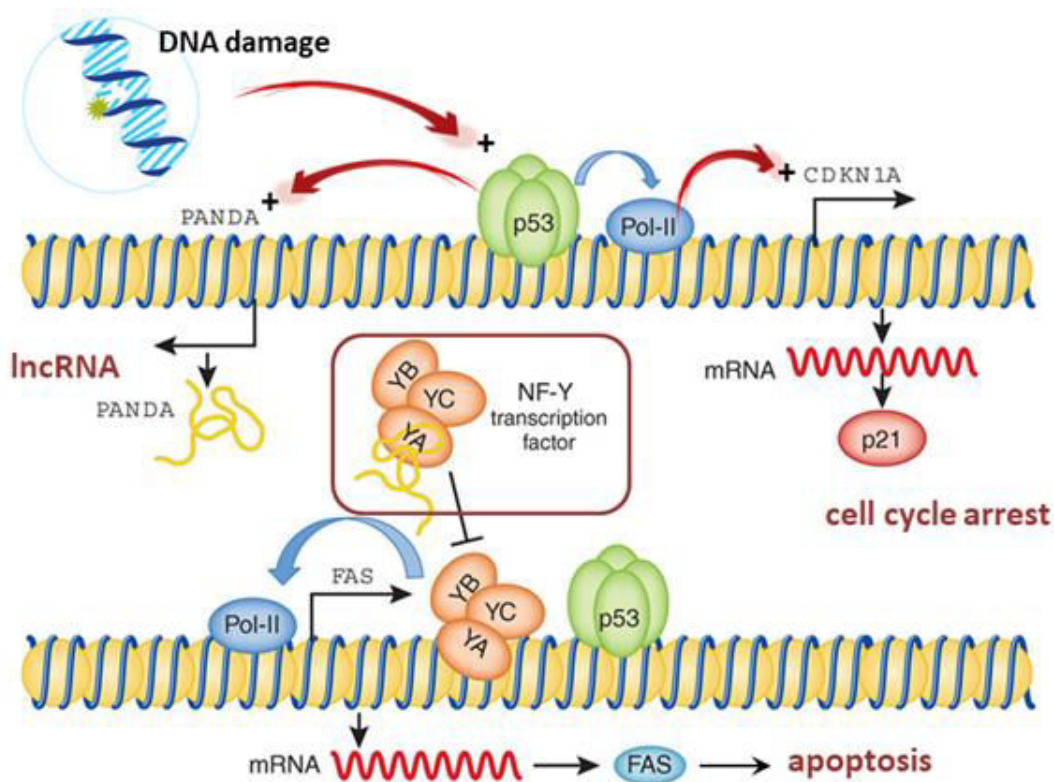


Figure 14: Schéma du mode d'action de PANDA. *Sotillo, Nat Genet, 2011* ⁸¹.

PANDA : p21-associated ncRNA DNA damage-activated. CDKN1A : cyclin-dependent kinase Inhibitor 1A. NF-Y : nuclear transcription factor Y. Pol-II : RNA polymerase II.

Dans le cytoplasme, les ARNlnc agissent au niveau post transcriptionnel (figure11).

Sur les ARNm, les ARNlnc peuvent agir comme :

- Régulateur de l'épissage des ARNm
- Protecteur de l'action des miARN (bloqueur de miARN)
- Régulateur de la traduction
- Régulateur de la dégradation ou de la stabilité des ARNm

Sur les miARN, les ARNlnc peuvent agir comme :

- Précurseur de miARN. Exemple : l'ARNlnc H19, qui est essentiel à la croissance tumorale du CHC, a sur son exon 1 un site précurseur de miR-675 (figure 15) ^{82,83}

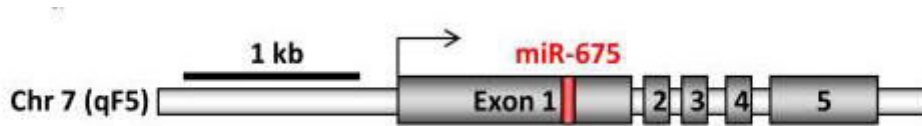


Figure 15 : Représentation schématique de l'unité transcriptionnelle de H19 avec miR-675 (en rouge) inclus dans l'exon 1 de H19. La flèche noire indique le site de départ de la transcription. *Keniry, Nat Cell Biol, 2012* ⁸³.

- Eponge à microARN, ce qui aura pour conséquence de réguler positivement leurs cibles. Exemple : l'ARNlnc phosphatase and tensin homolog pseudogene 1 (PTENP1) séquestre le microARN miR-21 (oncomiR) et l'empêche d'inhiber le suppresseur de tumeur PTEN ce qui inhibe la prolifération et bloque le cycle cellulaire via le blocage de la voie de signalisation AKT (figure 16) ⁸⁴.

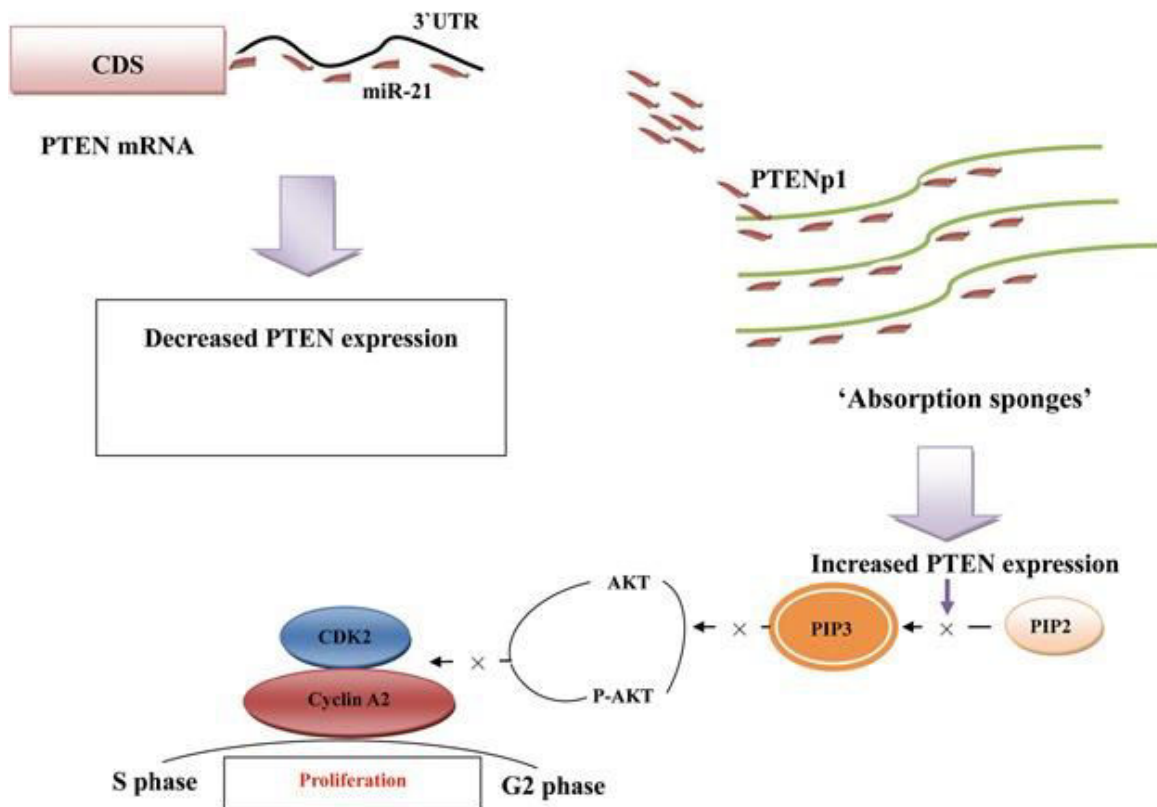


Figure 16 : Représentation schématique de l'action de PTENP1. *Gao, Mol Carcinog, 2017* ⁸⁴. CDS : coding DNA sequence. PTEN : phosphatase and tensin homolog. PIP: prolactin-inducible protein. CDK2: cyclin-dependent kinase 2.

ii. *ARNInc et cholangiocarcinome*

Dans le cholangiocarcinome, l'altération d'expression de plusieurs ARNInc a également été montré ⁸⁵. En 2014, Wang et al. ont montré par analyse transcriptomique de 77 CCI et tissus non tumoraux adjacents humains que 2773 ARNInc étaient surexprimés et 2392 sous exprimés ⁸⁶. L'analyse récente du profil d'expression des ARNInc de CCI humains a été réalisée par plusieurs équipes à partir de la base de données *The Cancer Genome Atlas* (TCGA). Ils ont également montré une différence d'expression d'ARNInc avec pour certains une corrélation à la survie des patients ^{87,88}. L'analyse des réseaux de co-expression des ARN codants et non-codants dans le CCI par Yang et al. en 2017 a identifié des ARNInc impliqués dans l'inflammation et la voie PPAR qui peut réguler la TEM ⁸⁹.

Au niveau épigénétique, l'interaction de l'ARNInc Small Nucleolar RNA Host Gene 1 (SNHG1) avec enhancer of zeste 2 (EZH2), la sous unité catalytique du PRC2, réduit l'expression de CDKN1A qui est suppresseur de tumeur ⁹⁰. L'ARNInc nuclear paraspeckle assembly transcript 1 (NEAT1) interagit également avec EZH2 pour diminuer l'expression d'E-cadhérine et favoriser la TEM dans le CCI ⁹¹.

Certains ARNInc ont également une action directe ou indirecte sur des régulateurs oncogéniques. Ainsi, l'ARNInc epigenetically-induced lncRNA1 (EPIC1) cible directement le facteur de transcription oncogénique MYC pour promouvoir la prolifération cellulaire ⁹². L'ARNInc metastasis associated lung adenocarcinoma transcript 1 (MALAT1) active la voie PI3/AKT et augmente la prolifération et l'invasion cellulaire ⁹³.

Le niveau d'expression de quelques ARNInc est corrélé au pronostic de patients opérés de CCI constituant ainsi de potentiels biomarqueurs. Par exemple, les ARNInc Colorectal Neoplasia Differentially Expressed (CRNDE) et taurine upregulated gene 1 (TUG1) sont surexprimés dans les CCI et leur surexpression est corrélée au mauvais pronostic ⁹⁴⁻⁹⁶. Une revue de la littérature est consacrée à ce sujet dans la partie résultat de ce manuscrit.

iii. ARNInc et TGFβ dans la cancérogénèse hépatique

Des ARNInc régulés par la voie TGFβ ont été identifiés dans la cancérogénèse. Au cours de la TEM, SNAI1 est un des régulateurs dont l'expression est sous la dépendance du TGFβ. L'ARNInc ZEB2 antisense RNA (ZEB2-AS) a été identifié comme médiateur de l'action de SNAI1 sur la synthèse de ZEB2 qui est un répresseur transcriptionnel de l'E-Cadhérine (figure 17)⁹⁷.

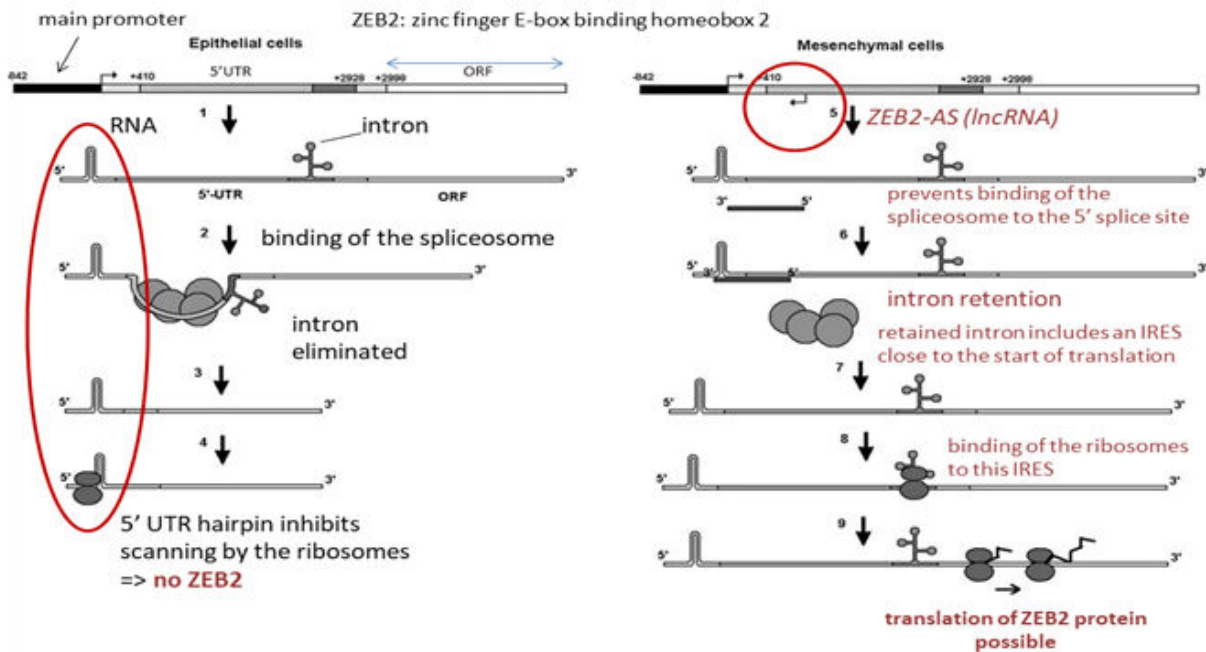


Figure 17: Schéma du mode d'action de ZEB2-AS. *Beltran, Genes Dev, 2008*⁹⁷.

SNAI1 n'influence pas directement la synthèse de ZEB2 mais prévient l'épissage d'un large intron situé sur son extrémité 5'UTR. L'intron contient un site d'entrée interne aux ribosomes nécessaire à l'expression de ZEB2. Le maintien de l'intron est dépendant de l'expression d'un transcrit naturel anti-sens (ZEB2-AS) qui chevauche le site de l'intron. L'expression ectopique de ZEB2-AS dans les cellules épithéliales prévient l'épissage de l'extrémité 5'UTR de ZEB2, augmente le niveau de la protéine ZEB2 et diminue l'expression de l'E-Cadhérine. ZEB: zinc finger E-box binding.

Jusqu'à présent au niveau hépatique, des ARNInc potentiels médiateurs de la voie TGFβ ont seulement été identifiés dans le CHC.

L'ARNInc activated by TGFβ (ATB) est induit par le TGFβ et sa surexpression est corrélée au mauvais pronostic du CHC. ATB régule ZEB1 et ZEB2 en agissant comme éponge à miR-200 et ainsi induit la TEM et l'invasion (figure 18)⁹⁸. ATB se lie également à l'IL11 ce qui induit une production autocrine d'IL11 qui va activer la voie STAT3 et le processus métastatique dans le CHC⁹⁸. ATB est également surexprimé dans de nombreux autres

cancers (e.g. colorectal, gastrique, rénal, pancréatique, pulmonaire) avec une corrélation à un mauvais pronostic ⁹⁹.

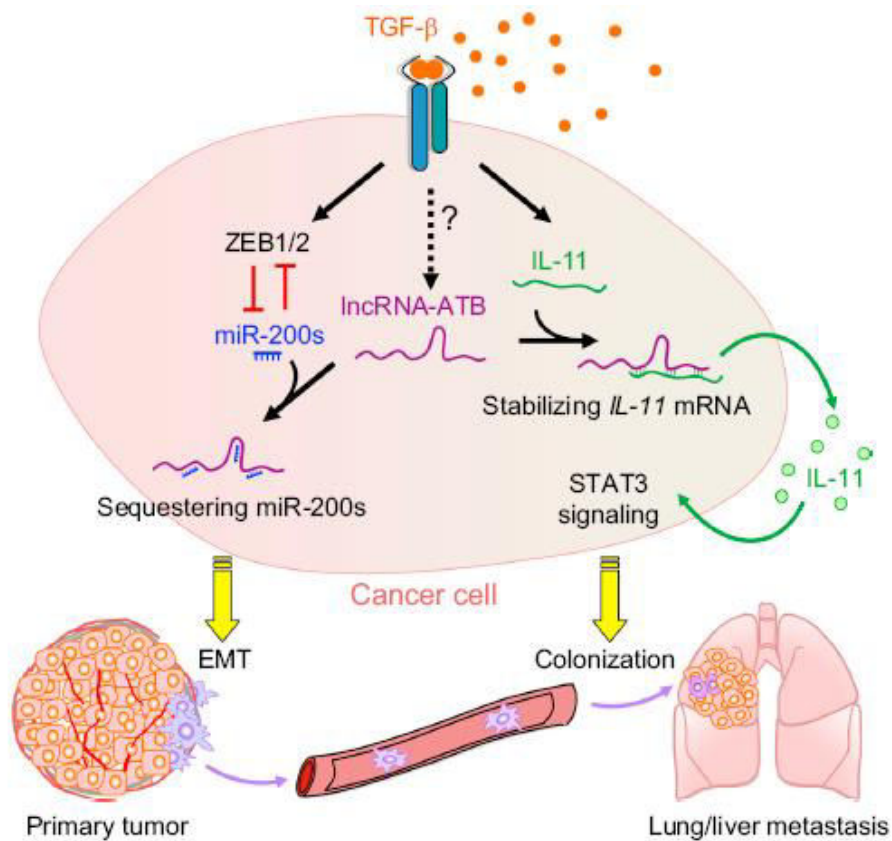


Figure 18 : Schéma du mode d'action de l'ARNinc ATB. Yuan, *Cancer Cell*, 2014 ⁹⁸.

Récemment, Zhang et al. ont montré que le TGFβ inhibe la transcription de l'ARNinc H19 par l'intermédiaire du facteur de transcription SOX2 dans les hépatocytes précurseurs de tumeurs. L'ARNinc H19 est impliqué dans la prolifération et la survie de ces hépatocytes ¹⁰⁰. Ces travaux ont donc permis d'identifier un ARNinc, H19, comme médiateur des effets suppresseurs de tumeur du TGFβ à un stade précoce du développement du CHC.

L'ARNinc Regulator Of Reprogramming (ROR) est induit par le TGFβ dans le CHC et intervient dans la chimiorésistance via les cellules exprimant CD133 ¹⁰¹.

Certains ARNinc peuvent également être régulateurs de la voie TGFβ.

L'ARNinc Maternally Expressed Gene 3 (MEG3), un ARNinc qui interagit avec la chromatine, régule la voie TGFβ par l'intermédiaire de structures ARN-ADN ¹⁰². Il joue un rôle dans le développement de la fibrose hépatique car son expression est diminuée en réponse au

TGF β dans la lignée de cellules hépatiques étoilées humaines LX2. Sa surexpression inhibe la prolifération cellulaire induite par le TGF β et augmente l'apoptose ¹⁰³. Son rôle dans le développement du CHC avait également été montré par l'intermédiaire de sa régulation par miR-29 ¹⁰⁴.

La surexpression de LINC00974 est corrélée à celle de la kératine 19 (KRT19) et à un mauvais pronostic dans le CHC. La répression de LINC00974 inhibe la prolifération cellulaire et l'invasion par l'activation de l'apoptose et de l'arrêt du cycle cellulaire. Son action est liée à son rôle d'éponge pour le miR-642 qui va induire une surexpression de KRT19 et une activation des voies de signalisation NOTCH et TGF β ¹⁰⁵.

L'ARNInc Plasmacytoma Variant Translocation 1 (PVT1) régule SMAD4 et les gènes associés à l'apoptose dans la voie TGF β ¹⁰⁶. Dans le CHC, PVT1 augmente la prolifération cellulaire et l'acquisition de propriétés de cellules souches ¹⁰⁷.

L'ARNInc Antisense Noncoding RNA In The INK4 Locus (ANRIL) est transcrit dans le sens opposé du locus INK4 codant pour les gènes suppresseurs de tumeurs p15INK4b, p14ARF et p16INK4a. ANRIL est un ARNInc dérégulé dans les maladies cardiovasculaires et de nombreux cancers. Il recrute les complexes PRC1 et PRC2 par l'intermédiaire des protéines polycomb chromobox7 (CBX7) et PRC2 Subunit (SUZ12), respectivement (figure 19). Ces interactions épigénétiques aboutissent à la répression du locus INK4 qui induit la prolifération cellulaire. Dans les cellules de cancer de la thyroïde et de cancer épidermoïde de l'œsophage, ANRIL réduit l'expression de p15/INK4 en inhibant la voie TGF β , ce qui aboutit à une augmentation de l'invasion et des métastases ^{108,109}.

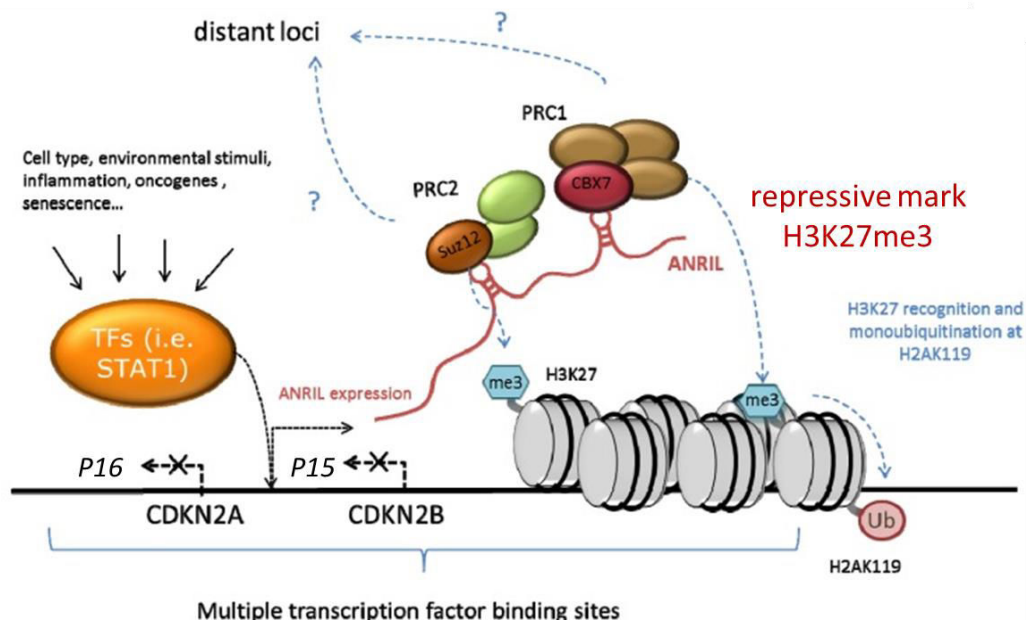


Figure 19 : Schéma du mode d'action d'ANRIL. *Congrains, Int J Mol Sci, 2013* ¹¹⁰.

CDKN2 : Cyclin Dependent Kinase Inhibitor 2. TFs : transcription factors. CBX7 : Chromobox 7.

Suz12 : Polycomb Repressive Complex 2 Subunit. H3K27me3 : trimethylated lysine 27 of histone H3.

ANRIL agit comme éponge pour plusieurs microARNs, notamment pour miR-122-5p dans le CHC. ANRIL est surexprimé dans le CHC où un lien a été observé avec une induction de la prolifération cellulaire, la capacité à former des colonies, des métastases et l'invasion ¹¹¹. Une partie des résultats de ce manuscrit est consacrée à l'identification d'ANRIL comme facteur prédictif de mauvais pronostic dans le CCI.

II. Objectifs

Le CCI est un cancer de mauvais pronostic avec un arsenal thérapeutique limité. Après une résection chirurgicale, seul traitement potentiellement curatif, la récurrence est fréquente et peu répondeuse aux chimiothérapies actuelles. Les axes de recherche actuels sur la prise en charge du CCI doivent donc se porter sur l'identification:

- des altérations d'expression génique présentes dans le CCI
- de biomarqueurs diagnostiques et pronostiques
- de potentielles cibles thérapeutiques

Des travaux antérieurs réalisés au sein de l'unité Inserm 1241 s'étaient intéressés au microenvironnement tumoral dans le CCI. L'expression de TGF β dans le stroma tumoral était corrélée à un mauvais pronostic ¹⁸. Comme décrit en introduction, plusieurs publications ont également confirmé cette implication de la voie TGF β dans le microenvironnement tumoral du CCI. Cependant, l'impact d'un environnement riche en TGF β sur le transcriptome des cellules de CCI a été peu étudié.

La dérégulation d'ARN non-codants dans la cancérogénèse est de plus en plus mise en évidence. La découverte de leur action régulatrice de nombreuses voies de signalisation permet d'imaginer de nouvelles cibles thérapeutiques.

L'hypothèse du projet est l'implication d'ARNInc dans le développement du CCI notamment comme médiateurs de la voie TGF β . Les objectifs du projet se sont focalisés sur:

- l'identification des ARNInc régulés par le TGF β dans le CCI et leur caractérisation fonctionnelle
- la recherche d'ARNInc potentiels biomarqueurs diagnostiques ou pronostiques

Les études ont été menées *in vitro* sur des lignées cellulaires de CCI et en parallèle *in vivo* sur tumeurs humaines. Les lignées cellulaires ont été choisies de phénotype différent pour étudier l'activation de la voie TGF β dans différents contextes cellulaires. Les tumeurs humaines ont été obtenues à partir du centre de ressources biologiques local du CHU de Rennes mais aussi via le réseau national des tumeurs du foie.

III. Résultats

1. Article n°1

Publié dans Hepatology Communications

« TLINC : un nouvel ARNInc induit par le TGF β favorise un microenvironnement inflammatoire dans le cholangiocarcinome intrahépatique »

L'objectif de ce travail était d'étudier l'impact du TGF β sur le transcriptome des cellules de CCI. Une analyse transcriptomique par microarrays de 2 lignées cellulaires de CCI de phénotype différent, HuCCT1 (épithélial) et HuH28 (mésenchymateux), a été réalisée avec et sans traitement par le TGF β . Une signature spécifique des gènes dérégulés par le TGF β a été identifiée incluant des gènes cibles connus de la voie TGF β mais également de nouveaux gènes cibles dont plusieurs ARNInc. Nous nous sommes focalisé sur l'étude d'un ARNInc connu sous le nom de CASC15/LINC00340, que nous avons renommé TLINC (*TGF β induced long non coding RNA*). En effet, nous montrons que TLINC est une cible du TGF β dans des lignées cellulaires hépatiques et non hépatiques. Dans les lignées de CCI, l'expression de 2 isoformes, longue et courte, était associée à un phénotype épithélial ou mésenchymateux respectivement. Les 2 isoformes ont été détectées dans le noyau et le cytoplasme. L'isoforme longue de TLINC était associée à un phénotype migratoire dans les lignées cellulaires de CCI et à l'induction de cytokines proinflammatoires, dont l'IL8, dans les lignées cellulaires et dans les CCI humains réséqués. TLINC a été identifié comme marqueur tumoral dans les cellules épithéliales et stromales. Dans les foies non tumoraux, TLINC est présent seulement dans les des espaces portes spécifiques avec des signes de réaction et d'inflammation ductulaire. Enfin, nous avons montré expérimentalement l'existence d'isoformes circulaires de TLINC dans les lignées cellulaires traitées avec le TGF β et dans les CCI humains réséqués.

A Novel Transforming Growth Factor Beta-Induced Long Noncoding RNA Promotes an Inflammatory Microenvironment in Human Intrahepatic Cholangiocarcinoma

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Intrahepatic cholangiocarcinoma (iCCA) is a deadly liver primary cancer associated with poor prognosis and limited therapeutic opportunities. Active transforming growth factor beta (TGF β) signaling is a hallmark of the iCCA microenvironment. However, the impact of TGF β on the transcriptome of iCCA tumor cells has been poorly investigated. Here, we have identified a specific TGF β signature of genes commonly deregulated in iCCA cell lines, namely HuCCT1 and Huh28. Novel coding and noncoding TGF β targets were identified, including a TGF β -induced long noncoding RNA (TLINC), formerly known as cancer susceptibility candidate 15 (CASC15). TLINC is a general target induced by TGF β in hepatic and nonhepatic cell types. In iCCA cell lines, the expression of a long and short TLINC isoform was associated with an epithelial and mesenchymal phenotype, respectively. Both isoforms were detected in the nucleus and cytoplasm. The long isoform of TLINC was associated with a migratory phenotype in iCCA cell lines and with the induction of proinflammatory cytokines, including interleukin 8, both *in vitro* and in resected human iCCA. TLINC was also identified as a tumor marker expressed in both epithelial and stroma cells. In nontumor livers, TLINC was only expressed in specific portal areas with signs of ductular reaction and inflammation. Finally, we provide experimental evidence of circular isoforms of TLINC, both in iCCA cells treated with TGF β and in resected human iCCA. **Conclusion:** We identify a novel TGF β -induced long noncoding RNA up-regulated in human iCCA and associated with an inflammatory microenvironment. (*Hepatology Communications* 2018;2:254-269)

Introduction

Cholangiocarcinomas (CCAs) comprise heterogeneous hepatobiliary tumors with cholangiocyte differentiation features. CCAs are classified as intrahepatic (iCCA), perihilar, and distal, based on their anatomic location.^(1,2) In the liver,

iCCA accounts for 5%-10% of all malignant primary cancers and ranks second after hepatocellular carcinoma (HCC). Importantly, both incidence and mortality rates of iCCA have increased significantly worldwide during the past decades.⁽²⁾ To date, surgery is the best potentially curative treatment for iCCA but is feasible in less than 40% of cases. For these

Abbreviations: CASC15, cancer susceptibility candidate 15; CCA, cholangiocarcinoma; circRNA, circular RNA; EMT, epithelial to mesenchymal transition; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; IL8, interleukin 8; ISH, *in situ* hybridization; LCM, laser capture microdissection; lncRNA, long non coding RNA; miRNA, miR, micro RNA; Q-RT-PCR, quantitative reverse-transcription polymerase chain reaction; RNase, ribonuclease; SMAD, SMAD family member; SNAIL1, snail family transcriptional repressor 1; TGF β , transforming growth factor beta; TLINC, transforming growth factor beta-induced long noncoding RNA; TLINC-L, long TLINC isoform; TLINC-S, short TLINC isoform.

Received November 1, 2017; accepted December 8, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1142/full.

Supported by INSERM, University of Rennes 1, Institut National du Cancer (INCa, Cancéropôles Ile-de-France and Grand-Ouest), Ligue contre le cancer (cd35, cd44, cd49), Novartis Oncology, and Association Française pour l'Etude du Foie, France (to L.S., C.C.); and by Deutsche Forschungsgemeinschaft DFG DO373/13-1 (to S.D.).

resectable early stage iCCAs, 5-year survival is about 40%. However, for unresectable advanced iCCA, the median survival is less than 15 months.⁽²⁾ Although significant progress in understanding the molecular basis of iCCA pathogenesis has been achieved by high throughput strategies, including the identification of genetic, epigenetic, and genomic alterations (e.g., KRAS proto-oncogene guanosine triphosphatase [KRAS], isocitrate dehydrogenase 1/2 [IDH1/2] mutations, fibroblast growth factor receptor 2 [FGFR2] fusions), there is no approved molecular targeted therapy that significantly improves patient survival in iCCA.^(1,3) Thus, iCCA remains characterized by limited therapeutic options and very poor prognosis.^(1,3) One histologic feature of iCCA is the presence of a prominent desmoplastic stroma that is known to contribute to tumor onset and progression.^(4,5) While previous studies largely focused on identifying molecular alterations in tumor cells, we recently characterized genomic alterations that occur in the tumor microenvironment of iCCA.⁽⁶⁾ Thus, by combining laser microdissection and gene expression profiling, 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 represent a potential therapeutic target in iCCA.⁽⁶⁾

TGF β is a pleiotropic cytokine that controls fundamental cellular processes associated with carcinogenesis (e.g., proliferation, apoptosis, invasion, angiogenesis, immunity).⁽⁷⁾ However, the actions of TGF β in cancer are complex because TGF β exhibits either tumor-suppressive or tumor-promoting properties, depending

on the tumor stage. At an early stage, TGF β exerts potent antiproliferative properties on precancerous epithelial cells, notably by inducing genes with cytostatic actions (e.g., cyclin-dependent kinase inhibitor 1A [CDKN1A]). At an advanced stage, cells become resistant to the cytostatic effects of TGF β , which then promotes tumor growth and metastatic progression, particularly as a potent inducer of epithelial–mesenchymal transition (EMT).^(7,8) To date, the molecular mechanisms switching the TGF β actions from tumor suppression toward tumor promotion are not fully understood and may depend on the cellular context.^(9,10) Gene mutations in the canonical TGF β signaling (e.g., SMAD family member 4 [SMAD4]) and crosstalk with proliferative pathways have been proposed to contribute to the loss of the tumor suppressor arm of TGF β in several cancers.⁽⁹⁾ In addition, recent evidence demonstrated that long noncoding RNA (lncRNA) could mediate the prometastatic properties of TGF β .^(11,12) LncRNAs form a large and diverse class of long RNA molecules (>200 nucleotides) that do not encode proteins. LncRNAs emerged as key regulators involved in human carcinogenesis, including liver cancers.^(13,14) Here, we characterized the transcriptional response of iCCA cell lines to TGF β and identified novel TGF β -regulated genes, including a lncRNA that we named TGF β -induced lncRNA (TLINC, formerly known as LINC00340 and cancer susceptibility candidate 15 [CASC15]). TLINC is induced by TGF β in several settings, and the expression of specific isoforms correlates with an EMT phenotype. TLINC is strongly up-regulated in human

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DOI 10.1002/hep4.1142

Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

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iCCA and is associated with an enhanced expression of proinflammatory cytokines, including interleukin 8 (*IL8*). Finally, circular isoforms of *TLINC* are highlighted as promising biomarkers in patients with iCCA.

Materials and Methods

CELL CULTURE AND REAGENTS

HuCCT1 (RCB-1960) and Huh28 (RCB-1943) CCA cell lines were purchased at the RIKEN BioResource Center (Tsukuba-shi, Japan). Cells were grown in Roswell Park Memorial Institute 1640 medium (HuCCT1) or minimal essential medium (Huh28) supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum. HCC cell lines were purchased from ATCC and cultured as described.⁽¹⁵⁾ LX2 cells were grown as described.⁽¹⁶⁾ Freshly isolated human hepatocytes were purchased from Biopredic International (St. Grégoire, France). Cells were cultured at 37 °C in a 5% CO₂ atmosphere, and overnight serum starvation was performed before each treatment. Unless otherwise specified, cells were treated with 1 ng/mL recombinant human TGF β (R&D Systems, Minneapolis, MN) and 10 μ M TGF β inhibitors (Sigma-Aldrich, St. Louis, MO).

TGF β SENSOR CELL LINES AND LUCIFERASE ACTIVITY

Stable TGF β sensor cell lines were generated by using a pCignal Lenti-SMAD Reporter assay (Qiagen, Courtabeuf, France) according to the manufacturer's instructions. Briefly, cells were transduced with lentiviral particles (multiplicity of infection, 10; 8 μ g/mL SureENTRY transduction reagent) and selected with 1 μ g/mL puromycin for 5 days. Luciferase activity was determined with the Firefly Luciferase Assay System (Promega, Madison, WI) and normalized by the number of viable cells evaluated by a PrestoBlue cell viability reagent (Invitrogen, Carlsbad, CA).

DNA TRANSFECTION

DNA constructs were kindly provided by Dr. Kristina A. Cole (Children's Hospital of Philadelphia, Philadelphia, PA) and Dr. Sumio Sugano (University of Tokyo, Tokyo, Japan). All constructs were amplified by transformation of One Shot TOP10 *Escherichia coli* (Invitrogen) and validated by DNA sequencing

(Supporting Table S1) after DNA purification (Nucleobond Xtra plasmid maxiprep kit; Macherey Nagel, Hoerd, France). DNA transfection was performed by using Lipofectamine 2000 (Invitrogen).

PROTEIN ANALYSIS

A Quantikine enzyme-linked immunosorbent assay human TGF β 1 immunoassay (R&D Systems) was used to evaluate the expression of TGF β in the supernatant of cell cultures collected 24 hours after serum starvation. Protein analysis by immunoblotting was performed as described.⁽¹⁷⁾ Human anti-E-cadherin (CDH1) (AF648; R&D Systems) and anti-vimentin (VIM) (B01P; Abnova) antibodies were used.

GENE EXPRESSION PROFILING

Total RNA, including lncRNA and micro RNA (miRNA, miR), was purified with an 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) as described.⁽¹⁵⁾ These microarrays include 26,083 Entrez genes and 30,606 lncRNAs. Gene expression data were processed using Feature Extraction and GeneSpring software (Agilent Technologies). Microarray data sets have been deposited into the public Gene Expression Omnibus database under accession numbers GSE102109 and GSE102110. 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.

QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) was performed as described⁽¹⁶⁾ by using a SYBR Green master mix (Applied Biosystems, Carlsbad, CA). A mixture of deoxythymidine oligomer (250 ng) and random hexamers (100 ng) was used to prime the RT reaction of 1 μ g total RNA (Superscript III RT; Invitrogen). A list of DNA primers used in this study is provided in Supporting Table S2.

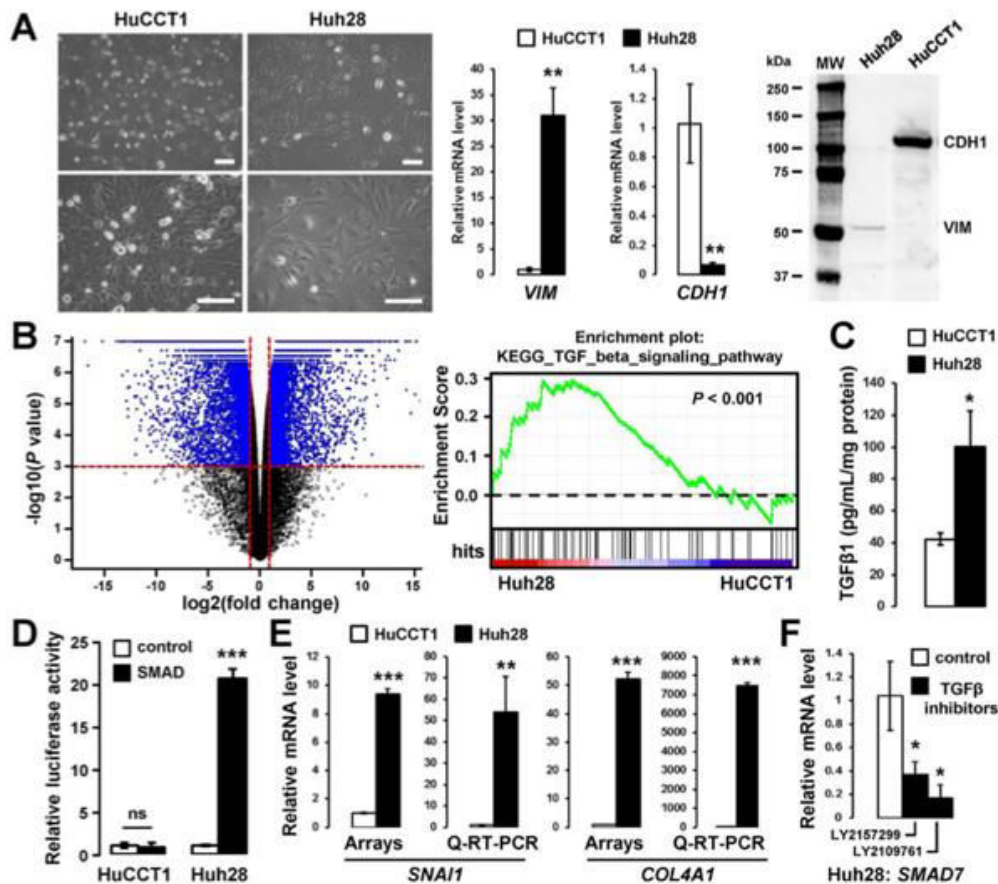


FIG. 1. HuCCT1 and Huh28 cell lines exhibit an epithelioid and a mesenchymal-like phenotype respectively, the later correlating with endogenous activation of the TGFβ pathway. (A) Phase contrast micrographs of HuCCT1 and Huh28 cells (left panel; scale bar, 100 μm) and expression of vimentin and E-cadherin at the RNA (middle panel) and protein level (right panel). (B) Volcano plot of genes differentially expressed between HuCCT1 and Huh28 (left panel) and GSEA highlighting a TGFβ signature enriched in the gene expression profile of Huh28. (C) Enzyme-linked immunosorbent assay demonstrating increased secretion of TGFβ1 in the supernatant of Huh28 cells 24 hours after overnight serum starvation. (D) Relative luciferase activity of HuCCT1 and Huh28 engineered cell lines in which a luciferase gene is under the transcriptional control of a TATA box alone (white bars) or with SMAD responsive elements (black bars). Luciferase activity was measured at the basal level without exogenous addition of TGFβ after overnight serum starvation. (E) Gene expression analysis of TGFβ target genes in HuCCT1 and Huh28 cells at the basal level by microarray and Q-RT-PCR. (F) Q-RT-PCR analysis of TGFβ target gene *SMAD7* in Huh28 cells treated with 10 μM TGFβ inhibitors (black bars). Statistical analysis was performed by *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$); $n \geq 3$ replicates. Data represent mean \pm SD. Abbreviations: mRNA, messenger RNA; MW, molecular weight.

FUNCTIONAL TESTS

Cell viability was evaluated using a PrestoBlue reagent (Invitrogen). Data were expressed as relative cell viability compared to untreated cells transfected with the control plasmid set as 1. Cell migration and invasion were evaluated using BD BioCoat Matrigel Invasion Chambers (BD Biosciences) as described.⁽¹⁶⁾ Briefly, 4×10^4 cells were plated in serum-free medium either on an 8-μm pore-size positron emission tomography membrane insert coated with a layer

of matrigel basement membrane matrix (invasion test) or on an 8-μm pore-size positron emission tomography control membrane insert (migration test). The lower compartment contained medium with 10% fetal bovine serum as a chemoattractant. After incubation for 48 hours, we removed the cells remaining on the upper surface of the membrane with a cotton swab, fixed cells in 4% paraformaldehyde that had passed through to the lower surface of the membrane, and stained the fixed cells with 1% crystal violet. We scored invasive and migrating cells per membrane under a

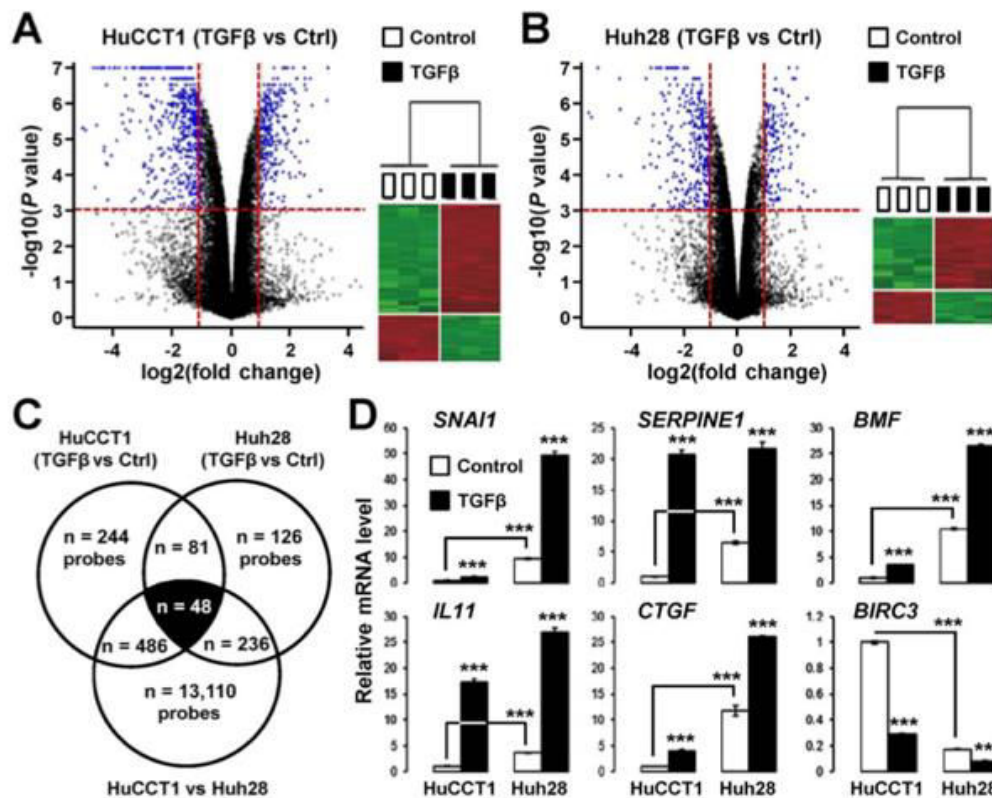


FIG. 2. A TGFβ-specific gene expression signature in cholangiocarcinoma cell lines. (A,B) Volcano plot and hierarchical clustering of genes differentially expressed by TGFβ (1 ng/mL, 16 hours) in (A) HuCCT1 and (B) Huh28 cells. Horizontal dashed lines on volcano plots, *P* < 0.001; vertical dashed lines, fold change > 2. (C) Venn diagram analysis of genes differentially expressed by TGFβ in HuCCT1 and Huh28 cells. A common TGFβ signature made of 48 probes (42 nonredundant well-annotated genes) was identified. (D) Expression of six well-known TGFβ target genes in HuCCT1 and Huh28 cells after 16 hours of culture in absence (white bars) or presence (black bars) of 1 ng/mL TGFβ. Statistical analysis was performed by *t* test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001); n ≥ 3 replicates. Data represent mean ± SD. Abbreviation: Ctrl, control.

light microscope at a 5× magnification (n = 3 independent experiments). The number of migrating and invasive cells after transfection of TLINC was normalized relative to the number of migrating and invasive cells after transfection of the control plasmid, which was set to 1.

HUMAN iCCA SAMPLES AND TISSUE MICROARRAY

Freshly frozen and formalin-fixed paraffin-embedded human iCCA samples were obtained through the national liver biobanks network. Written informed consent was obtained from all patients. The study protocol fulfilled national laws and regulations and was approved by the local ethics committee and the institutional review board of the Institut National de la Santé

et de la Recherche Médicale (IRB00003888). Tissue microarrays were performed using a Minicore 3 Tissue Arrayer (Excilone, Vicq, France) as described.⁽⁶⁾

LASER CAPTURE MICRODISSECTION

Laser capture microdissection (LCM) was performed using an Arcturus Veritas Microdissection system (Applied Biosystems) as described.⁽⁶⁾ Briefly, serial sections of 10-μm frozen tissues were mounted onto PEN membrane glass slides prior to LCM of biliary epithelial cells and fibrous tissue from tumor and nontumor parts. Total RNA was purified using an Arcturus Picopure RNA isolation kit (Applied Biosystems).

TABLE 1. TGFβ SIGNATURE IN CHOLANGIOCARCINOMA CELL LINES

Agilent Probe	Gene Name	Gene Symbol	Accession No.	HuCTT1 (TGFβ/Ctr)		Huh28 (TGFβ/Ctr)		Huh28/HuCTT1		Known TGFβ Target
				P Value	FC	P Value	FC	P Value	FC	
A_24_P411561	Hepatitis A virus cellular receptor 2	HAVCR2	NM_032782	1.00E-07	38.46	5.00E-07	7.14	1.40E-06	11.25	yes
A_24_P158089	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	NM_000602	< 1e-07	20.83	7.00E-07	3.33	1.00E-07	6.63	yes
A_21_P0011334	na	XLOC_I2_004757	BC043257	4.50E-05	18.52	< 1e-07	5.88	8.21E-04	5.20	no
A_33_P3243887	Interleukin 11	IL11	NM_000641	< 1e-07	17.24	< 1e-07	7.14	1.40E-06	3.54	yes
A_21_P0001676	na	XLOC_000166	na	< 1e-07	15.15	5.10E-06	2.08	< 1e-07	300.67	no
A_23_P153571	IGF-like family member 2	IGFL2	NM_01002915	3.40E-06	12.82	5.35E-05	2.27	4.69E-05	5.42	no
A_33_P3243454	IGF-like family member 3	IGFL3	NM_207393	5.30E-06	11.76	1.00E-07	3.70	4.00E-07	57.37	no
A_33_P3255384	BPI Fold Containing Family C	BPFC	NM_174932	1.00E-07	10.20	7.56E-05	2.78	2.00E-07	12.20	no
A_32_P185637	Collagen, type XX, alpha 1	COL20A1	NM_020882	1.50E-06	10.10	< 1e-07	6.25	3.10E-06	8.09	no
A_23_P110777	Leukocyte cell-derived chemotaxin 2	LECT2	NM_002302	2.57E-04	8.33	1.23E-05	7.69	2.30E-06	7.21	no
A_33_P3345534	Keratin 14	KRT14	NM_000526	1.00E-07	7.69	9.30E-06	2.70	1.00E-07	10.82	no
A_23_P96158	Keratin 17	KRT17	NM_000422	< 1e-07	7.69	8.70E-06	2.63	< 1e-07	9.50	no
A_24_P887857	na	na	na	< 1e-07	7.69	1.65E-05	2.70	1.00E-07	9.67	no
A_23_P205074	Solute carrier family 46, member 3	SLOC46A3	NM_181785	3.15E-04	7.69	1.00E-06	2.33	3.55E-04	7.54	no
A_24_P882732	na	na	DR007925	< 1e-07	7.14	2.28E-05	2.70	1.00E-07	9.50	no
A_21_P0011578	Keratin 42 pseudogene	XLOC_I2_006021	DB117598	3.00E-07	7.14	3.00E-05	2.70	4.00E-07	8.77	no
A_33_P3857239	na	KRT42P	NR_033415	< 1e-07	6.67	9.70E-06	2.86	1.00E-07	8.69	no
A_33_P3235905	na	na	na	6.20E-05	6.67	6.82E-05	2.44	3.22E-04	3.90	no
A_21_P0008663	Keratin 16 pseudogene 2	XLOC_011331	na	2.90E-06	6.67	< 1e-07	5.56	3.00E-07	19.21	no
A_32_P62963	Long intergenic nonprotein coding RNA 313	KRT16P2	NR_029392	1.00E-07	5.88	2.19E-05	2.70	4.00E-07	5.88	no
A_23_P381489	Long intergenic nonprotein coding RNA 312	LINC00313	NR_026863	4.10E-06	5.88	8.91E-05	2.08	2.19E-04	2.29	no
A_23_P166779	Long intergenic nonprotein coding RNA 312	LINC00312	NR_024065	3.06E-05	4.17	1.00E-07	5.00	1.36E-04	2.86	no
A_23_P19663	Connective tissue growth factor	CTGF	NM_001901	4.00E-07	4.00	1.91E-05	2.22	1.00E-07	11.67	yes
A_19_P00321671	na	na	na	1.56E-04	4.00	3.60E-06	3.03	5.06E-05	5.57	no
A_21_P0012601	na	XLOC_I2_010751	na	1.20E-06	4.00	6.60E-06	3.03	5.00E-07	5.23	no
A_32_P76627	na	na	CR597597	1.00E-07	3.70	2.60E-06	2.78	3.00E-07	4.00	no
A_23_P379649	Bcl2 modifying factor	BMF	NM_001003940	1.65E-05	3.57	3.00E-07	2.56	8.00E-07	10.75	yes
A_21_P0009087	na	XLOC_012049	na	1.72E-04	3.45	1.06E-05	3.03	2.81E-05	5.86	no
A_19_P00318323	Long intergenic nonprotein coding RNA 340	LINC00340	NR_015410	2.06E-05	3.33	8.60E-06	2.27	1.00E-07	19.17	no
A_23_P107744	Sphingosine-1-phosphate receptor 5	S1PR5	NM_030760	6.04E-04	3.03	1.50E-06	3.57	1.35E-05	12.24	no
A_32_P309404	Solute carrier family 22 (extraneuronal monoamine transporter), member 3	SLOC22A3	NM_021977	5.00E-07	3.03	1.78E-05	2.04	1.60E-06	3.48	no
A_24_P838448	Long intergenic nonprotein coding RNA 340	LINC00340	NR_015410	1.04E-04	2.86	1.30E-06	2.44	2.00E-07	12.50	no

(A) Genes induced by TGFβ

TABLE 1. CONTINUED

(A) Genes induced by TGFβ										
Agilent Probe	Gene Name	Gene Symbol	Accession No.	HuCTT1 (TGFβ/Ctrl)		Huh28 (TGFβ/Ctrl)		Huh28/HuCTT1		Known TGFβ Target
				P Value	FC	P Value	FC	P Value	FC	
A_23_P124642	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	RASGRP1	NM_005739	1.40E-05	2.70	1.32E-05	2.08	< 1e-07	27.00	no
A_23_P344531	Synaptopodin	SYNPO	NM_007286	7.10E-06	2.50	1.60E-06	2.56	< 1e-07	24.38	no
A_23_P303548	Nuclear protein 4-like	NOL4L	XM_003403733	2.34E-04	2.44	5.14E-05	2.08	4.81E-04	2.31	no
A_24_P239606	Growth arrest and DNA-damage-inducible, beta	GADD45B	NM_015675	3.00E-06	2.33	6.10E-05	2.44	3.69E-05	2.93	yes
A_23_P131846	Snail homolog 1 (<i>Drosophila</i>)	SNAI1	NM_005985	4.20E-06	2.33	1.00E-07	5.26	< 1e-07	9.48	yes
A_21_P0009196	na	XLOC_012515	na	6.91E-04	2.27	2.06E-05	2.08	8.00E-06	7.67	no
(B) Genes repressed by TGFβ										
Agilent Probe	Gene Name	Gene Symbol	Accession No.	HuCTT1 (TGFβ/Ctrl)		Huh28 (TGFβ/Ctrl)		Huh28 / HuCTT1		Known TGFβ Target
				P Value	FC	P Value	FC	P Value	FC	
A_33_P3663974	Thyroid hormone receptor beta	THRB	NM_001128177	8.10E-06	0.49	1.75E-04	0.47	1.70E-06	0.42	no
A_21_P0010449	na	XLOC_014399	XR_109732	2.70E-06	0.47	1.05E-04	0.38	1.00E-07	0.08	no
A_21_P0000841	Hypothetical LOC100505483	LOC100505483	NR_038926	7.60E-06	0.45	3.13E-05	0.40	2.25E-05	0.43	no
A_23_P121064	Penitoxin 3, long	PTX3	NM_002852	1.50E-06	0.45	2.00E-06	0.32	4.90E-06	0.42	yes
A_23_P121657	Heparan sulfate (glucosamine)	HS3ST1	NM_005114	3.00E-07	0.42	3.07E-04	0.45	< 1e-07	0.00	no
A_23_P52266	3-O-sulfotransferase 1 protein with tetratricopeptide repeats 1	IFT1	NM_001548	1.19E-04	0.40	1.66E-05	0.36	1.72E-05	0.27	no
A_23_P345460	Pleckstrin homology domain-containing, family G (with RhoGef domain)	PLEKHG4	NM_015432	6.00E-07	0.36	2.90E-04	0.50	1.00E-07	0.21	no
A_23_P98350	Baculoviral inhibitor of apoptosis protein repeat-containing 3	BIRC3	NM_001165	1.00E-07	0.29	2.70E-06	0.45	< 1e-07	0.17	yes
A_33_P3398181	Hypothetical LOC100127909	LOC100127909	XR_111781	8.81E-05	0.26	7.13E-05	0.35	1.89E-05	0.27	no
A_23_P415021	Methyltransferase-like 7A	METTL7A	NM_014033	2.00E-07	0.22	4.00E-06	0.32	1.00E-07	0.19	no

Abbreviations: Ctrl, control; FC, fold change; na, not applicable.

IN SITU HYBRIDIZATION

Specific *in situ* hybridization (ISH) riboprobes were designed to evaluate the expression of TLINC-S and TLINC-T transcripts (Supporting Table S1). Sense and antisense riboprobes were generated by *in vitro* transcription from PCR products incorporating the promoter of T7 RNA polymerase. *In vitro* transcription was performed with 40 units T7 RNA Polymerase (Agilent) in the presence of 0.35 mM digoxigenin-11-UTP (Roche Diagnostics, Meylan, France). ISH was performed on the Discovery Automated IHC Stainer using the Ventana Detection Kit (Ventana Medical Systems, Tucson, AZ). Staining was performed using an anti-digoxigenin-horseradish peroxidase antibody (Roche Diagnostics), and signal detection was performed using a nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate or a Discovery purple kit (Ventana Medical Systems).

CIRCULAR RNA ANALYSIS

RT-PCR using convergent and divergent primers combined with ribonuclease (RNase) R (Epicentre, Middleton, WI) treatment (1 μ /g, 37 °C, 1 hour) was used to detect linear and circular transcripts of *TLINC* and actin beta (*ACTB*). The sequence of DNA primers is provided in Supporting Table S2.

Results

ENDOGENOUS TGF β ACTIVATION IN Huh28 CELLS

HuCCT1 and Huh28 cells were used to study the impact of TGF β on iCCA cells. The two cell lines have different phenotypic features that reflect tumor heterogeneity. HuCCT1 cells exhibit an epithelioid-like phenotype associated with a cobblestone-like morphology and numerous cell-to-cell junctions compared to Huh28 cells, which exhibit a mesenchymal-like phenotype associated with a spindle and polygonal morphology together with prominent cytoskeleton fibers (Fig. 1A). Epithelioid and mesenchymal-like phenotypes were further characterized by the expression of *CDH1* and *VIM* markers in HuCCT1 cells and Huh28 cells, respectively, both at the RNA and protein level (Fig. 1A). Marked differences in pangenomic expression profiles were also highlighted between HuCCT1 and Huh28 cells and validated by Q-RT-PCR (Fig. 1B; Supporting Fig. S1A).

Interestingly, GSEA, using the Kyoto Encyclopedia of Genes and Genomes signaling pathways, demonstrated that a TGF β signature was significantly enriched ($P < 0.001$) at the basal level in mesenchymal Huh28 cells (Fig. 1B). Endogenous activation of the TGF β pathway in Huh28 cells was further validated by a significantly enhanced secretion of TGF β (Fig. 1C) that correlated with enhanced luciferase activity as detected in engineered SMAD sensor cell lines (Fig. 1D). A higher expression of TGF β target genes (e.g., snail family transcriptional repressor 1 [*SNAIL1*], collagen type IV alpha 1 chain [*COL4A1*], *SMAD7*) in Huh28 cells was also validated by Q-RT-PCR (Fig. 1E; Supporting Fig. S1A) as well as a reduced expression of *SMAD7*, a well-known TGF β transcriptional target, when cells were cultured in the presence of TGF β inhibitors (Fig. 1F). Altogether, these data demonstrate that Huh28 cells exhibit a mesenchymal phenotype associated with basal and endogenous activation of the TGF β pathway.

NEW TGF β TARGET GENES IN iCCA CELLS

HuCCT1 and Huh28 cells were treated with 1 ng/mL TGF β for 16 hours. Gene expression profiling using pangenomic microarrays identified 859 probes in HuCCT1 cells (Fig. 2A) and 491 probes in Huh28 cells (Fig. 2B) that were significantly deregulated by TGF β ($P < 0.001$; fold change > 2). A Venn diagram analysis, including genes differentially expressed between HuCCT1 and Huh28 cells at a basal level (Fig. 1B), identified a set of 48 probes (42 nonredundant annotated genes) that were recurrently deregulated by TGF β in all three experimental settings (Fig. 2C; Table 1). The deregulation of well-known positive (e.g., serpin family E member 1 [*SERPINE1*], connective tissue growth factor [*CTGF*]) and negative (e.g., baculoviral inhibitor of apoptosis protein repeat containing 3 [*BIRC3*]) TGF β target genes was confirmed by Q-RT-PCR on independent RNA samples (Fig. 2D; Supporting Fig. S1B). GSEA was also performed in all specific gene subsets and demonstrated that the 42 common genes were significantly associated with TGF β and SMAD2/3 and related to EMT (Supporting Fig. S2). Importantly, this common signature comprised genes not known to be regulated by TGF β , including several lncRNAs (e.g., *LINC00312*, *LINC00340*). Notably, two microarray probes for *LINC00340* were induced by TGF β (Table 1).

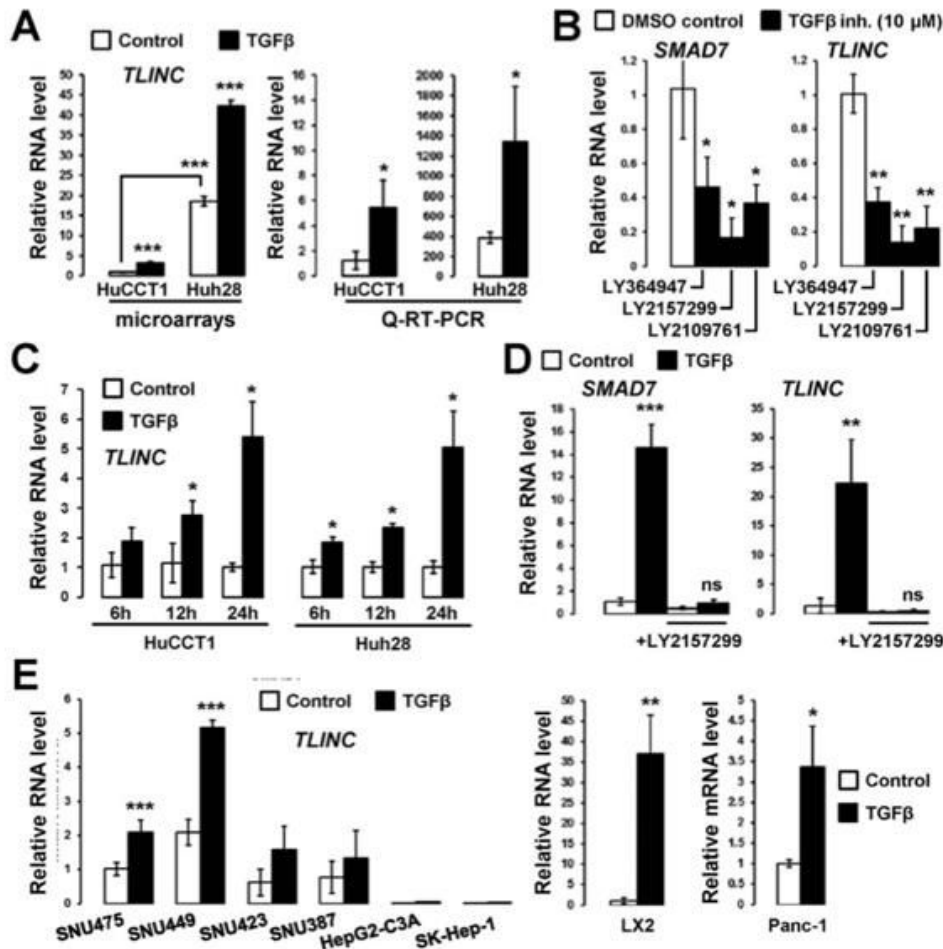


FIG. 3. LINC00340/CASC15/TLINC is a novel TGFβ-induced lncRNA. (A) Analysis of *LINC00340* expression in HuCCT1 and Huh28 cells after 16 hours of culture in absence (white bars) or presence (black bars) of 1 ng/mL TGFβ, as evaluated by microarray and Q-RT-PCR. (B) Analysis of *SMAD7* and *LINC00340* expression in Huh28 cells after 72 hours treatment with 10 μM TGFβ inhibitors. (C) Time-course analysis of *LINC00340* expression in HuCCT1 and Huh28 cells (1 ng/mL TGFβ). (D) Analysis of *SMAD7* and *LINC00340* expression in primary human hepatocytes (n = 3 independent donors) in the presence of 1 ng/mL TGFβ and 10 μM TGFβ inhibitor LY2157299 for 16 hours. (E) Analysis of *LINC00340* expression in HCC cell lines (left panel) and LX2 and Panc-1 (pancreatic cancer) cells (right two panels) (1 ng/mL TGFβ for 16 hours). Statistical analysis was performed by *t* test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001); n ≥ 3 replicates. Data represent mean ± SD. Abbreviations: DMSO, dimethyl sulfoxide; inh., inhibitor; mRNA, messenger RNA; ns, not significant.

LINC00340/CASC15/TLINC IS A NOVEL TGFβ-INDUCED lncRNA

LINC00340 is known as cancer susceptibility candidate 15 (*CASC15*) located on chromosome 6p22. *CASC15* was reported to act as a tumor suppressor in neuroblastoma⁽¹⁸⁾ and to promote tumor progression and phenotype switching in melanoma.⁽¹⁹⁾ To further characterize *LINC00340* biological activities in iCCA, we first validated the microarray expression data by Q-RT-PCR. *LINC00340* was induced by TGFβ in

both HuCCT1 and Huh28 cells, and a significant higher expression in Huh28 cells at the basal level was confirmed (Fig. 3A). Repression of *LINC00340* and *SMAD7* was observed in Huh28 cells treated with inhibitors of the TGFβ pathway (Fig. 3B). Time-course experiments also demonstrated increased expression of *LINC00340* by TGFβ, both in HuCCT1 and Huh28 cells (Fig. 3C). Interestingly, *LINC00340* was induced by TGFβ in nontransformed human primary hepatocytes, and the induction was abolished in the presence of a TGFβ inhibitor

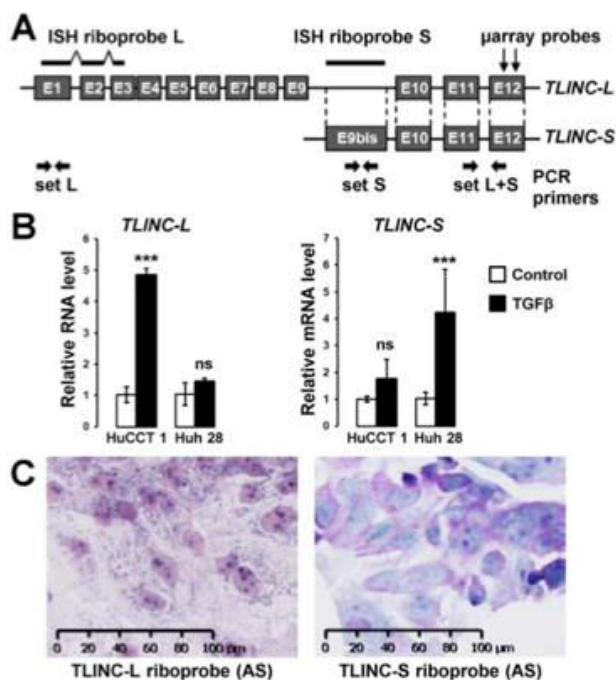


FIG. 4. Expression and cellular localization of TLINC isoforms. (A) Simplified representation of TLINC locus highlighting two reported isoforms: TLINC-L and TLINC-S. ISH riboprobes, sets of PCR primers, and microarray probes are also represented. (B) Analysis of *TLINC-L* and *TLINC-S* expression in HuCCT1 and Huh28 cells after 16 hours of culture in the absence (white bars) or presence (black bars) of 1 ng/mL TGF β , as evaluated by Q-RT-PCR using specific sets of primers. (C) *In situ* hybridization using TLINC-L and TLINC-S riboprobes highlights a nuclear and cytoplasmic location of both TLINC isoforms (HuCCT1 cells treated with 1 ng/mL TGF β). Scale, 100 μ m. Statistical analysis was performed by *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$); $n \geq 3$ replicates. Data represent mean \pm SD. Abbreviations: (AS), antisense; mRNA, messenger RNA; ns, not significant.

(Fig. 3D). In addition, *LINC00340* was induced by TGF β in several human HCC cell lines as well as in LX2-activated hepatic stellate cells (Fig. 3E). Finally, induction by TGF β occurred also in nonhepatic cell lines, such as pancreatic Panc-1 (Fig. 3E). Altogether, these data demonstrate that *LINC00340* is a novel and general transcriptional target of TGF β . We therefore named it TGF β -induced lncRNA or *TLINC*.

INDUCTION OF TWO TLINC ISOFORMS LINKED TO AN EMT PHENOTYPE

Previous studies demonstrated that *TLINC* is located on a complex locus that produces multiple

transcripts by alternative splicing, including a short (TLINC-S) and a long (TLINC-L) isoform, as shown in Fig. 4A.^(18,19) These two isoforms have been functionally associated with either tumor suppressive (TLINC-S)⁽¹⁸⁾ or tumor promoting (TLINC-L)⁽¹⁹⁾ functions in neuroblastoma and melanoma, respectively. Because our previous gene expression analysis by microarray (Fig. 2) and Q-RT-PCR (Fig. 3) could not distinguish the two isoforms (primer set L + S), we next evaluated TLINC-S and TLINC-L with specific sets of primers (Fig. 4A). TLINC-L was induced by TGF β in HuCCT1 cells and TLINC-S in Huh28 cells, as demonstrated by Q-RT-PCR (Fig. 4B). ISH demonstrated that the two isoforms were expressed in both the cytoplasm and the nucleus (Fig. 4C).

FUNCTIONAL CHARACTERIZATION OF TLINC ISOFORMS

Gain of function experiments were performed using DNA constructs designed to specifically overexpress TLINC-L and TLINC-S (Supporting Fig. S3A-C). No significant effect was observed on cell viability (Supporting Fig. S3D) and cell invasion (Fig. 5A); however, overexpression of TLINC-L increased cell migration (Fig. 5A). In order to get insights into the molecular mechanisms involved, gene expression profiling was performed on HuCCT1 cells after TLINC-L overexpression. Numerous genes were repressed (Fig. 5B), whereas several inflammation-associated genes, including cytokines and chemokines (e.g., *IL6*, *IL8*, C-X-C motif chemokine ligand 1 [*CXCL1*]) were induced together with *LINC00340/TLINC* (Fig. 5B). Accordingly, an immune response gene signature was significantly enriched in the gene expression profiles of HuCCT1 cells overexpressing TLINC-L (Fig. 5C; Supporting Table S3). The enrichment of a described⁽²⁰⁾ cell migration signature supported the phenotype observed after TLINC-L overexpression (Fig. 5C). Although no obvious change was observed on cell morphology after TLINC-L transfection, EMT-associated *SNAI2* was enriched in the gene expression profiles of TLINC-L transfected cells (Supporting Table S4). A significant enrichment of TGF β target genes was also observed, suggesting that TLINC-L may act as a feedback regulator of the TGF β pathway (Fig. 5C). However, experimental data using SMAD sensor cell lines refuted this hypothesis (Supporting Fig. S3E). In agreement

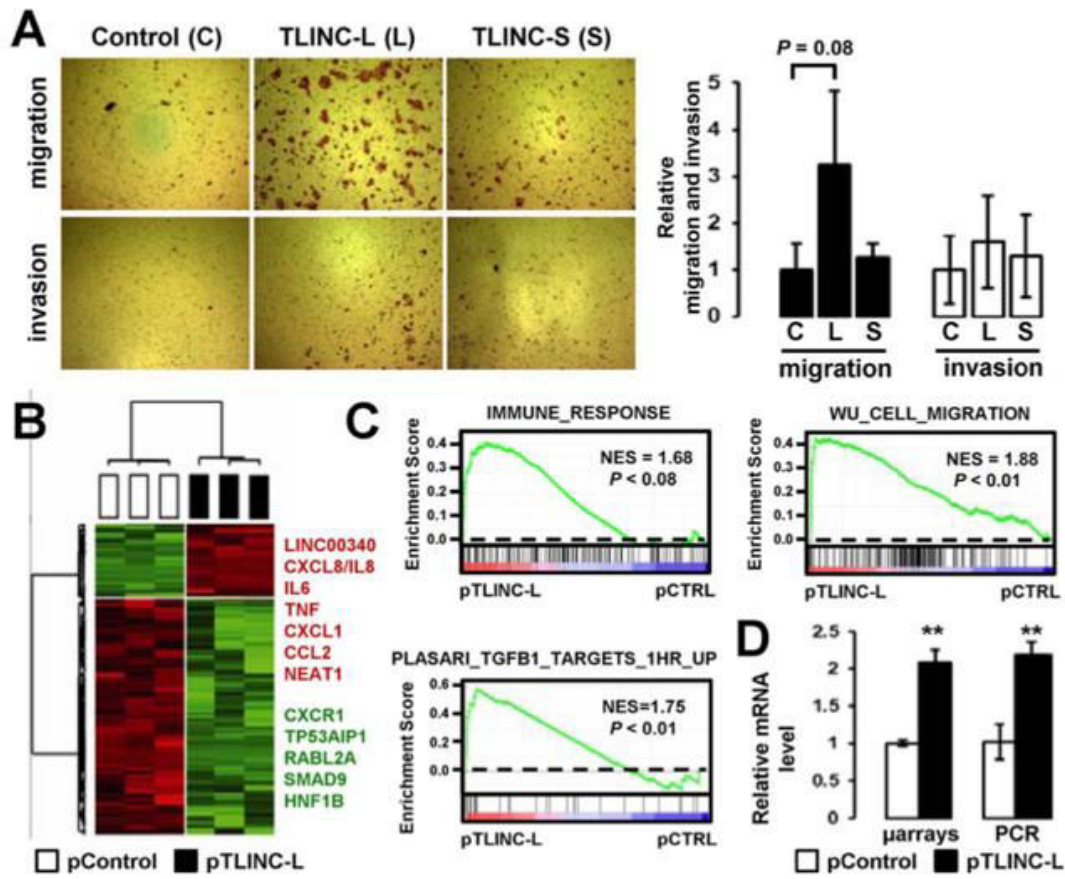


FIG. 5. Functional analysis of TLINC. (A) Effects of TLINC-L and TLINC-S overexpression on migration and invasion of HuCCT1 cells 48 hours after transfection of the DNA constructs. (B) Gene expression analysis of HuCCT1 cells 24 hours after transfection of the *TLINC-L* DNA construct. Hierarchical clustering was based on the expression of 431 genes ($P < 0.01$; fold change > 1.5). (C) GSEA of specific signatures significantly enriched in the gene expression profile of HuCCT1 cells overexpressing TLINC-L. (D) Analysis of *IL8* expression in HuCCT1 cells after transfection of the *TLINC-L* DNA construct, as evaluated by microarray and Q-RT-PCR. Statistical analysis was performed by *t* test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$); $n \geq 3$ replicates. Data represent mean \pm SD. Abbreviations: C, control; CCL2, chemokine (C-C motif) ligand 2; CTRL, control; CXCR1, chemokine (C-X-C motif) receptor 1; HNF, hepatocyte nuclear factor; L, long; mRNA, messenger RNA; NES, normalized enrichment score; p, plasmid [for pControl and pTLINC-L]; RABL2A, RAB, member of RAS oncogene family like 2A; S, short; TP53AIP1, tumor protein p53 regulated apoptosis inducing protein 1; TNF, tumor necrosis factor.

with the identified immune signature, increased expression of *IL8* was confirmed after TLINC overexpression (Fig. 5D). As a potent inducer of cell motility, *IL8* may contribute to the observed phenotype by creating a gradient for the migration of cancer cells.

INCREASED EXPRESSION OF TLINC IN HUMAN iCCA

We next evaluated the clinical relevance of TLINC expression in human resected iCCA. TLINC was shown to be induced both in tumor biliary epithelial

cells and in the stroma compared to their nontumor counterparts (Fig. 6A). Overexpression of TLINC in iCCA tumors was validated by ISH on tissue microarrays (Fig. 6A). Interestingly, Q-RT-PCR on 22 resected human iCCA demonstrated that the expression of TLINC-L and TLINC-S was tightly correlated (Fig. 6B). A detailed analysis by ISH on human resected iCCA confirmed that both TLINC isoforms were expressed in tumor cholangiocytes and in cells from the tumor microenvironment. In the nontumor liver, TLINC was only expressed in remodeled portal areas with signs of ductular reaction and inflammation (Fig. 6C; Supporting Fig. S4).

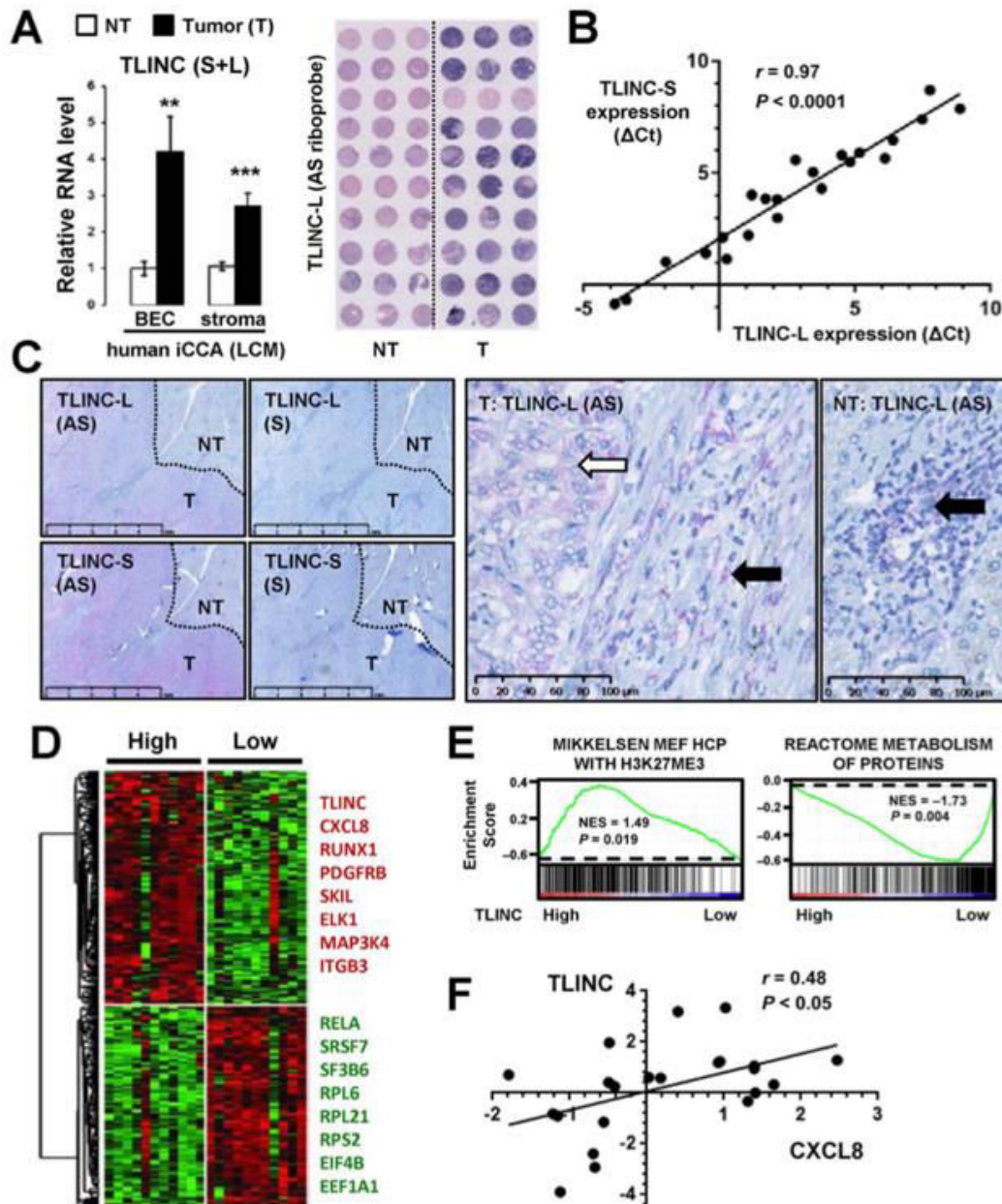


FIG. 6. Clinical relevance of *TLINC* expression in human resected iCCA. (A) Analysis of *TLINC* expression in biliary epithelial cells and stroma isolated by laser capture microdissection from nontumor (white bars) and tumor (black bars) tissues ($n = 10$ patients, left panel). Data represent mean \pm SD. ISH on human iCCA tissue microarrays using *TLINC-L* antisense riboprobes demonstrating higher expression in the tumor than in the nontumoral tissues (tissue microarray, right panel). (B) Correlation between *TLINC-L* and *TLINC-S* expression in human iCCA resected tumors, as evaluated by Q-RT-PCR ($n = 22$ patients). (C) ISH analysis of *TLINC-L* and *TLINC-S* expression using specific antisense and sense riboprobes. In the tumor part, *TLINC* was expressed in both epithelial cells (white arrow) and in the stroma (black arrow). In the nontumor part, *TLINC* was only expressed in remodeled portal areas with signs of ductular reaction. Scale bars: 5 mm (left panels) and 100 μ m (right panels). (D) Expression of *TLINC* identifies human iCCA with specific gene expression profiles. Median expression of *TLINC* was used to define high and low expression groups ($n = 22$ patients). (E) Example of signatures significantly enriched in the gene expression profiles of iCCA with high versus low *TLINC* expression, as evaluated by GSEA. (F) Correlation between the expression of *TLINC* and *CXCL8* in 22 human iCCAs. Abbreviations: (AS), antisense; BEC, biliary epithelial cells; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; EIF4B, eukaryotic translation initiation factor 4B; ELK1, ELK1, ETS transcription factor; ITGB3, integrin subunit beta 3; HCP, high-CpG-density promoters; MAP3K4, mitogen-activated protein kinase kinase 4; MEF, mouse embryonic fibroblast; NF-kB subunit; NES, normalized enrichment score; NT, nontumor; PDGFRB, platelet derived growth factor receptor beta; RELA, RELA proto-oncogene; RPL6, ribosomal protein L6; RPL21, ribosomal protein L21; RPS2, ribosomal protein S2; RUNX1, runt related transcription factor 1; (S), sense; SF3B6, splicing factor 3b subunit 6; SKIL, SKI like proto-oncogene; SRSF7, serine and arginine rich splicing factor 7; T, tumor.

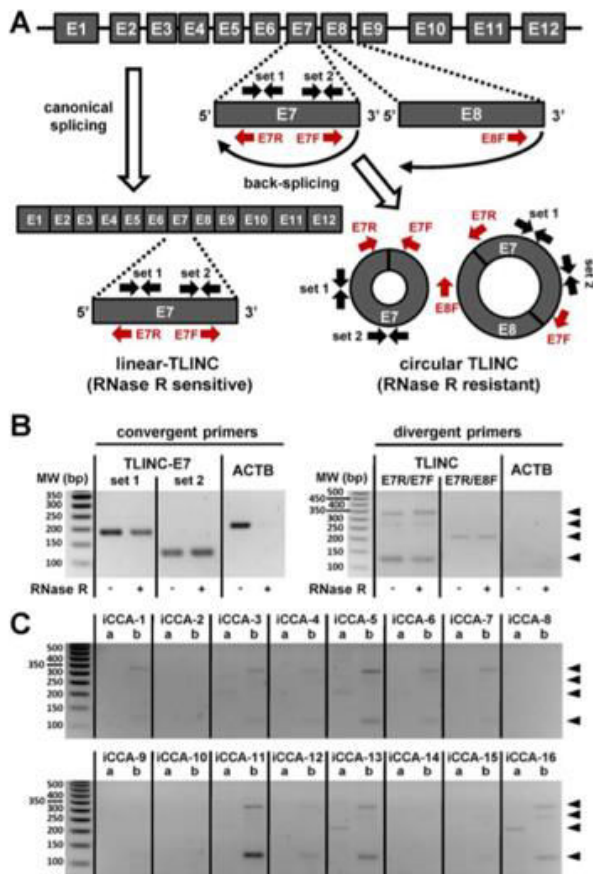


FIG. 7. Experimental evidence of TLINC circular isoforms. (A) Schematic representation of TLINC RNA processing into linear RNA (canonical splicing) or circular RNA (back-splicing). Several sets of convergent and divergent PCR primers were designed to detect specific linear and circular isoforms. (B) RNA was extracted from Huh28 cells after 16 hours of treatment with 1 ng/mL TGFβ. RNase R treatment was performed prior to RT-PCR analysis. Convergent primers (left panel) detected specific TLINC-E7 transcripts in both RNase R untreated and treated samples. For ACTB (a housekeeping gene highly expressed), no amplification was observed after RNase R treatment. By using a specific set of divergent primers located on exons E7 and E8, several TLINC circular isoforms were highlighted (black arrowheads). (C) RNAs were extracted from 16 human iCCA tumors. RT-PCR analysis was performed using a specific set of divergent primers located on exons E7 and E8, as indicated at the top of the panels (a, E7R/E8F; b, E7R/E7F). Black arrowheads highlight TLINC circular isoforms. Abbreviations: bp, base pair; MW, molecular weight.

TLINC EXPRESSION DEFINES SPECIFIC SUBSETS OF HUMAN iCCA

Gene expression profiling was performed on 22 resected human iCCA, and a specific signature was

defined on the basis of TLINC expression (Fig. 6D). High expression of TLINC correlated with a high expression of TGFβ target genes and inflammation-associated genes, as previously observed in cell lines (Fig. 6D; Supporting Fig. S5). In addition, GSEA demonstrated that up-regulated genes in high-TLINC-expressing tumors were significantly associated with the H3K27Me3 repressive epigenetic mark signature, while down-regulated genes were linked to protein metabolism and included numerous genes encoding ribosomal components (Fig. 6D,E; Supporting Fig. S6). Among the chemokines induced in HuCCT1 cells directly overexpressing TLINC (Fig. 5B) and possibly contributing to a migration phenotype, IL8 was found to be significantly correlated with TLINC expression in human iCCA tumors (Fig. 6F).

EXPERIMENTAL EVIDENCE OF TLINC CIRCULAR ISOFORMS

A genome-wide bioinformatic analysis of RNA-sequencing data previously reported a 204-nucleotide circular isoform of LINC00340/TLINC, probably derived from exon E7. This isoform was one of the most highly expressed circular RNAs (circRNAs) in H1-human embryonic stem cells.⁽²¹⁾ To evaluate the putative expression of such circular isoforms in iCCA, we designed specific sets of convergent and divergent primers from exons E7 and E8 of TLINC (Fig. 7A). ACTB was used as a housekeeping gene control that was highly expressed and without prior evidence of a circular isoform. Interestingly, by combining RNase R treatment and RT-PCR experiments, we provide evidence of the existence of several RNase R-resistant circular TLINC isoforms not only in iCCA cells treated with TGFβ (Fig. 7B) but also in resected human iCCA tumors (Fig. 7C).

Discussion

The molecular mechanisms involved in iCCA carcinogenesis are complex and involve multiple interactions between tumor cells and their microenvironment.⁽¹⁾ We previously demonstrated that active TGFβ signaling is an important feature in the iCCA microenvironment⁽⁶⁾; however, a deep characterization of the impact of TGFβ on tumor cells at the transcriptome level was lacking in iCCA. In this study, we first validated suitable experimental cellular models to study TGFβ signaling in iCCA cells. Particularly, we provided strong experimental evidence that Huh28 cells

exhibit an endogenous activation of TGF β , which correlates with a mesenchymal phenotype. This cell line may thus be relevant to test TGF β inhibitors for therapeutic purposes, as was suggested in liver cancers.⁽²²⁾ In addition, we provided the first TGF β transcriptional signature in iCCA cells, which allowed us to identify novel TGF β transcriptional targets, including coding and noncoding genes, both *in vitro* and in resected human iCCA.

Few studies have reported a deregulation of lncRNA in iCCA.^(23,24) As an example, oncogenic metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) lncRNA was shown to promote proliferation and invasion of CCA cell lines by activating the phosphoinositide 3-kinase/protein kinase B signaling pathway.⁽²⁴⁾ Here, we provide the first report of lncRNA directly regulated by TGF β in iCCA, supporting the previous description of TGF β -regulated lncRNA in other cancer types.^(11,12) Notably, lncRNA activated by TGF- β was shown to induce EMT and to promote HCC cell invasion through a TGF β /miR-200/zinc finger E-box binding axis.⁽¹²⁾ Similarly, TGF β -induced lncRNA-HIT was shown to promote EMT in breast cancer, notably by decreasing the expression of CDH1.⁽¹¹⁾ By comparing the transcriptome profiles of HuCCT1 and Huh28 cells at the basal level and after TGF β treatment, we identified a robust signature made of 42 nonredundant genes. This signature was validated by including well-known TGF β target genes (e.g., *SERPINE1*, *IL11*, *CTGF*, *BCL2* modifying factor [*BMF*], *SNAIL1*) reported in multiple cell types, including hepatocytes.⁽²⁵⁾ Importantly, this approach highlighted several novel TGF β -induced lncRNA, including LINC00312, LINC00313, and LINC00340 (TLINC), possibly involved in TGF β signaling. LINC00312 has been reported to inhibit the migration and invasion of bladder cancer cells by targeting miR-197-3p.⁽²⁶⁾ High expression of *LINC00313* has been associated with a poor prognosis and metastasis in lung cancer.⁽²⁷⁾ Experimental data on TLINC were more complex given that several isoforms were reported to exhibit either tumor-suppressive or tumor-promoting features in various types of cancer.^(18,19) Thus, in neuroblastoma, a short isoform was associated with tumor suppression by hampering cell growth and migration, and a lower expression of this short isoform was found in advanced tumors associated with a poor survival.⁽¹⁸⁾ Conversely, a long isoform of TLINC was reported in metastatic melanoma and associated with tumor progression.⁽¹⁹⁾ The differences in the basal expression

and induction by TGF β of TLINC isoforms in HuCCT1 and Huh28 cell lines suggest that the function of TLINC is highly dependent on the cellular context, as known for several TGF β target genes.⁽¹⁰⁾

Although TLINC deregulation has been reported in cancer,^(18,19) we provide the first evidence that TLINC is a common transcriptional target of TGF β not only in liver cancer (iCCA and HCC) but also in other cancer types, including pancreatic cancer. Interestingly, we demonstrated that TLINC is also up-regulated by TGF β in cells from the tumor microenvironment, as evidenced by the analysis of its expression in activated hepatic stellate cells (LX2) and in human iCCA tumors by ISH and LCM of the stroma. These data suggest that TLINC may play a role in TGF β -induced remodeling of the tumor microenvironment. Supporting this hypothesis, our experimental data show that ectopic expression of TLINC directly impacts the expression of several inflammatory mediators, including chemokine *IL8*. Increased expression of *IL8* has been reported in cancer and is associated with important changes within the tumor microenvironment, including promotion of angiogenesis, infiltration of neutrophils and tumor-associated macrophages, and migration of cancer cells.^(28,29) Interestingly, a functional link has been established between the expression of *IL8* and nuclear paraspeckle assembly transcript 1 [*NEAT1*], another lncRNA that we found to be induced in cells transfected with the TLINC DNA construct (Fig. 5B). Indeed, it was demonstrated that NEAT1-dependent relocation of a splicing factor proline/glutamine-rich from the promoter region of the *IL8* gene to paraspeckle led to the transcriptional activation of *IL8*.⁽³⁰⁾ One can hypothesize that TLINC acts as a structural component of paraspeckles and that interaction with NEAT1 may result in *IL8* expression. This hypothesis is in agreement with the partial nuclear location of TLINC. The enrichment of a gene signature associated with repressive marks H3K27me3 in iCCA tumors characterized by a high expression of TLINC may also suggest a role of TLINC in epigenetic silencing, as was described for other lncRNAs, such as *Kcnq1ot1* antisense.⁽³¹⁾ Similarly, enrichment of a gene signature associated with protein metabolism in iCCA with low TLINC expression together with the cytoplasmic expression of TLINC may suggest a role of TLINC in translation, as described for lncRNA growth arrest specific 5 (*GAS5*), which interacts with the translational machinery to inhibit c-Myc translation.⁽³²⁾ Another interesting aspect is the restricted expression of TLINC in remodeled portal areas with

signs of ductular reaction in nontumor livers. These areas are usually associated with activation of the cancer stem cell compartment.⁽³³⁾ Supporting a putative role of TLINC in cancer stem cell biology, TLINC has been reported to be highly expressed in human embryonic stem cells.^(21,34) At the functional level, we showed that TLINC-L overexpression in HuCCT1 cells resulted in increased cell migration. Although the underlying molecular mechanisms are still to be clarified, *SNAI2* was found to be enriched in the gene expression profiles of TLINC-L transfected cells, suggesting that the type of migration could be partly related to EMT. Other genes found to be tightly correlated with TLINC expression in human iCCA tumors may also contribute to the observed phenotypes. As an example, platelet-derived growth factor receptor beta (*PDGFRB*) has been associated with the aggressive behavior of several types of tumors.⁽³⁵⁾ Notably, *PDGFRB* has been shown to promote liver metastasis formation of mesenchymal-like colorectal tumor cells.⁽³⁶⁾

Finally, we provide experimental evidence of circular isoforms of TLINC that may represent putative, relevant, noninvasive biomarkers for iCCA. Indeed, recent data demonstrated that circRNAs are enriched in extracellular vesicles, including exosomes, compared to their linear counterparts.^(37,38) The circular structure of circRNAs renders them more stable and resistant to degradation by exonucleases. Thus, pangenomic profiling of circRNA abundance is emerging in cancer, and several expression signatures have been reported, notably in HCC.⁽³⁹⁾ Our study provides the first report of circRNA expression in iCCA. The function of circRNA-TLINC remains to be explored, but several experimental data demonstrated that circRNAs may act as sponges for miRNA and RNA binding proteins. With respect to the TGF β pathway, circRNA_010567 has been recently shown to promote TGF β -induced myocardial fibrosis by directly suppressing miR-141, which naturally targets TGF β .⁽⁴⁰⁾

In conclusion, our study identifies novel TGF β transcriptional target genes in iCCA, including an lncRNA that may represent a promising biomarker for iCCA.

Acknowledgment: We thank the core facilities of Biosit and Biogenouest (CRB Santé BB-0033-00056, GEH, H²P², ImPACcell) and the French liver cancer biobanks network (BB-0033-00085), including Beaujon, Bordeaux, Mondor, Nantes, Paris-Sud, and Rennes biobanks. C.C. thanks K.A. Cole (Children's

Hospital of Philadelphia) and S. Sugano (University of Tokyo) for providing the CASC15 DNA constructs; S. Dréano (Institut de génétique et développement de Rennes [IGDR]) for DNA sequencing; and C. Père for reporter sensor cell line analysis. We thank D. Gilot, M.D. Galibert (IGDR, Rennes), B. Fromenty (NuMeCan, Rennes), and S. Diederichs (German Cancer Research Center [DKFZ], Heidelberg) for helpful discussions.

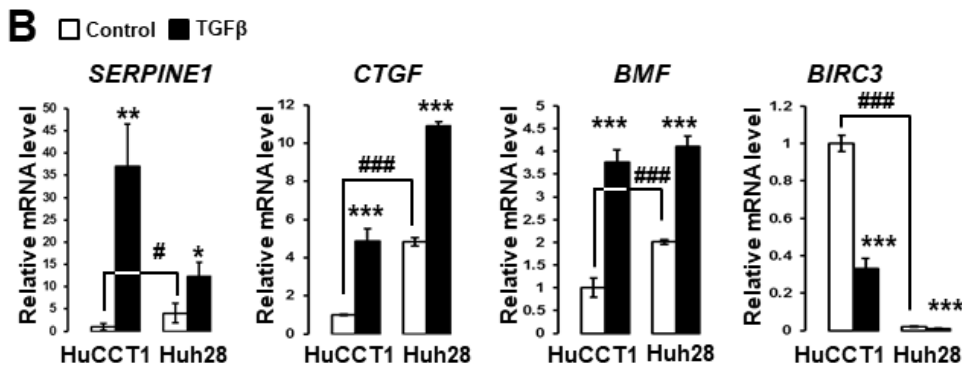
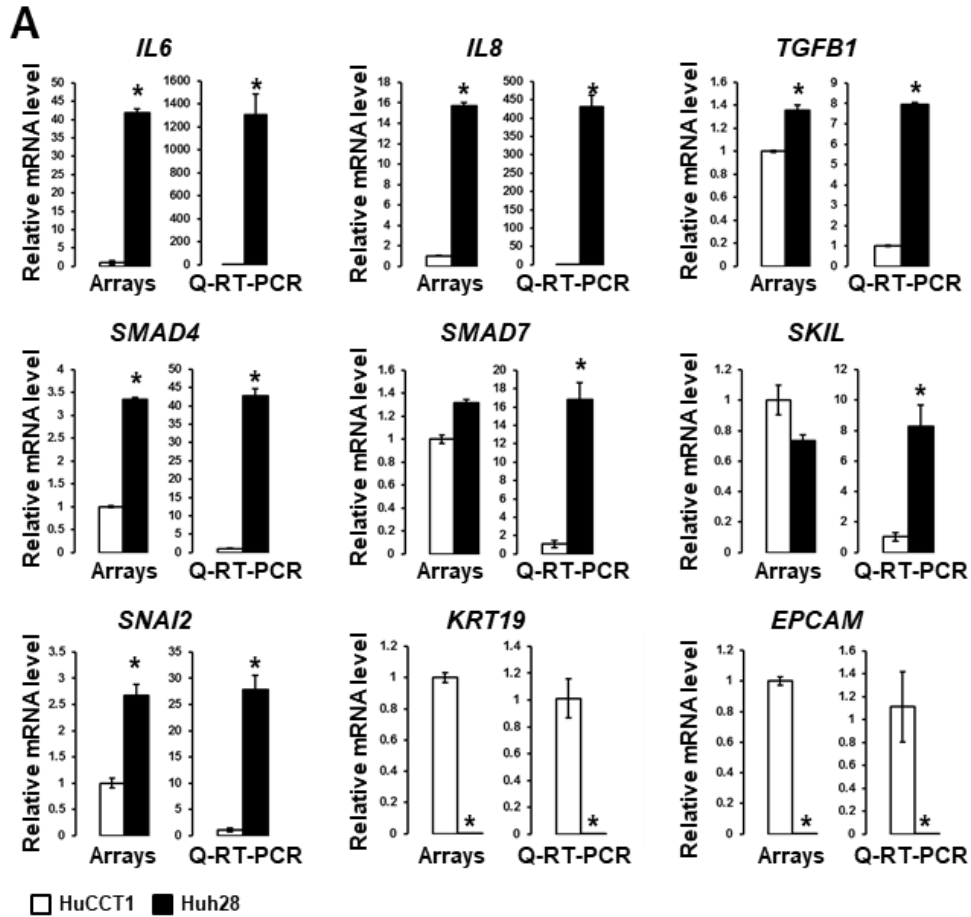
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Supporting Information

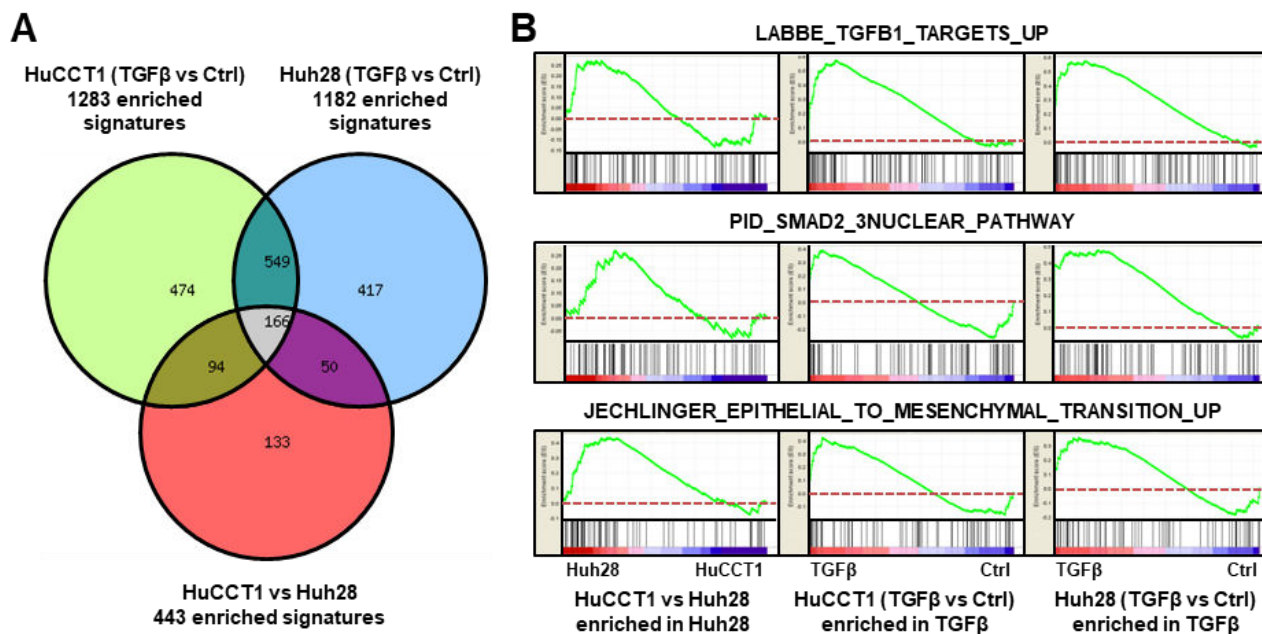
Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1142/full.



SI Figure 1: Expression of TGFβ target genes in HuCCT1 and Huh28 cells at basal condition and after a 16hrs TGFβ treatment

A: Expression of TGFβ target genes in HuCCT1 (white bars) and Huh28 (black bars) cells at basal level, as evaluated by DNA microarrays (left panels) and Q-RT-PCR (right panels). Statistical analysis was performed by a t-test (* P<0.05; n=3 replicates)

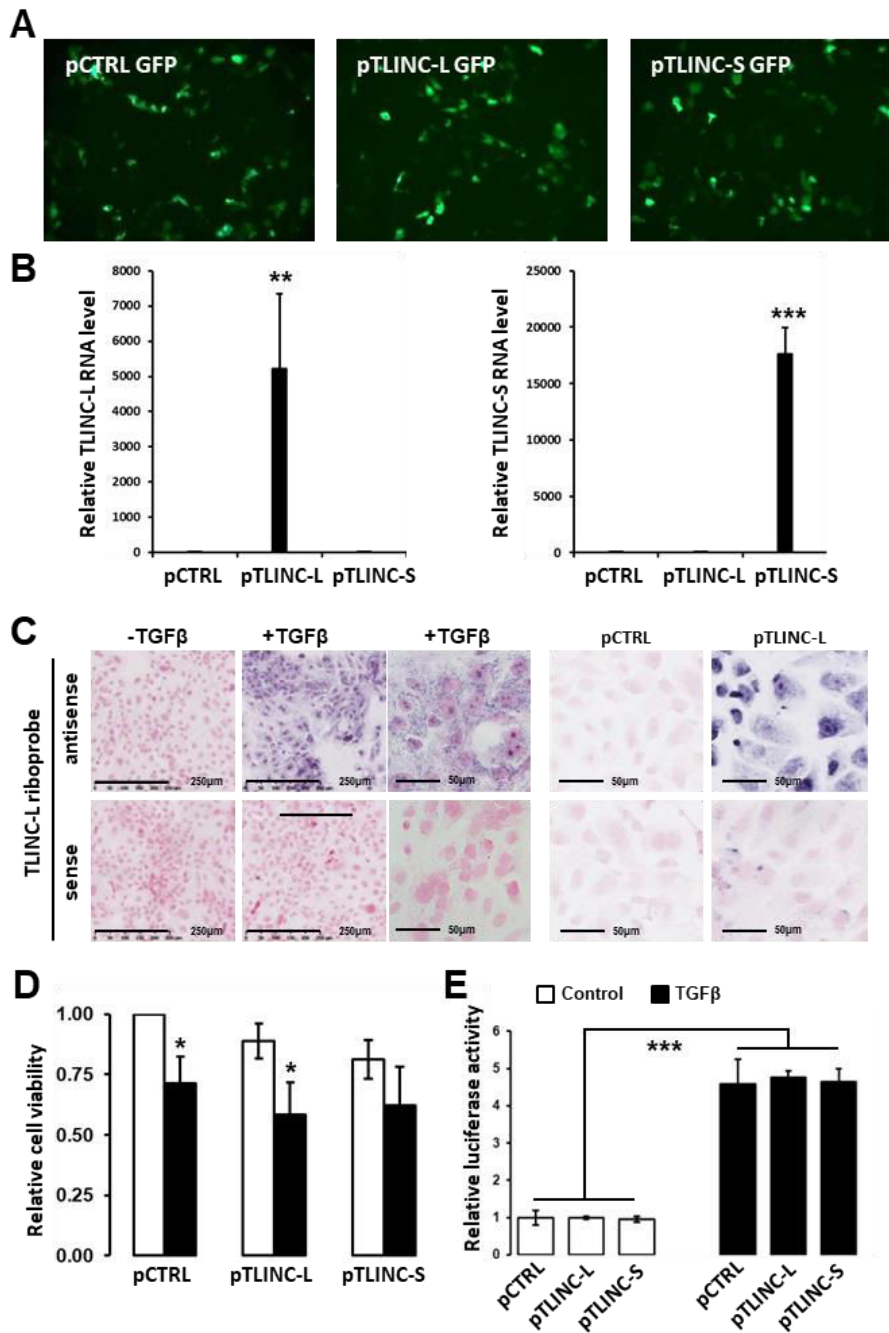
B: Expression of well-known TGFβ target genes in HuCCT1 and Huh28 cells after 16hrs of culture in absence (white bars) or presence (black bars) of 1ng/mL TGFβ, as evaluated by Q-RT-PCR. Statistical analysis was performed by a t-test (TGFβ vs Control: * P<0.05; ** P<0.01; *** P<0.001 / HuCCT1-Control vs Huh28-Control: # P<0.05; ### P<0.001) ; n=3 replicates.



SI Figure 2: Enrichment of specific gene expression signatures in TGFβ-associated gene sets identified in HuCC1 and Huh28 cholangiocarcinoma cell lines

A: Venn diagram analysis of gene signatures significantly enriched ($P < 0.05$) in TGFβ-associated gene sets identified in HuCC1 and Huh28 (as in the main Figure 2), evaluated by unsupervised GSEA.

B: Examples of 3 gene signatures associated with TGFβ, SMAD2/3 and EMT, significantly enriched in Huh28 at basal level as compared to HuCC1 (left panels), or in TGFβ-treated HuCC1 (middle panels) and Huh28 (right panels) cells, as compared to untreated cells.



SI Figure 3. Functional analysis of TLINC-L and TLINC-S isoforms

A: Green fluorescent protein (GFP) image analysis ($\lambda=509\text{nm}$) of HuCCT1 cells transfected with specific DNA constructs designed to overexpress TLINC-L and TLINC-S isoforms with GFP (100ng plasmid / 24hrs post-transfection).

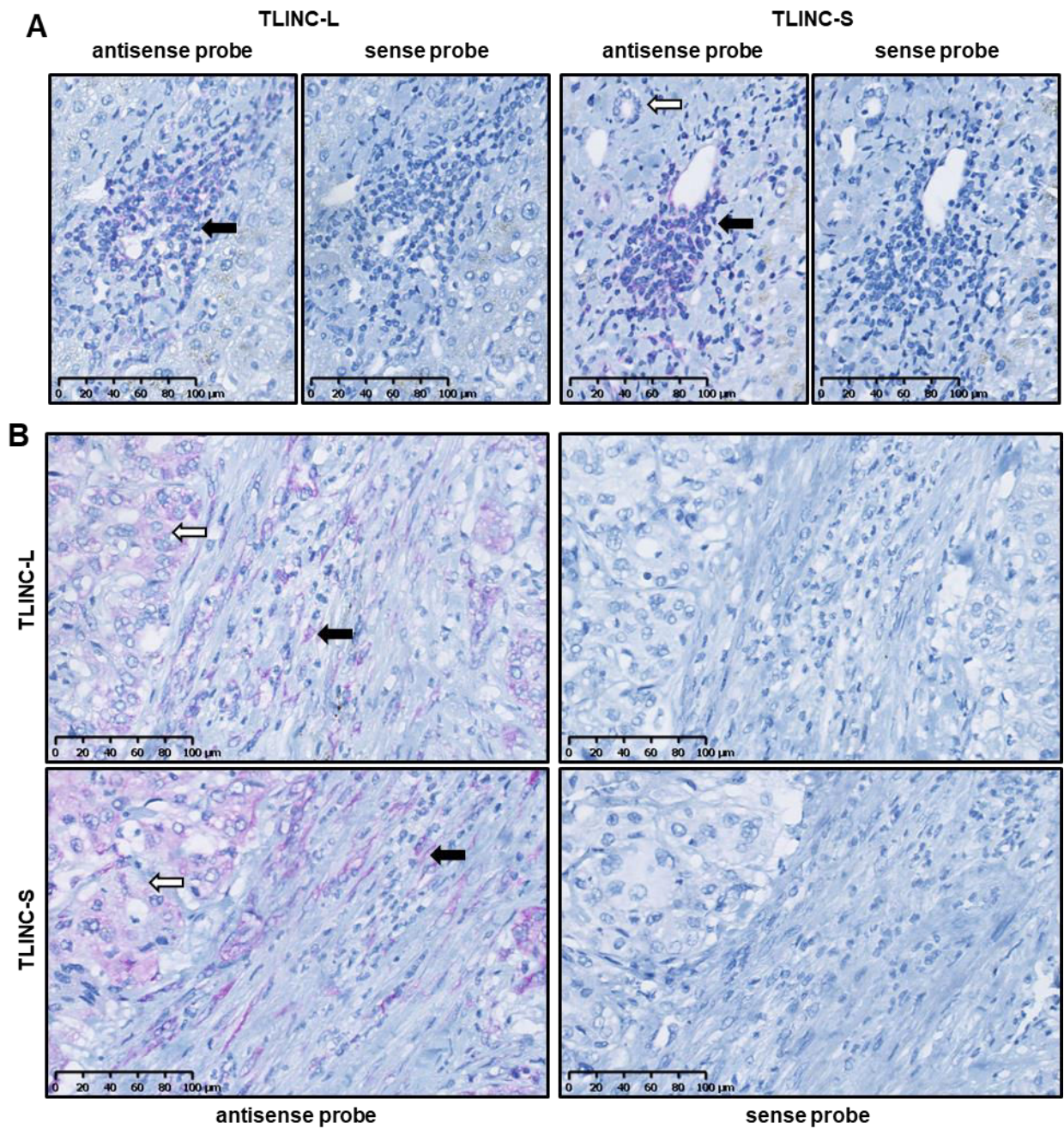
B: Validation of specific TLINC-L and TLINC-S overexpression by Q-RT-PCR using specific sets of primers, as in Figure 4A.

C: ISH using TLINC-L riboprobes (antisense, upper panels ; sense, lower panels) on HuCCT1 cells transfected with pTLINC-L DNA construct demonstrating a similar localization than after a treatment with TGF β (Figure 4).

D: Cell viability of HuCCT1 cells transfected with pLINC DNA constructs after 72hrs of culture in absence (white bars) or presence (black bars) of 1ng/mL TGF β .

E: Luciferase activity of HuCCT1 engineered cell lines in which a luciferase gene is under the transcriptional control of SMAD responsive elements. Luciferase activity was measured in cells transfected with TLINC-L and TLINC-S DNA constructs in absence (white bars) or presence (black bars) of TGF β . No significant difference was observed between the different TLINC DNA constructs.

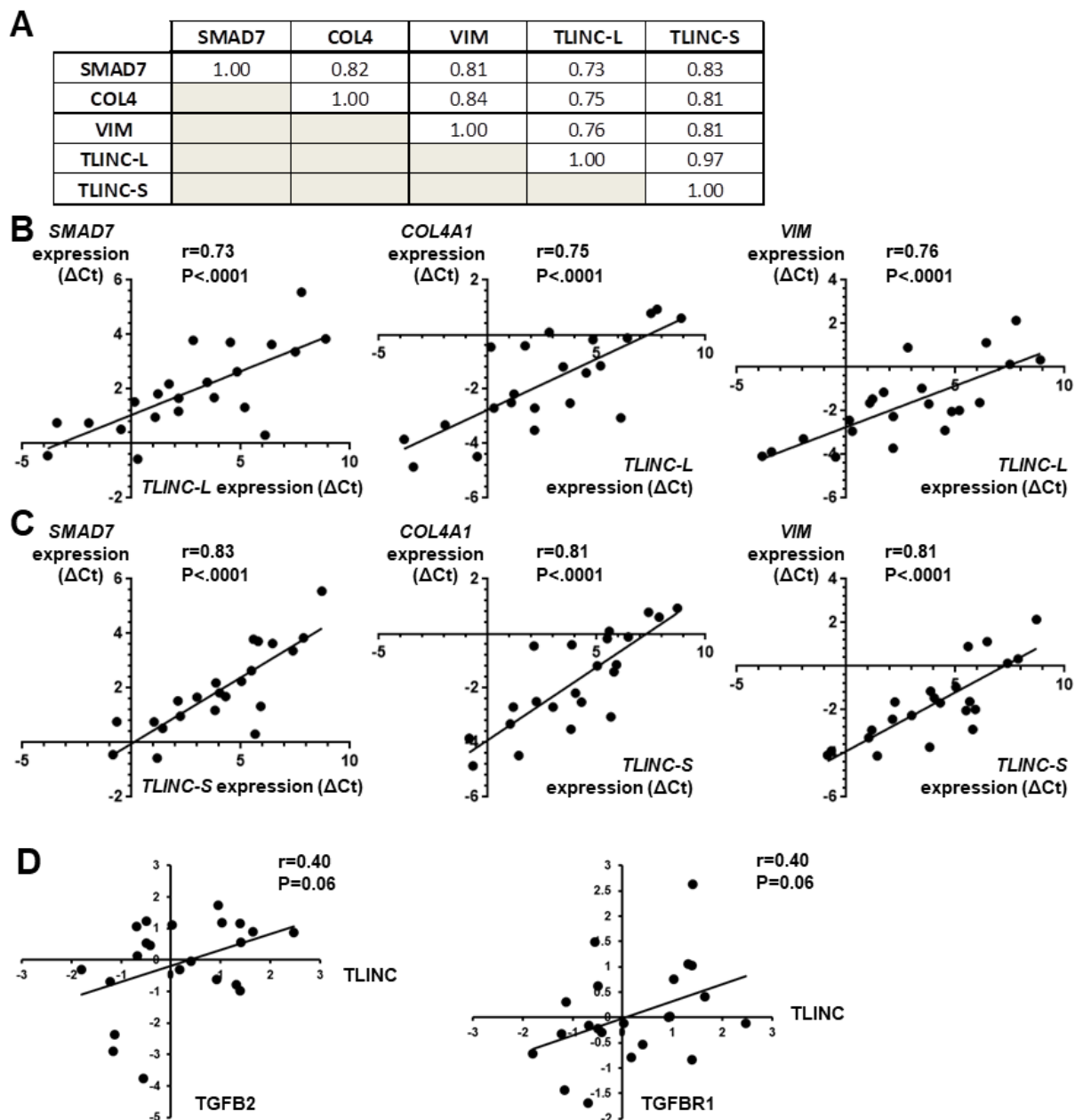
Statistical analysis was performed by a t-test (* $P<0.05$; ** $P<0.01$; *** $P<0.001$) ; n=3 replicates.



SI Figure 4: Analysis of *TLINC-L* and *TLINC-S* expression by ISH on human resected iCCA

A: ISH analysis of *TLINC-L* and *TLINC-S* expression using specific antisense and sense riboprobes in non-tumor liver. Signal was observed in remodeled portal areas (black arrow) but not in normal bile ducts (white arrow) or normal hepatocytes. No signal was observed with the sense riboprobes.

B: ISH analysis of *TLINC-L* and *TLINC-S* expression using specific antisense and sense riboprobes in the tumor part. *TLINC* was expressed in both epithelial cells (white arrow) and in the stroma (black arrow). No signal was observed with the sense riboprobes. Scale bar: 100 μ m.



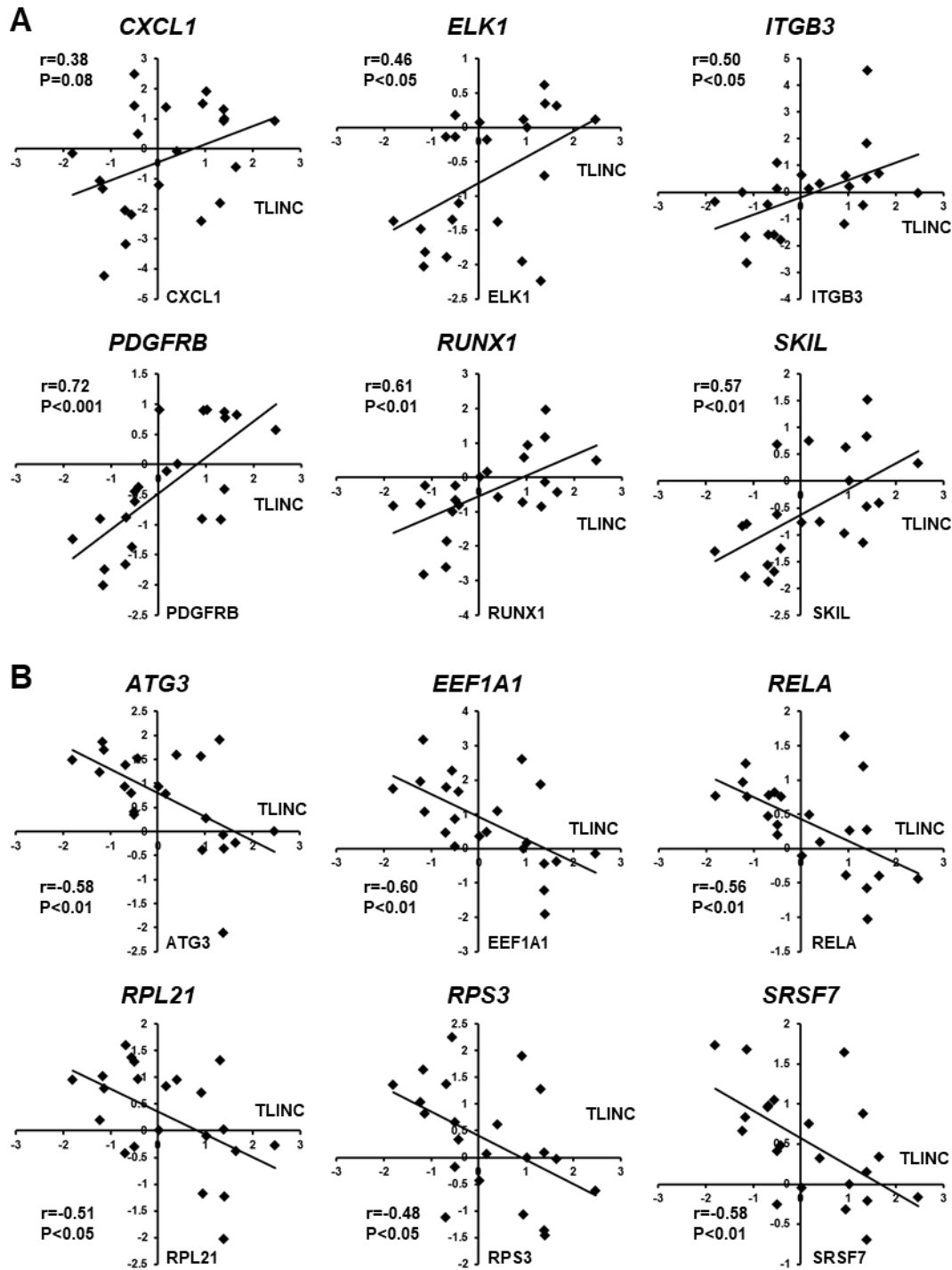
SI Figure 5: Correlation of gene expression between TLINC isoforms and TGF β target genes in resected human iCCA tumors

A: Correlation coefficient between the expression of TLINC-L, TLINC-S and TGF β -associated genes, as detected by Q-RT-PCR in 22 resected human iCCA tumors; $P < 0.001$ for all correlations.

B: Correlation plot between the expression of TLINC-L and well-known TGF β target genes, as detected by Q-RT-PCR in 22 resected human iCCA tumors.

C: Correlation plot between the expression of TLINC-S and well-known TGF β target genes, as detected by Q-RT-PCR in 22 resected human iCCA tumors.

D: Correlation plot between the expression of TLINC and TGFB2 and TGFB1, as detected by microarray in 22 resected human iCCA tumors.



SI Figure 6: Correlation of gene expression between TLINC and genes significantly induced or repressed in resected human iCCA tumors characterized by high and low TLINC expression

A: Examples of genes positively correlated with TLINC.
B: Examples of genes negatively correlated with TLINC.
 n=22 human iCCA

Supplementary Table 1. Sequence of TLINC-L and TLINC-S isoforms

Alternating of black and blue colors delineates successive exons. Underlined nucleotides refer to the sequence of riboprobes used for in situ hybridization experiments.

A) Sequence of TLINC-Long isoform (TLINC-L): E1-E2-E3-E4-E5-E6-E7-E8-E9-E10-E11-E12

GTCTGCTCCGGGACTTGAACAAAAGGGGAAGCTCTGATGAAGCTCTTTCTCCCTCTCCCCGGGACGC
CGGGGTATCTCCCTCTCGCAACTTTGCCGCCCGACTTTCTCTGCTGTCAGGCCGGGAAAAAGTGTCCGAA
CGCCTCGTGACTGCAGCGGGGAAAATGTCCCTTAAAAGTGCGACGAAGTGGGAAGAAGGTGTAATTACT
ATTATCAGCATCTAGAAAGCATCATGAATTTGCTGGAGTACTTCTAGCACTGACCTCCTTCACTTGCCTTGT
TCTTACTGGATCTTTCCATCAGCCAACAATATGGAAGTACCAATACAAGGTCAAATCATTCTGGATTCATCTG
GAGTTGCTTAAAAGTTAAATCATTGGAATTTTTGATGATACTTTTCTATATGGATTACAATTTGATCGCTGGG
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CTTTCTGGATGGGGAAGATTTCCAGGAATTGGCAACAAGCTCATTTCCTGGTGGGTTTGTGAAGCATTATC
ACAAGACAGTCAGAATGACTGATGAGTGTCTTTCAGGTGTGAATCATGGCAATACAGTAAAAGACAGTGATTT
ACTGCTTTGAGGGCGTGCATGTATATGATTAACGGATGGAAGTGCAGGACTCCAAGATTTACTTCCCTCCCT
TTCCAGCAGAATTACCTGAGACGAGTAAAATCTACTGGTGGAGTCACTCCATTATTCTTATCTGTGGAGATCT
AGATCTTGATTTGAAAGTTTCTGAGAAAAATCTTCAGCTCAGACTTGAGGGTCAACTTTACCAGCTGAAGGATC
TGCATTTACTGCTCAACCACATCTAATTTGATGTCTCTGCAGATTTAAAATGTGTGCCTTCTTTCCGTCACC
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ATGGAAAACCCAGAGGAAATGAGTGAGGATCAATGGGAAGAAGAGAGCCAGCCAGGAAGTTGAAGATTTGT
CCAGGAGCAGATAGCTGAAGAGAGAGAGAGAAGAGAGAACGGCTTACAGCTCAGGTCTCTCTCCATGC
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TGCAACAGTATGGGCTCACAGGCAGAAGGAATTTTCTTGTCTTGGATGAGACTTTTGACTTGGACTTTTGGG
TTAAGTTTCTGGAGACCAGAAGGCCAAAATCAAAAGTATGGGCAGGCTTGATTTCTTTAGAAGACTCCAGCGG
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AACACAACCTCAGCCAAGAAGAGTATGCAGAGAAAGGAGTCCCCTACCTGCCACAAAAGTGTGTCTGAAAA
CTGTCTCATATTGTCTCAAGTTGTCAATTCATTGTGAATTAGACCTGTTTAAACATGTAATCTGCAACATGCTTCA
CTGTCTAATTTTCCAGAGCCCTCATATAAGGAAGTGTATTATTGGTATAATCATCATGGTGAAGAAGTTGGTA
TGTGGGGGAGAGATGACAGAAACAGAGAGTAAGTCAGAGCTGGCTGCCTGACAGATAAAAAGGAAATGACC
AA

B) Sequence of TLINC-Short isoform (TLINC-S): E9bis-E10-E11-E12

GGGCACGCAAGCTTCCCTTGTTACCTGAGCTGCTCCTGCCGTCTCCCGCTGGGCTTCGCCGTGGTGCAC
CCGATCCCGAATCGTGCGTCTGCGCCCTGCGAAAGAAGGACCTGCTGGCGGAGCTCCCGCCGGGTCTC
CTGCCTCGCAGCTGGGCGAGGGGACTTGGAGGACAGGGTGAAGCTGCAGAAGACCTGGGGTGGGATGGCT
AGAGAGGACGCCAAGGACTGGGGAAGGGGAAGTTAGGAATACCTTACATCCAATGCCACCCCGTGTCCGC
AGGGCAAGGGCAGCCGTGCCTCGGCCGCGTGCACCCAGCTCAGGCTGTTCCAGGGATTTAGTCTGGGG
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GAATGCCGGGTGATTGTCTGCCGCTTGCTGTGCATTCTCATTCTAGGGAGGATGGCATAATTTATAACCCAG
CACATGGATAGAGGAACTGTAAATTGTAGTCTGGATGTCCCGGCGCTGTGACGAAGGAACTGATGGGCT
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CAGACCCCTCATATAAGGAACTGTATTATTGGTATAATCATCATGGTGAAGAAGTTGGTATGTGGGGGAGA
GATGACAGAAACAGAGAGTAAGTCAGAGCTGGCTGCCTGACAGATAAAAAGGAAATGACCAA

Supplementary Table 2. Sequence of DNA primers used in the study

Gene	Forward primer (5' to 3' sequence)	Reverse primer (5' to 3' sequence)
ACTB	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
BIRC3	TTTCCGTGGCTCTTATTCAAAC	GCACAGTGGTAGGAACTTCTCAT
BMF	GCGCGGAGCCCTGGCATCAC	GCTGGTGTGCTGCACATGAAGCCG
CDH1	CGAGAGCTACACGTTCCACGG	GGGTGTCGAGGGAAAAATAGG
CDKN1A	GGCGTTTGGAGTGGTAGAA	TGTCACTGTCTTGTACCCTTG
COL4A1	ACTCTTTTGTGATGCACACCA	AAGCTGTAAGCGTTTGCCTA
CTGF	TCACTGACCTGCCTGTAG	GCTGAGTCTGCTGTTCTG
CXCL8	CTGGCCGTGGCTCTCTTG	CCTGGCAAACCTGCACCTT
GAPDH	GACCACAGTCCATGCCATCA	TCCACCACCCTGTTGCTGTA
EPCAM	CCTGCTCTGAGCGAGTGAGAACC	GATCTCCTTCTGAAGTGCAGTCCGC
IL11	ACAGCTGAGGGACAAATTCC	AGCTGTAGAGCTCCCAGTGC
IL6	ACCCCAAAGTTTGTCTCAAGGA	AAAGCAGAACAGCACTCCCA
KRT19	TTTGAGACGGAACAGGCTCT	AATCCACCTCCACACTGACC
NEAT1	GCTCACTCCACCCCTTCTTC	CACATCACTCCCCACCCTC
SERPINE1	AAGCCTAATCAGCCCACCAT	TAGCATTTGACACCACCCT
SKIL	CAGATGCACCATCAGGAATG	TTGCCTCTGTCTTTGTGAGC
SMAD4	GCTGCTGGAATTGGTGTGATG	AGGTGTTTCTTTGATGCTCTGTCT
SMAD7	CCAAGTGCAGACTGTCCAGA	GGGCTCCTGGACACAGTAGA
SNAI1	CACTATGCCGCGCTCTTTC	GGCTGCTGGAAGGTAAACTC
SNAI2	TCGGACCCACACATTACCTT	TTGGAGCAGTTTTTGCCTG
TBP	GAGCTGTGATGTGAAGTTTCC	TCTGGGTTTGATCATTCTGTAG
TGFB1	CACGTGGAGCTGTACCAGAA	CCGGTAGTGAACCCGTTGAT
TLINC (S+L)	CAAGAGGAATCCAGCAAAGC	AGCATGGAGAGAGGACCTGA
TLINC-S	GAGAAGGCAGAGCTGGAGAA	CTAGCCCATCAGTTCCTTCG
TLINC-L	CAAAGGGGGAACTCTGATG	CCTGACAGCAGAGAAAGTCG
VIM	GACGCCATCAACACCGAGTT	CCTTGTGCTTGGTTAGCTGGT

Primers used to detect circular isoforms of TLINC (as in Figure 7)

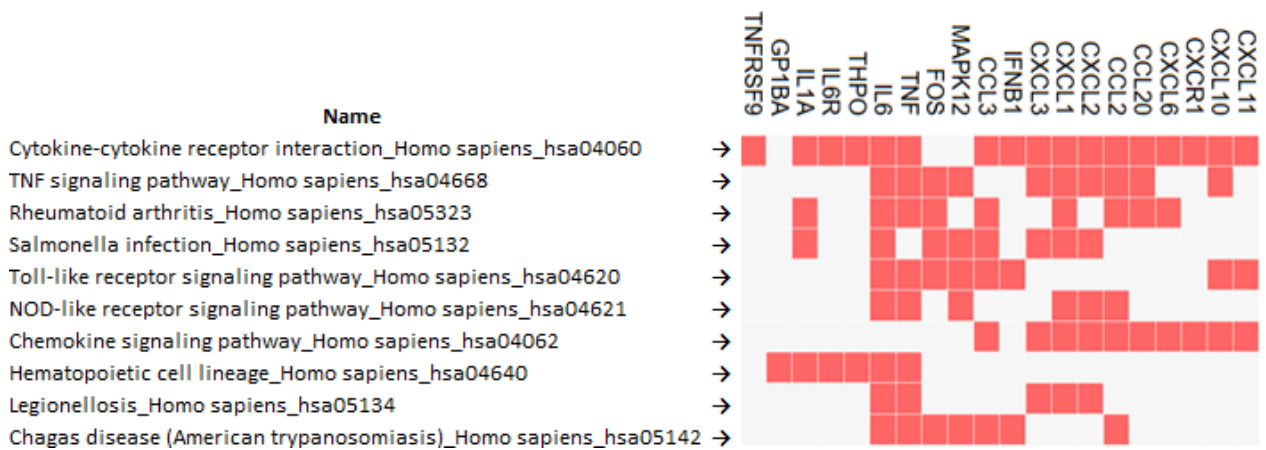
	Forward primer (5' to 3' sequence)	Reverse primer (5' to 3' sequence)
Convergent primers		
TLINC-E7 set1	GATCTGCATTTACTGCTCAACC	TGTTTGTGTTTGGAGACATCTGG
TLINC-E7 set2	CCTGGGTTACTACTGAACATCC	TGTCTTCTTCCTTCACCGATTAC
Divergent primers		
E7F/E7R	GACAGAAGAAGGAAGTGGCTAA	GTAGTAACCCAGGGATGACTTG
E8F/E7R	CAAGAGGAATCCAGCAAAGC	GTAGTAACCCAGGGATGACTTG

Supplementary Table 3. Data mining of genes differentially expressed by TLINC-L

A) Enrichment of KEGG pathways and genes involved

Name of KEGG pathway	P-value	Z-score	Combined score
Cytokine-cytokine receptor interaction_Homo sapiens_hsa04060	0.000067	-1.88	18.03
TNF signaling pathway_Homo sapiens_hsa04668	0.000134	-1.89	16.83
Rheumatoid arthritis_Homo sapiens_hsa05323	0.000139	-1.72	15.25
Salmonella infection_Homo sapiens_hsa05132	0.000533	-1.64	12.33
Toll-like receptor signaling pathway_Homo sapiens_hsa04620	0.002089	-1.77	10.9
NOD-like receptor signaling pathway_Homo sapiens_hsa04621	0.001387	-1.65	10.86
Chemokine signaling pathway_Homo sapiens_hsa04062	0.002517	-1.77	10.61
Hematopoietic cell lineage_Homo sapiens_hsa04640	0.002915	-1.58	9.24
Legionellosis_Homo sapiens_hsa05134	0.006526	-1.54	7.75
Chagas disease (American trypanosomiasis)_Homo sapiens_hsa05142	0.007288	-1.56	7.68

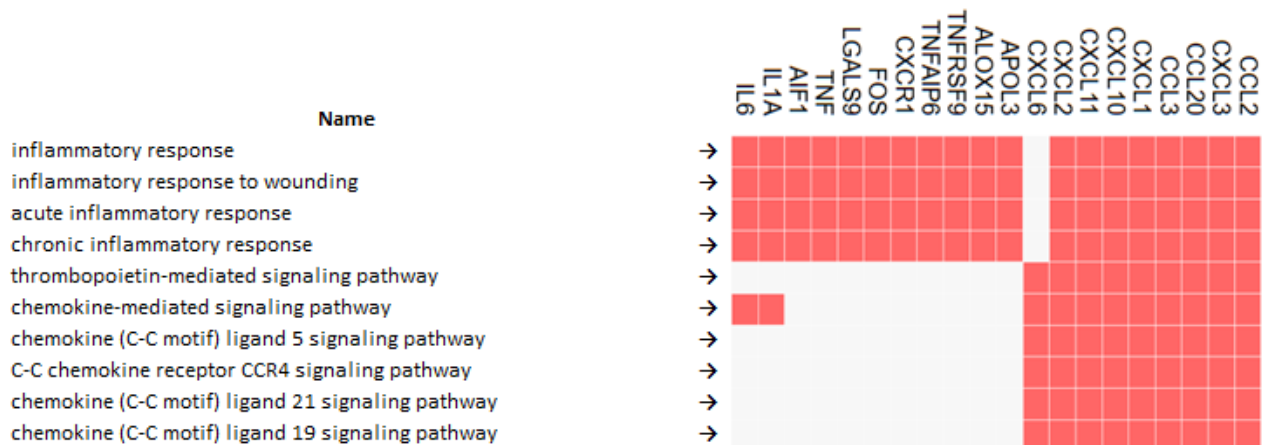
Genes involved



B) Enrichment of GO Biological Process and genes involved

Name of GO pathway	P-value	Z-score	Combined score
inflammatory response	1.70E-07	-3.71	57.9
inflammatory response to wounding	1.70E-07	-3.71	57.86
acute inflammatory response	1.83E-07	-3.73	57.85
chronic inflammatory response	1.70E-07	-3.71	57.82
thrombopoietin-mediated signaling pathway	1.13E-07	-2.53	40.4
chemokine-mediated signaling pathway	1.911E-06	-3.05	40.15
chemokine (C-C motif) ligand 5 signaling pathway	8.83E-07	-2.51	34.99
C-C chemokine receptor CCR4 signaling pathway	8.83E-07	-2.51	34.97
chemokine (C-C motif) ligand 21 signaling pathway	8.83E-07	-2.5	34.88
chemokine (C-C motif) ligand 19 signaling pathway	8.83E-07	-2.5	34.84

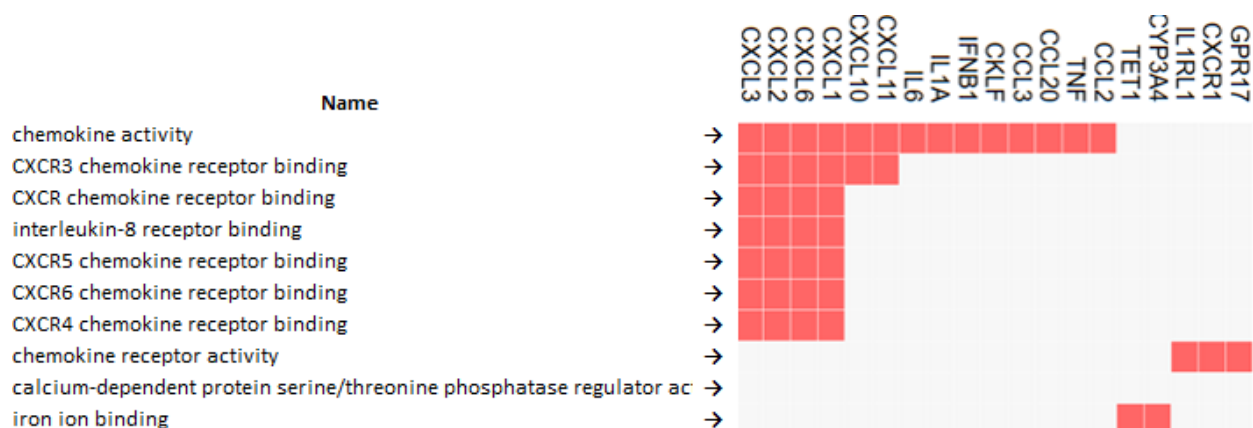
Genes involved



C) Enrichment of GO Molecular Function and genes involved

Name of GO pathway	P-value	Z-score	Combined score
chemokine activity	4.713E-06	-2.59	31.8
CXCR3 chemokine receptor binding	2.51E-07	-1.16	17.62
CXCR chemokine receptor binding	0.000315	-1.26	10.2
interleukin-8 receptor binding	0.000062	-0.82	7.97
CXCR5 chemokine receptor binding	0.000040	-0.75	7.62
CXCR6 chemokine receptor binding	0.000024	-0.69	7.33
CXCR4 chemokine receptor binding	0.000024	-0.69	7.3
chemokine receptor activity	0.02398	-1.83	6.81
calcium-dependent protein serine/threonine phosphatase regulator activity	0.01981	-1.62	6.36
iron ion binding	0.03363	-1.69	5.74

Genes involved



Supplementary Table 4. Migration-related genes significantly enriched in the gene expression profile of HuCCT1 cells overexpressing TLINC-L

GENE SYMBOL	RANK IN GENE LIST	RANK METRIC SCORE	RUNNING ES	CORE ENRICHMENT *
OLR1	6	4.458	0.0679	Yes
NEDD9	46	2.793	0.1086	Yes
PTGS2	47	2.772	0.151	Yes
HIST1H2BK	53	2.681	0.1917	Yes
CYR61	62	2.576	0.2307	Yes
TXNIP	84	2.359	0.2656	Yes
UGCG	89	2.337	0.3012	Yes
MT1E	96	2.282	0.3358	Yes
MT2A	204	1.7	0.3561	Yes
CXCL1	236	1.62	0.3793	Yes
IL1A	247	1.582	0.4029	Yes
SNAI2	365	1.346	0.4173	Yes
MSN	371	1.331	0.4374	Yes

* from Wu et al., Oncogene, 2008

2. Article n°2

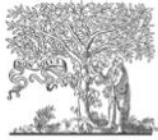
Accepté pour publication dans « Digestive Liver Disease »

« L'expression de l'ARNInc ANRIL prédit un mauvais pronostic dans le CCI »

Le but de ce travail était d'identifier des ARNInc dont l'expression était significativement associée à la survie des patients opérés d'une résection chirurgicale curative d'un CCI.

Le profil d'expression génique et des q-RT-PCR ont été effectuées sur l'ARN extrait de 39 CCI issus d'une cohorte avec les données cliniques correspondantes. Un modèle de Cox univarié avec statistique de Wald a été utilisé pour identifier les ARNInc significativement associés à la survie sans récurrence et la survie globale des patients. Une méta-analyse des bases de données du TCGA a été réalisée pour évaluer la pertinence clinique de l'expression des ARNInc dans d'autres cancers.

Une signature de 9 ARNInc a été identifiée comme significativement associée ($p < 0.05$) avec la survie sans récurrence et la survie globale. Parmi ces ARNInc, quatre d'entre eux (Inc-CDK9-1, XLOC_I2_009441, CDKN2B-AS1, HOXC13-AS) étaient très exprimés dans les CCI de mauvais pronostic et cinq d'entre eux (Inc-CCHCR1-1, Inc-AF131215.3.1, Inc-CBLB-5, COL18A1-AS-2, Inc-RELL2-1) plus exprimés dans les CCI de meilleur pronostic. Nous avons validé CDKN2B-AS1, aussi connu sous le nom d'ANRIL, comme biomarqueur de mauvais pronostic, non seulement dans le CCI mais aussi dans le CHC, le cancer du rein à cellules claires et le carcinome de l'endomètre.

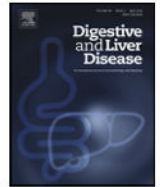


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Digestive and Liver Disease

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Oncology

Expression of long non-coding RNA ANRIL predicts a poor prognosis in intrahepatic cholangiocarcinoma

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ARTICLE INFO

Article history:
Received 7 February 2019
Accepted 22 March 2019
Available online xxx

Keywords:
ANRIL
Cholangiocarcinoma
Long non-coding RNA
Prognosis

ABSTRACT

- Q4 **Background:** Intrahepatic cholangiocarcinoma (iCCA) is a deadly cancer worldwide associated with an increased incidence, limited therapeutic options and absence of reliable prognostic biomarkers. Long non-coding RNAs (lncRNA) emerge as relevant biomarkers in cancer being associated with tumor progression.
- Q5 **However,** lncRNA have been poorly investigated in iCCA.
- Aim:** To identify lncRNA significantly associated with the survival of patients with iCCA after tumor resection for curative intent.
- Methods:** Gene expression profiling and Q-RT-PCR were performed from a cohort of 39 clinically well-annotated iCCA. Univariate Cox proportional hazards model with Wald Statistic was used to identify lncRNA significantly associated with overall (OS) and/or disease-free (DFS) survival.
- Q6 **Results:** A signature made of 9 lncRNA was identified to be significantly ($P < 0.05$) associated with OS and DFS, including 4 lncRNA (lnc-CDK9-1, XLOC.L2.009441, CDKN2B-AS1, HOXC13-AS) highly expressed in poor prognosis iCCA and 5 lncRNA (lnc-CCHCR1-1, lnc-AF131215.3.1, lnc-CBLB-5, COL18A1-AS2, lnc-RELL2-1) highly expressed in better prognosis iCCA. We further validated CDKN2B-AS1 (ANRIL) as a poor prognosis biomarker, not only in iCCA, but also in hepatocellular carcinoma, kidney renal clear cell carcinoma and uterine corpus endometrial carcinoma.
- Q7 **Conclusions:** We report a prognosis lncRNA signature in iCCA and the clinical relevance of CDKN2B-AS1 (ANRIL) overexpression in several cancers.

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1. Introduction

Q8 Cholangiocarcinomas (CCA) comprise several types of tumors arising from the malignant transformation of cholangiocytes lining different part of the biliary tree. Based on the anatomical location, intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) CCA are recognized [1,2]. Each of these CCA subtypes exhibits specific biological and pathological features, e.g. in term of clinical presentation, risk factors, prognosis and therapeutic strategies [2]. In the liver, iCCA represents the second most common type of malignant primary tumor after hepatocellular carcinoma (HCC), encompassing 10% to 15% of cases. However, an increase in iCCA incidence and mortality has been observed worldwide over the last decades. Today, surgical resection still remains the best curative treatment strategy of CCA, although it is associated with a high risk of tumor

recurrence [1]. For patients with unresectable or metastatic CCA, mostly as a result of a late diagnosis, systemic chemotherapy (usually a gemcitabine and cisplatin combination) is proposed as a palliative treatment [1]. Recently, adjuvant chemotherapy was demonstrated effective in resected CCA [3]. However, defining the optimal population that could benefit from adjuvant chemotherapy is particularly important. From a clinical point of view, the identification of innovative biomarkers for early diagnosis, prognosis and response to therapies, may improve the management and the survival of patients with iCCA. Although carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) levels may be elevated in some patients, there is no specific serum biomarkers for the diagnosis of iCCA. Several prognostic tissue biomarkers have been proposed based on genomic analyses. Notably, by combining laser capture microdissection and unsupervised gene expression profiling, we previously established clinically relevant signatures specific of the tumor microenvironment in iCCA [4]. Thus, an increased expression of osteopontin (OPN) and lysyl oxidase like 2 (LOXL2) in the stroma was identified as a significant prognostic factor associated with a reduced survival of patients after tumor resection [4,5].

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<https://doi.org/10.1016/j.dld.2019.03.019>
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Please cite this article in press as: Angenard G, et al. Expression of long non-coding RNA ANRIL predicts a poor prognosis in intrahepatic cholangiocarcinoma. Dig Liver Dis (2019), <https://doi.org/10.1016/j.dld.2019.03.019>

More recently, we identified a novel long non-coding RNA (lncRNA) up-regulated by the transforming growth factor beta (TGFβ) in human iCCA and associated with an inflammatory microenvironment, indicating that lncRNAs are also actively involved in iCCA carcinogenesis [6].

lncRNAs belong to an emerging class of regulatory RNAs that play critical roles in modulating a large variety of biological processes and cell signaling pathways. Accordingly, altered lncRNA expression has been reported in several cancers, including CCA [7]. Recent meta-analyses of The Cancer Genome Atlas (TCGA) database identified differentially expressed lncRNA in CCA (e.g. LINC00313, NEXN-AS1, COL18A1-AS1, HULC), the expression of some of them being significantly associated with overall survival (OS) of patients [8,9]. Mechanistically, co-expression network analysis of coding and non-coding RNA in resected CCA tumors identified lncRNA (e.g. APOC1P1, PVT1) possibly regulating inflammation and oxidative stress in malignant cholangiocytes [10,11]. Notably, pvt1 oncogene (PVT1) was shown to promote cell proliferation and migration by epigenetically silencing angiopoietin like 4 (ANGPTL4), a tumor suppressor gene candidate [12]. Cyclin dependent kinase inhibitor 1A (CDKN1A), another well-described tumor suppressor gene, was shown to be epigenetically silenced in CCA through the interaction of small nucleolar RNA host gene 1 (SNHG1) lncRNA with enhancer of zeste 2 (EZH2), the catalytic subunit of the polycomb repressive complex 2 (PRC2) [13]. Nuclear paraspeckle assembly transcript 1 (NEAT1) lncRNA may interact with EZH2 as well to repress the expression of E-cadherin (CDH1), thus promoting epithelial-to-mesenchymal transition (EMT) and metastasis [14]. Accordingly, an increased expression of SNHG1 and NEAT1 has been associated with aggressive tumorigenesis features in CCA [13,14]. NEAT1 has been also reported to contribute to gemcitabine sensitivity [15]. Conversely, lncRNA may positively affect the expression of oncogenic regulators, either directly or indirectly. Thus, epigenetically-induced lncRNA-1 (EPIC1) was reported to promote CCA cell growth and cancer progression by physically

interacting with MYC oncogene and possibly acting as a guide to direct MYC onto its target genes involved in cell cycle progression [16]. Interestingly, LINC01296 lncRNA was demonstrated to stabilize MYCN oncogene by sponging microRNA-5095, which naturally targets MYCN mRNA for degradation [17]. Urothelial cancer associated 1 (UCA1) lncRNA was reported to promote CCA formation and progression by activating a proliferative AKT/GSK-3β/CCND1 axis and by inducing EMT, migration and invasion [18]. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1), a lncRNA induced in several cancers, promotes cell proliferation and invasion of CCA cells by activating the PI3K/AKT pathway as well [19]. While interesting mechanisms of lncRNA action have been elegantly described in CCA carcinogenesis, reports on their clinical relevance are scarce. The expression of few individual lncRNA (e.g. CRNDE, UCA1, TUG1) was reported to correlate with clinical progression (e.g. tumor grade, lymph node metastasis, TNM stage) and was identified as risk factors for an unfavorable overall survival (OS) in iCCA [16,18,20-23]. Circulating lncRNAs, including prostate cancer associated transcript 1 (PCAT1) and MALAT1, have been also reported in the plasma of patients with pCCA [24]. However, besides individual lncRNA, there is no exhaustive report on the clinical relevance of lncRNA expression as prognostic factors in iCCA so far [7,23]. Thus, this study was specifically designed to establish an unsupervised robust signature of lncRNA predictive of overall (OS) and/or disease-free (DFS) survival, by using pan-genomic microarrays and a cohort of clinically well-annotated iCCA.

2. Methods

2.1. Human iCCA samples

A cohort of 39 patients with iCCA was studied. Freshly frozen tumor samples were obtained through the French liver cancer biobanks network - INCa (BB-0033-00085). These patients underwent liver tumor resection for curative intent from January 2007 to July 2014. None of the patient underwent liver transplantation. Six patients underwent chemo- and/or radio-therapy prior surgery. For all cases, iCCA diagnosis was confirmed by a pathological examination. Tissue sections were evaluated after hematoxylin-eosin and Sirius Red staining (SI Fig. 1 in Supplementary material). Written informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008), as reflected in a priori approval by the local institution's human research committee and the institutional review board of Inserm (IRB00003888, IORG0003254). Clinical and pathological features of patients are reported in Table 1.

2.2. Gene expression profiling

Total RNA was extracted from tumor tissue sections and purified with a miRNAeasy kit (Qiagen, Courtaboeuf, France). Genome-wide expression profiling was performed using the low-input QuickAmp labeling kit and human SurePrint G3 8 × 60 K pangenomic microarrays (Agilent Technologies, Santa Clara, USA). Gene expression data were processed using Feature Extraction and GeneSpring softwares (Agilent Technologies), as previously described [6]. Clustering analysis was performed using Gene Cluster and TreeView softwares [25].

2.3. Q-RT-PCR

Quantitative-RT-PCR was performed by using a SYBR Green master mix (Applied Biosystems, Carlsbad, CA) as previously described [6]. A mixture of oligo-dT (250 ng) and random hexamers (100 ng) was used to prime the reverse transcription of

Table 1
Clinical and pathological features of iCCA patients.

Clinical and pathological features	n = 39
Age (years, mean ± SD)	61 ± 14
Gender (male/female)	27/12
BMI	26 ± 3
Tobacco use	18 (46%)
Diabetes	7 (18%)
Etiology	
HBV or HCV infection	5 (13%)
Alcohol	3 (8%)
Hemochromatosis	1 (3%)
Primary biliary cirrhosis	1 (3%)
Histologically normal liver	3 (8%)
Association of factors	6 (15%)
Undetermined	20 (51%)
Metavir fibrosis score (F0/F1/F2/F3/F4/na)	7/6/6/4/6/10
Cirrhosis	6 (15%)
Steatosis	21 (54%)
Tumor size (mm, mean ± SD)	63 ± 27
Tumor ≥ 50 mm	27 (69%)
OMS differentiation grade	
Well-differentiated	15 (38%)
Moderately-differentiated	17 (44%)
Poorly-differentiated	7 (18%)
Satellite nodules > 1	19 (49%)
Tumor necrosis	16 (41%)
Microvascular tumor invasion	20 (51%)
Portal invasion	6 (15%)
Biliary tract invasion	10 (26%)
Perineural invasion	15 (38%)
Capsular invasion	8 (21%)
Lymph nodes invasion	11 (28%)
Extrahepatic tissue invasion	10 (26%)

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Table 2
lncRNA signature predictive of overall (OS) and disease-free (DFS) survival.

Probe ID	Gene	Cytoband	OS		DFS	
			HR ^a	p-value ^b	HR ^a	p-Value ^b
lncRNA up-regulated in poor prognosis iCCA						
A.21.P0006229	lnc-CDK9-1	hs 9q34.11	3.83	0.007	3.65	0.03
A.21.P0012328	XLOC.J2.009441	hs 22q11.1	1.99	0.018	2.01	0.01
A.19.P00322815	CDKN2B-AS1	hs 9p21.3	1.86	0.008	2.06	0.002
A.22.P00019478	HOXC13-AS	hs 12q13.13	1.83	0.047	1.75	0.048
A.21.P0014397	RALY-AS1	hs 20q11.22	24.36	0.002		ns
A.22.P00018189	lnc-ZNF609-1	hs 15q22.31	3.39	0.036		ns
A.22.P00001518	LOC101929064	hs 4q21.3	3.23	0.051		ns
A.23.P431569	LOC100049716	hs 12p13.33	2.12	0.004		ns
A.21.P0010648	XLOC.J2.001086	hs 1q21.1	2.03	0.044		ns
A.32.P850562	ZNF337-AS1	hs 20p11.1	1.88	0.022		ns
A.21.P0004202	lnc-C5orf38-1	hs 5p15.33		ns	3.13	0.009
A.21.P0009376	lnc-NLGN2-1	hs 17p13.1		ns	2.82	0.005
lncRNA down-regulated in poor prognosis iCCA						
A.21.P0005160	lnc-CCHCR1-1	hs 6p21.33	0.72	0.001	0.71	0.005
A.24.P312325	lnc-AF131215.3.1	hs 8p23.1	0.65	0.005	0.56	0
A.21.P0003146	lnc-CBLB-5	hs 3q13.12	0.6	0.02	0.56	0.024
A.22.P00025222	COL18A1-AS2	hs 21q22.3	0.55	0.002	0.55	0.001
A.22.P00012968	lnc-RELL2-1	hs 5q31.3	0.39	0.003	0.38	0.001
A.21.P0005161	lnc-CCHCR1-1	hs 6p21.33	0.59	0.001		ns
A.22.P00004717	lnc-CTDSP2-2	hs 15q21.1		ns	0.37	0.034
A.22.P00014082	HAND2-AS1	hs 4q34.1		ns	0.46	0.049
A.22.P00015084	lnc-SNURF-3	hs 15q11.2		ns	0.49	0.014
A.21.P0008621	lnc-EIF2AK4-2	hs 15q15.1		ns	0.64	0.032
A.22.P00010275	lnc-MTMR9-1	hs 8p23.1		ns	0.68	0.04
A.22.P00017999	lnc-ZNF132-1	hs 19q13.43		ns	0.71	0.046

^a Hazard ratio.^b p-Value, as determined by a log-rank test; ns: not significant.

149 1 µg total RNA (Superscript III RT, Invitrogen, Carlsbad, CA).
 150 Quantitative analysis of PCR data was conducted with the 2^{-ΔΔCt}
 151 method using actin beta (ACTB) Ct values for normalization.
 152 Melting analysis was conducted to validate the specificity of
 153 PCR products. PCR analysis was performed using the following
 154 primers: CDKN2B-AS1-Forward, 5'-TCCAGTGCAAGTATGGTCTGTG-
 155 3'; CDKN2B-AS1-Reverse, 5'-TAGCAGAAAGCTGCAAAGGC-3';
 156 ACTB-Forward, 5'-GGACTTCGACGAAGAGATGG-3'; ACTB-Reverse,
 157 5'-AGCACTGTGTTGGCGTACAG-3'.

158 2.4. Statistical analysis

159 Statistical analysis of microarray data was as previously
 160 described [26]. Notably, lncRNAs significantly associated with OS
 161 and/or DFS were identified using an univariate Cox proportional
 162 hazards model with Wald Statistic [26,27]. Up- and down-regulated
 163 lncRNAs were determined by the ratio of their expression in poor
 164 versus better prognosis iCCA. A meta-analysis of RNA-sequencing
 165 data generated by The Cancer Genome Atlas (TCGA) was also per-
 166 formed to determine the clinical relevance of CDKN2B-AS1/ANRIL
 167 expression in independent datasets. The Kaplan–Meier method
 168 was used to estimate OS and DFS, and group differences were ana-
 169 lyzed with the log-rank test [4,5,28].

170 3. Results

171 3.1. Patient characteristics

172 Thirty nine patients (27 men) who underwent liver resection
 173 with curative intent for iCCA were included in the profiling study
 174 (Table 1). The median age was 61 ± 14 years. Similar to our pre-
 175 vious cohort [5], 69% patients had a tumor with a size >5 cm. The
 176 cohort included 15 (38%) well-differentiated, 17 (44%) moderately-
 177 differentiated, and 7 (18%) poorly-differentiated tumors. Satellite

178 nodules were detected in 49% patients and lymph nodes invasion
 179 in 28% patients.

180 3.2. A lncRNA signature predictive of OS and DFS

181 Gene expression profiling was performed on RNA extracted
 182 from the 39 iCCA cases. In order to identify innovative prognos-
 183 tic biomarkers, statistical analysis was restricted to the differential
 184 expression of lncRNAs. By using an univariate Cox proportional
 185 hazards model with Wald Statistic, 16 and 17 lncRNAs were iden-
 186 tified to be significantly (P < 0.05) associated with OS and DFS,
 187 respectively (Fig. 1A and Table 2). Among those, 9 were predic-
 188 tive of both OS and DFS, including 4 up- and 5 down-regulated
 189 lncRNA in poor prognosis iCCA (Fig. 1A and Table 2). CDKN2B-AS1
 190 and COL18A1-AS2 are two examples of such prognostic lncRNAs.
 191 Indeed, CDKN2B-AS1 median expression was able to segregate iCCA
 192 tumors into two groups, where a high expression of CDKN2B-
 193 AS1 was significantly associated with a reduced OS (P = 0.008)
 194 and DFS (P = 0.002). Conversely, highly COL18A1-AS2 expressing
 195 iCCA tumors were significantly associated with a better prognosis
 196 (Fig. 1B). In addition, clustering analysis of 39 iCCA tumors based
 197 on the expression of the 9 lncRNA expression signature identi-
 198 fied two clusters: cluster A and cluster B (Fig. 1C). Validating this
 199 prognostic lncRNA signature, patients included in clusters A and B
 200 exhibited a significant difference in OS and DFS, as demonstrated
 201 by Kaplan–Meier plot analysis and log-rank testing (Fig. 1D). Clus-
 202 ter A was also associated with a significantly higher number of
 203 poorly-differentiated iCCA (SI Table 1 in Supplementary material).

204 3.3. CDKN2B-AS1 is a poor prognosis biomarker in cancer

205 Quantitative RT-PCR was performed to validate the microarray
 206 data. We focused on CDKN2B-AS1 that exhibited a high variabil-
 207 ity among the samples and the more robust statistical significance
 208 (Fig. 1C and Table 2). Thus, from our initial cohort of 39 iCCA,
 209 two groups were defined based on the expression of CDKN2B-AS1,

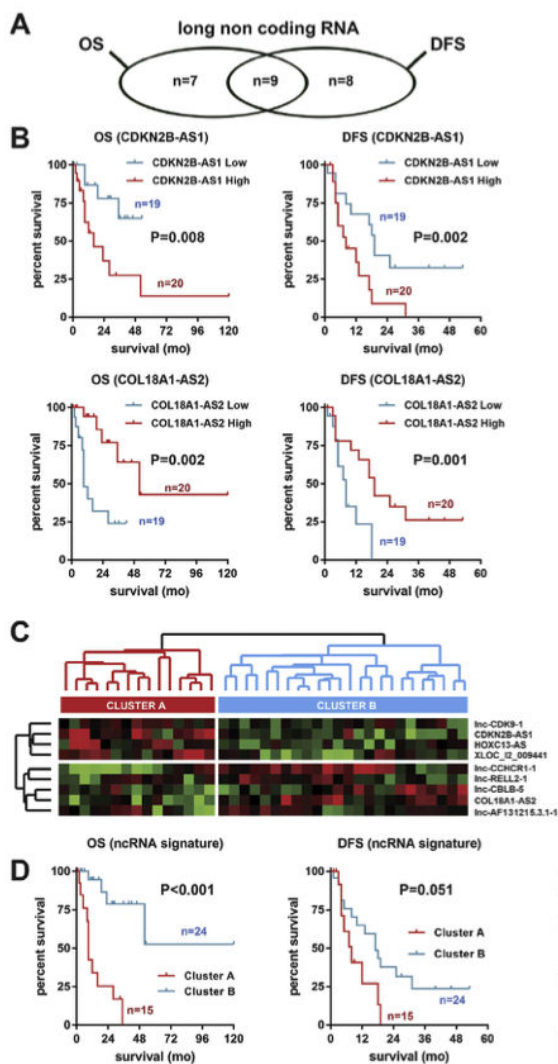


Fig. 1. Identification of a prognostic lncRNA signature in iCCA. Gene expression profiling of lncRNA was performed from 39 resected iCCA tumors. (A) Venn diagram analysis of lncRNA significantly ($P < 0.05$) associated with overall (OS) and/or disease-free (DFS) survival. (B) Example of Kaplan–Meier plots for CDKN2B-AS1 and COL18A1-AS2 expression significantly associated with OS and DFS. (C) Clustering analysis of 39 iCCA samples based on the expression of the 9-lncRNA signature significantly associated with both OS and DFS. Two clusters, A and B, were identified. (D) Kaplan–Meier plots for iCCA samples from cluster A and cluster B.

including 15 highly expressing tumors and 15 tumors with low or no *CDKN2B-AS1* expression (Fig. 2A). Validating our previous observation, a marked difference was observed in the OS of these two groups of patients (Fig. 2A). No significant association with other clinical and pathological variables was identified in our cohort of iCCA (SI Table 2 in Supplementary material). More interestingly, a high expression of *CDKN2B-AS1* was shown to be significantly ($P < 0.05$) associated with a reduced survival of patients with hepatocellular carcinoma, kidney renal clear cell carcinoma and uterine corpus endometrial carcinoma (Fig. 2B), identifying *CDKN2B-AS1* as a poor prognosis biomarkers in several cancers.

4. Discussion

CCA is a deadly cancer worldwide waiting for innovative biomarkers and therapeutic strategies to improve the survival of patients [1,2]. In iCCA, several clinical variables have been identified

as important prognostic factors after surgical resection for curative intent, including tumor size, pathological lymph node involvement and vascular invasion [1,29,30]. Omics approaches (e.g. genomics, proteomics, metabolomics) have been extensively used to screen for novel candidate serum, bile and tissue molecular biomarkers in iCCA [30]. As an example, by proteomic analysis, a high expression of SSP411 in the serum and/or the bile has been significantly associated with iCCA diagnosis [31]. Serum and bile CA 19-9 level has been also associated with lymph node metastasis and OS although the specificity and sensitivity was highly heterogeneous [30]. By focusing more spec molecular biomarkers in iCCA [30]. As an example, by proteomic analysis, a high expression of SSP411 in the serum and/or the bile has been significantly associated with iCCA diagnosis [31]. Serum and bile CA 19-9 level has been also associated with lymph node metastasis and OS although the specificity and sensitivity was highly heterogeneous [30]. By focusing more specifically on the alterations of the gene expression in the stroma of iCCA [4,32], we previously identified and validated specific mRNA and associated proteins, including OPN, LOXL2 and epithelial cell adhesion molecule (EPCAM), as independent prognostic tissue biomarkers associated with a reduced OS of patients with iCCA after tumor resection [4,5,28]. Here, we specifically focused on the expression of lncRNA and we identified a set of lncRNA predictive of OS ($n = 16$) and/or DFS ($n = 17$), among those 9 were predictive of both OS and DFS. Except for *HAND2-AS1*, *HOXC13-AS*, and *CDKN2B-AS1*, the identified lncRNA were poorly characterized. Our data indicated that *HAND2-AS1* down-regulation in poor prognosis iCCA is predictive of DFS. Accordingly, *HAND2-AS1* has been recently shown to inhibit cancer cell proliferation, migration, and invasion in esophagus squamous cell carcinoma and colorectal cancer by sponging oncogenic microRNA, including miR-21 [33,34]. *HOXC13-AS* (up-regulated in poor prognosis iCCA from our lncRNA signature) has been shown to positively affects cell proliferation and invasion in nasopharyngeal carcinoma by sponging miR-383-3p [35]. *CDKN2B-AS1*, also known as antisense non-coding RNA in the INK4 locus (*ANRIL*), was identified as a prognostic biomarker, not only in iCCA, but also in hepatocellular, kidney and uterine corpus carcinomas. Very promisingly, the low expressing group in iCCA showed a very high long term survival. In clinical context, one can question the need for adjuvant chemotherapy in a population with such high survival, potentially sparing toxicity for patients who do not require such therapy.

ANRIL is located at the *CDKN2 A/B* genomic locus on chromosome 9p21.3 in human [36]. Interestingly, this locus is associated with an increased risk of cancer and metabolic disease, including type 2 diabetes, obesity and cardiovascular diseases [37]. *ANRIL* expression and function have been extensively investigated in several cancers but not in iCCA [38]. Here, we demonstrated that *ANRIL* expression is associated with a dismal outcome. Although clinically relevant differences in the expression of *ANRIL* expression have been highlighted in iCCA tumor tissues in our study, it remains to be determined whether *ANRIL* is up-regulated in iCCA as compared to normal biliary epithelium, as it was previously reported in other cancers, including HCC [39]. Such an induction of *ANRIL* may suggest a key role in iCCA carcinogenesis. Indeed, gain and loss of function experiments in cancer cells demonstrated that *ANRIL* lncRNA favors cell proliferation and invasion. *In vivo*, *ANRIL* expression is associated with an increase of tumor size and growth, as well as invasive and metastatic phenotypes. Mechanistically, *ANRIL* was initially shown to promote cell proliferation and to inhibit apoptosis by epigenetically silencing the *CDKN2 A/B* genomic locus [40]. In non-small cell lung cancer *ANRIL* was reported to silence krupel like factor 2 (*KLF2*) and *CDKN1 A/P21* expression as well [41]. In gastric cancer, the impact of *ANRIL* on proliferation and apoptosis was mediated by the epigenetic silencing of miR-99a and miR-449a, two miRNAs known to target mTOR and *CDK6/E2F1* pathways

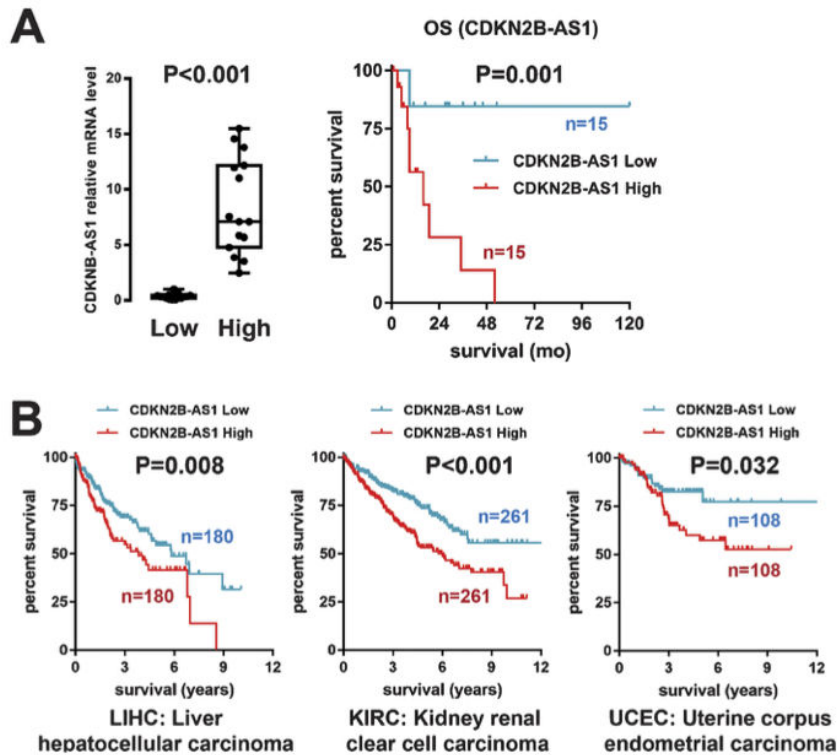


Fig. 2. CDKN2B-AS1 expression predicts a poor prognosis in several cancers. (A) Q-RT-PCR of CDKN2B-AS1 was performed in iCCA cases that were further ranked into low (n = 15) and high (n = 15) expressing tumors. Kaplan-Meier plots for iCCA classified into low vs. high CDKN2B-AS1 expressing tumors. (B) Kaplan-Meier plot analysis (OS) based on the expression of CDKN2B-AS1 in independent gene expression datasets from the TCGA consortium.

[42]. In ovarian [43] and bladder [44] cancers, ANRIL may induce BCL2 apoptosis regulator (BCL2) and repress CDKN2B/P15, BCL2 associated X, apoptosis regulator (BAX) and cleaved caspase-9, to promote cell cycle progression and inhibit apoptosis and senescence. In nasopharyngeal carcinoma, pro-tumorigenic features of ANRIL were associated with a glucose metabolism reprogramming and an induction of side-population stem-like cancer cells [45]. In thyroid cancer, ANRIL promotes tumor cell invasion and metastasis through TGF- β /Smad signaling and EMT [46]. In HCC, sponging of liver enriched miR-122-5p by ANRIL induces cell proliferation, metastasis and invasion [47]. Interestingly, ANRIL was reported to contribute to paclitaxel resistance in lung cancer, partly through poly(ADP-ribose) polymerase (PARP) and BCL2 modulating mitochondrial pathway [48].

The clinical relevance of ANRIL expression has been evaluated in several types of cancers. Thus, a high ANRIL expression has been associated with TNM stage, advanced lymph node metastasis, tumor size, and was highlighted as an independent predictor of OS and/or DFS, particularly in nasopharyngeal [45], ovarian [43] or lung [41,49] cancers. Interestingly, it was recently reported that ANRIL was significantly up-regulated in lung cancer tissues and serum samples compared with normal controls, suggesting that ANRIL may serve as a relevant circulating lncRNA biomarker [50]. Given the significant association of ANRIL and prognosis factors, it would be interesting to evaluate its expression in body fluids, including serum, urine and/or bile. Another point of interest is to understand the mechanisms by which the expression of ANRIL is increased in poor prognosis tumors. External risk factors, including lifestyle, nutrition or environment, have been shown to influence the expression of lncRNA [51]. Thus, HOTAIR and MALAT1 have been the most studied lncRNA in the context of tobacco use in lung cancer [51]. However, in our analysis, no statistically significant

association was identified between the expression of ANRIL and lifestyle-associated factors (e.g. obesity, diabetes, tobacco, alcohol). Interestingly, ANRIL was shown to be transcriptionally induced by the cell cycle associated E2F1 transcription factor [42], notably in an ATM-dependent manner following DNA damage [52], suggesting a positive regulation in highly proliferative tumors. In addition, MYC oncogene was shown to positively regulate ANRIL expression in lung cancer [53]. Epigenetic regulation of ANRIL through promoter methylation has been also reported [54].

Altogether, these data suggest that ANRIL may represent not only a clinically relevant biomarker for cancer diagnosis, prognosis and response to treatment, but also an innovative therapeutic target due to the well described pro-tumorigenic features of this lncRNA.

Conflicts of interest
None declared.

Funding

This work was supported by Inserm, Université de Rennes 1, INCa, and ITMO Cancer AVIESAN (Alliance Nationale pour les Sciences de la Vie et de la Santé) dans le cadre du Plan cancer (Non-coding RNA in cancerology: fundamental to translational; [Grant C18007NS to CC]). CL is supported by a PhD fellowship from Université de Rennes 1.

Ethical statement

The study was approved by the institutional review board of Inserm (IRB00003888).

Please cite this article in press as: Angenard G, et al. Expression of long non-coding RNA ANRIL predicts a poor prognosis in intrahepatic cholangiocarcinoma. Dig Liver Dis (2019), <https://doi.org/10.1016/j.dld.2019.03.019>

Acknowledgements

The authors thank the core facilities of Université de Rennes 1, Biosit (US18, UMS 3480) and Biogenouest (CRB Santé BB-0033-00056, GEH and H2P2), and the French liver cancer biobanks network – Institut National du Cancer (INCa) BB-0033-00085, including Beaujon, Bordeaux, Mondor, Nantes, Paris-Sud and Rennes biobanks. CC and JE are members of the European Network for the Study of Cholangiocarcinoma (ENSCCA) and participate in the initiative COST Action EURO-CHOLANGIO-NET granted by the COST Association (CA18122).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.dld.2019.03.019>.

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3. Article n°3

Revue de la littérature en préparation

«ARN longs non-codants et cholangiocarcinome »

Cette revue de la littérature présente les ARNInc identifiés dans le cholangiocarcinome selon leur rôle dans la cancérogénèse. Les ARNInc identifiés jusqu'à présent sont tous surexprimés. Les mécanismes d'action de certains d'entre eux, e.g. NEAT1, par modulation épigénétique ont été mis en évidence. Des liens entre des ARNInc et plusieurs voies de signalisation agissant sur la TEM et l'apoptose ont été décrits. Quelques variations génomiques d'ARNInc ont été identifiées. Enfin, la pertinence clinique des ARNInc comme biomarqueurs ou cibles thérapeutiques a été recherchée dans de nombreuses études.

Review

Long non-coding RNAs in cholangiocarcinoma

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Acknowledgements

Funding: This work was supported by Inserm, Université de Rennes 1, and ITMO Cancer AVIESAN (Alliance Nationale pour les Sciences de la Vie et de la Santé) dans le cadre du Plan cancer (Non-coding RNA in cancerology: fundamental to translational; [Grant C18007NS to CC]). CC is a member of the European Network for the Study of Cholangiocarcinoma (ENSCCA) and participates in the initiative COST Action EURO-CHOLANGIO-NET granted by the COST Association (CA18122).

Introduction

Cholangiocarcinomas (CCA) are malignant tumors originating from the bile ducts and exhibiting cholangiocyte differentiation features. CCA are classified into three types according to the anatomical location: intrahepatic, perihilar and distal CCA. Intrahepatic CCA represent the second primary hepatic malignancy after hepatocellular carcinoma (HCC). CCA are aggressive and poor prognosis neoplasms with a median survival of 24 months after the initial diagnosis. Despite the recent identification of molecular alterations involved in CCA pathogenesis, there is no established molecular targeted therapy that significantly increases patient survival yet. Surgical resection remains the only potential curative treatment when feasible and performed at early stage of the disease ¹. Thus, a better understanding of molecular pathogenesis of CCA together with the identification of robust biomarkers and therapeutic targets is still necessary to improve the management of CCA.

Non coding RNAs (ncRNAs) belong to a heterogeneous class of RNA molecules. They account for the large majority of RNAs produced in human cells and tissues and are involved in numerous biological and pathological processes, including cancer onset and progression ². To date, ncRNAs are usually classified based on their size and structure and included microRNAs (miRNAs), long non coding RNAs (lncRNAs) and circular RNAs (circRNAs). miRNAs have been largely studied in CCA. For example, miR-21 is a well-known oncogenic miRNA overexpressed in CCA tumors and involved in the response to chemotherapy. miR-21 modulates gemcitabine-induced apoptosis by phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-dependent activation of PI3K signaling ^{3,4}. Its overexpression in plasma makes it a potential circulating biomarker for CCA diagnosis and prognosis ⁵. The first lncRNAs implicated in liver carcinogenesis were described in HCC (e.g. lncRNAs HULC, H19, MALAT1) and have been further studied in many others organs and pathologies ⁶.

However, lncRNAs remain poorly studied in CCA. lncRNAs are defined by a size greater than 200 nucleotides and the lack of a functional open reading frame. They are transcribed

by RNA polymerase II and can be polyadenylated. Transcriptome analysis by deep sequencing and bioinformatics approaches have shown that the number of lncRNAs is greater than the one of protein coding mRNAs. Genomic location and organization of lncRNAs is complex and can be described according to their relationship to protein-coding genes: intergenic, sense, antisense, intronic and bidirectional ⁷. Several lncRNAs have been functionally involved in pluripotency, differentiation, proliferation or cell survival although the function of most of the lncRNAs remains largely unknown. They show tissue specific expression and are tightly regulated at multiple levels, involving chromatin marks, independent promoters, regulation by transcription factors and splicing of multiple exons into a mature transcript. lncRNAs are located into the nucleus, the cytoplasm and could be also secreted, notably through extracellular vesicles (EVs). Based on their cellular location, lncRNAs have been shown to modulate gene expression, notably by acting as chromatin remodeling factors or by interacting with transcription factors ⁸. lncRNAs have been also reported to modulate post-transcriptional mRNA processing, protein function or localization and intercellular signaling through EVs ⁹. In the cytoplasm, lncRNAs and circRNAs could interact directly with miRNA at post transcriptional level as competing endogenous RNA (ceRNA) (sponge for miRNAs) ^{10,11}.

This review presents lncRNAs identified in CCA according to their role in carcinogenesis.

lncRNAs and epigenetic modulation

In the nucleus, lncRNAs could act at epigenetic level, mainly as scaffolding for chromatin or histone modifiers. Polycomb repressive complex 2 (PRC2) is a protein complex involved in methylating lysine 27 on histone H3 (H3K27). EZH2 is the catalytic subunit of PRC2. BAP1, as a chromatin modulator, can involve interactions with several methylation and deacetylase components and result in modulation of gene expression. lncRNA NEAT1, a lncRNA upregulated in CCA and a downstream-target of BAP1, is involved in responses to therapy as well as in maintenance of phenotypic characteristics such as the proliferative, migratory and invasive capabilities of CCA cells ¹². Exogenous silencing of NEAT1 expression using

siRNA in CCA cells reduced cell proliferation, migration, invasion and colony-forming abilities¹². The effect of NEAT1 on modulating EZH2 expression further supports an effect of this lncRNA on tumor growth¹². EZH2 recruitment by NEAT1 and subsequent involvement of the complex in the repression of E-cadherin promote CCA migration and invasion. Depletion of NEAT1 causes a reduction in EZH2 binding to the E-cadherin promoter and increases E-cadherin transcription. EZH2 suppresses gene expression through H3K27 trimethylation (H3K27me3). ChIP using an H3K27me3 antibody found decreased enrichment of H3K27me3 at the E-cadherin promoter. Taken together, these data suggest that EZH2 is recruited by NEAT1 to the promoter of E-cadherin and that together they repress E-cadherin expression¹³. NEAT1 can also regulate gene expression through its involvement in paraspeckle nuclear body formation¹⁴.

LncRNA SNHG1, upregulated in CCA, promotes CCA malignancy through a bond with EZH2, followed by repressing the expression of the CDKN1A. Knockdown of the SNHG1 lowered the binding of EZH2 as well as H3K27me3 levels at CDKN1A promoter, resulting in CDKN1A increased transcription and subsequent protein level, thus decreasing CCA growth through cell cycle inhibition¹⁵.

LncRNA SPRY4-IT1 might epigenetically repress underlying targets expression at transcriptional level. SPRY4-IT1, up regulated in CCA, exerts oncogenic properties partly dependent on repressing tumor suppressors KLF2 and LATS2 expression by scaffolding EZH2¹⁶. SPRY4-IT1 also functioned as a molecular sponge for miR-101-3p, antagonizing its ability to repress EZH2 protein translation¹⁶.

LncRNAs and deregulation of signaling pathways

Epithelial-mesenchymal transition, migration and invasion

Several ncRNAs have been shown to play a key role in migration, invasion and metastatic process in CCA, notably by contributing to epithelial-mesenchymal transition (EMT). As

described above, lncRNA NEAT1 is one of the lncRNAs involved in the repression of E-cadherin to promote CCA migration and invasion¹³. In vitro, the knockdown of numerous upregulated lncRNAs (e.g. HOTAIR, CRNDE, PANDAR, TUG1, CCAT1, CCAT2, SPRY4-IT1, UCA1) also reduced the expression of mesenchymal markers (Vimentin, N-cadherin), and increased the expression of epithelial marker E-cadherin, resulting in EMT reversion¹⁶⁻²³. The oncogenic effects of lncRNAs through EMT are frequently mediated by acting as ceRNA (e.g. CCAT1, SPRY4-IT1, TUG1)^{16,21,24}. As an example the oncogenic effects of CCAT1 in ICC cells are at least partly through inhibiting miR-152²¹. miR-152 inhibitor rescued the cell migration and invasion capability of ICC cells after CCAT1 knockdown. In CCAT1 silenced cells, miR-152 inhibitor increased the expression of N-cadherin and Vimentin, but reduced the expression of E-cadherin²¹.

Multiple lncRNAs have been identified as regulators or mediators of oncogenic pathways implicated in EMT. lncRNA MALAT1 has a key role in multiple human cancers and is upregulated in CCA. In hilar cholangiocarcinoma cells (HCCA), MALAT1 knockdown could significantly suppress proliferation, migration and invasion²⁵. A positive correlation between MALAT1 expression and CXCR4 expression has been demonstrated in HCCA tissues. Acting as a ceRNA, MALAT1 regulates negatively miR-204 expression in HCCA cells. MALAT1 exerts its promotive functions in HCCA cell proliferation, migration and invasion by miR-204-dependent CXCR4 regulation²⁵.

In 2016, Wang et al. analyzed 5 RNAseq datasets in order to identify lncRNAs implicated in inflammation and oxidative stress in CCA. H19 and HULC were up-regulated after both short and long-term oxidative stress, implying their pivotal roles in inflammation promotion and CCA pathogenesis. H19 and HULC regulate cell migration and invasion by increasing pivotal inflammation cytokine IL-6 and chemokine receptor CXCR4 through sponging let-7a/let-7b and miR-372/miR-373, respectively²⁶. Xu et al. confirmed in 2017 that H19 promotes cell migration and invasion. Knockdown of H19 decreased proliferation, increased apoptosis and reversed EMT in CCA cells²⁷.

Transforming Growth Factor beta (TGF β) pathway is tightly implicated in EMT. TLINC is a lncRNA induced by TGF β and upregulated in human intrahepatic CCA. Its long isoform is associated with a migratory phenotype in CCA cells and promotes an inflammatory microenvironment through upregulation of IL8 both in vitro and in resected human intrahepatic CCA ²⁸.

LncRNA PCAT1 is upregulated in extrahepatic cholangiocarcinoma and regulates its progression via the Wnt/ β -catenin signaling pathway ²⁹. Downregulation of PCAT1 following transfection with silencing RNA reduced ECC cell growth, migration and invasion. PCAT1 acts as a ceRNA against miR-122. WNT1 mRNA levels were significantly reduced upon miR-122 mimic transfection, whereas transfection of a miR-122 inhibitor increased WNT1 mRNA level. In ECC cells, PCAT1 depletion dramatically decreased β -catenin levels, and increased GSK3 β levels. In ECC cells co-transfected with miR-122 inhibitor and PCAT1 siRNA, protein level of β -catenin was restored and colony formation increased. PCAT1 regulates Wnt/ β -catenin-signaling pathway and cell growth via miR-122 ²⁹.

Apoptosis and survival

The majority of lncRNAs promote cell proliferation and downregulate cell apoptosis in CCA. Thus, knockdown of several upregulated lncRNAs, including HOTAIR, PANDAR, CCAT2, PCAT1 and TP73-AS1, increases the expression of caspase-3 and caspase-9 ^{17,19,22,30}. LncRNAs PANDAR and TUG1 also restrained the expression of Bcl-2 and increased the expression of Bax in CCA cells ^{19,20}. However, the exact mechanisms are not described.

LncRNA UCA1, upregulated in CCA, affects EMT but also cell apoptosis and cell cycle in vitro in CCA cells through the activation of AKT/GSK-3 β /CCND1 signaling pathway ²³. PI3K/AKT pathway is also activated by MALAT1 and SOX2-OT to promote cholangiocarcinoma cell proliferation and invasion ³¹⁻³³.

MAP kinase pathway has a critical role in regulating cell apoptosis and proliferation. LncRNA AFAP1-AS1 is upregulated in CCA. AFAP1-AS1 silencing resulted in a significant decrease in cell migration and invasion, cell proliferation and colony formation with downregulation of MMP2 and MMP9. Thus, AFAP1-AS1 silencing resulted in suppression of proliferation by arresting cell cycle via decreases of the protein levels of Cyclin D1 and c-Myc^{34,35}. Both tumor volume and tumor weight were significantly decreased in mice xenografted with CCA cells with knocked down AFAP1-AS1. Tumors had also weaker Ki67 and PCNA expression. The tumor metastatic effects of AFAP1-AS1 may be mediated by the altered AFAP1 protein levels³⁵.

LncRNAs could also alter gene expression by modulating transcription factors. Li et al. demonstrated by RNA immunoprecipitation that lncRNA EPIC1, upregulated in CCA, directly targets the transcription factor MYC in CCA cells. MYC is a key transcription factor regulating the expression of a wide range of genes involved in cell growth, proliferation and survival. Accordingly, MYC targets, including Cyclin A/D and CDK9, were downregulated in cells transfected with EPIC1 siRNA. MYC knockout also suppresses CCA cell growth, colony formation and apoptosis³⁶.

LINC01296 was demonstrated to sponge miR-5095, which targets MYCN mRNA (MYCN proto-oncogene bHLH transcription factor) in human CCA. By inhibition of miR-5095, LINC01296 overexpression upregulated the expression of MYCN and promoted cell viability, migration and invasion in CCA cells³⁷.

LncRNAs and genetic instability

Genomic variations and/or alterations such as single nucleotide polymorphisms (SNPs), mutations and gene fusions referring to coding regions are common events in human carcinogenesis. LncRNAs which are less conserved across species are even more susceptible to genomic variations. Fujimoto et al. provided in 2016 a comprehensive whole-genome mutational analysis of 300 liver tumors from Japanese patients including CCA,

combined CCA-HCC and a majority of HCC ³⁸. The analysis revealed NEAT1 and MALAT1 to be prominently mutated among the non-coding genes in liver tumors. The lncRNASNP is a database of SNPs in lncRNAs in human and mouse ³⁹. HULC and MALAT1 SNPs were found implicated in HCC carcinogenesis ⁴⁰. However, no lncRNA SNPs were reported so far in CCA carcinogenesis.

Clinical relevance of lncRNA expression

LncRNAs as biomarkers

Multiple studies focused on evaluating miRNA abundance in tissues, serum and bile as diagnostic biomarkers in CCA ⁴¹. Some of them were also identified as promising prognostic biomarkers in serum ^{42,43}. To date, all the variations reported in the expression of lncRNAs in CCA tissues are an overexpression compared to the adjacent non tumoral tissues. Thus, most of the lncRNAs are overexpressed in CCA cells and tissues and were identified as regulators of carcinogenesis processes (e.g. epigenetic deregulation, EMT, apoptosis). This overexpression is also frequently associated with a poor prognosis (H19, SOX2OT, CRNDE, UCA1, HOTAIR, GAPLINC) ^{17,18,23,27,33,44} and poor histologic features (CCAT1, AFAP1-AS1, PANDAR, TUG1, SPRY4-IT1, TP73-AS1, LINC01296, CPS1-IT1) ^{16,20,21,24,30,34,35,37,45,46,47}. Some lncRNAs are not yet relevant for prognosis and just potential diagnostic biomarker (e.g. NEAT1, SNHG1, PCAT1, EPIC1, TLINC, MALAT) ^{13,15,29,36}. However, ideal biomarkers are those obtained with non-invasive tests from body fluids, such as serum. In a recent meta-analysis, lncRNAs were found to be of high diagnostic value for HCC and their expression could potentially be used as auxiliary biomarkers in confirming HCC diagnosis (e.g. UCA1, MALAT1, SPRY4-IT1) ⁴⁸. A recent study found circulating PCAT1, MALAT1 and CPS1-IT1 significantly increased in plasma samples of HCCA patients ⁴⁹.

LncRNAs as therapeutic targets

The capability of lncRNAs to regulate gene expression makes them potential targets for therapeutic research. However, actual drugs are unable to directly target lncRNAs. Promising strategies are in development including siRNA mediated silencing and lncRNA knockdown by antisense oligonucleotides to target oncogenic lncRNAs⁸. The ability of lncRNAs to sponge miRNAs could be also exploited to sequester oncogenic miRNAs. This mechanism has been used experimentally with an artificial lncRNA to overcome sorafenib resistance in HCC cells⁵⁰. In CCA, few lncRNAs were found to influence current therapeutics. NEAT1 is a downstream target of BAP1, a frequently mutated chromatin modulator, and contributes to sensitivity to gemcitabine in CCA cells¹². Inhibition of NEAT1 restored cells response to gemcitabine and could be a potential sensitizer.

New players in the field: circRNAs

In our work, we highlighted circular isoforms of TLINC, a TGF β induced lncRNA, in CCA tissues²⁸. CircRNAs are particular ncRNAs mostly greater than 200 nucleotides characterized by covalently closed loop structures with neither 5' to 3' polarity nor polyadenylated tail⁵¹. This structure is formed by a back-splicing that ligates a downstream splice donor site reversely with an upstream splice acceptor site⁵². This circular structure renders circRNA particularly resistant to the action of RNAses. Thus, circRNAs may represent interesting stable biomarkers in tissues or circulating in EVs such as exosomes detectable in serum⁵⁶.

Some circRNA were already identified as potential molecular biomarker in CCA e.g. CircRNA Cdr1as and Hsa_circ_0001649^{53,54}. Hsa_circ_0001649 is aberrantly downregulated in CCA tissues and cells, and its downregulation is associated with tumor size and differentiation grade in CCA⁵⁴. Overexpressed circRNA Cdr1as may serve as a potential molecular biomarker to predict the aggressive tumor progression and worse prognosis for CCA patients

⁵³. Circ2174, an intergenic circular RNA, can act as a sponge to regulate the expression of miR149 ⁵⁵.

Conclusion

CCA are aggressive neoplasms resulting from a complex multifactorial carcinogenesis process. LncRNAs are implicated in a large variety of functions in cancer development. Their potential interactions with DNA, RNA and protein result in regulation at epigenetic, transcriptional and post-transcriptional levels. They act as regulators of key processes in cancer, including EMT, apoptosis, and proliferation. Thus, lncRNAs represent a promising class of biomarkers for diagnosis and prognosis of CCA, as well as interesting therapeutic targets.

Table: List of lncRNAs induced in ICC

LncRNA name	Mecanismos of action	Biological functions	Related pathways	Targets	Biomarker	References
NEAT1	epigenetic, mutation	proliferation, migration, invasion, EMT, resistance to therapeutic		EZH2, E-cadherin	diagnostic	12,13,14
SNHG1	epigenetic, ceRNA	proliferation, invasion, cell cycle	TLR4/NF-kB	EZH2, CDKN1A, miR-140	diagnostic	15
SPRY4-IT1	epigenetic, ceRNA	proliferation, migration, invasion, EMT, apoptosis		EZH2, KLF2, LATS2, miR-101-3p	diagnostic	16
HOTAIR		migration, invasion, EMT, apoptosis			diagnostic, prognostic	17
CRNDE		proliferation, migration, invasion, EMT			diagnostic, prognostic	18
PANDAR		proliferation, migration, invasion, EMT, apoptosis			diagnostic, prognostic	19,46
TUG1	ceRNA	proliferation, migration, invasion, EMT, apoptosis		miR-145, Sirt3, GDH	diagnostic, prognostic	20,24
CCAT1	ceRNA	migration, invasion, EMT		miR-152	diagnostic, prognostic	21,45
CCAT2		migration, invasion, EMT, apoptosis			diagnostic	22
UCA1		proliferation, migration, invasion, EMT, apoptosis, cell cycle	AKT/GSK-3β/CCND1		diagnostic, prognostic	23
MALAT1	mutation, ceRNA	proliferation, migration, invasion	CXCR4, PI3K/AKT	miR-204	diagnostic	25,31
H19	ceRNA	proliferation, migration, invasion, EMT, apoptosis	CXCR4/IL6	let-7a, miR-372	diagnostic, prognostic	26,27
HULC	ceRNA	migration, invasion	CXCR4/IL6	let-7b, miR-373	diagnostic	26,4
TLINC /CASC15		migration	TGFβ		diagnostic	28
PCAT1	ceRNA	proliferation, migration, invasion, apoptosis	Wnt/β-catenin	miR-122	diagnostic	29
TP73-AS1		migration, invasion, EMT, apoptosis			diagnostic	30
SOX2-OT		proliferation, invasion	PI3K/AKT		diagnostic, prognostic	32,33
AFAP1-AS1		proliferation, migration, invasion, cell cycle	MAPK		diagnostic, prognostic	34,35
EPIC1	transcription factor	proliferation, apoptosis		MYC, cyclin A/D, CDK9	diagnostic	36
LINC01296	ceRNA	migration, invasion		MYC, miR-5095	diagnostic, prognostic	37
GAPLINC		proliferation, migration, invasion			diagnostic, prognostic	44
CPS1-IT1		proliferation, apoptosis			diagnostic, prognostic	47

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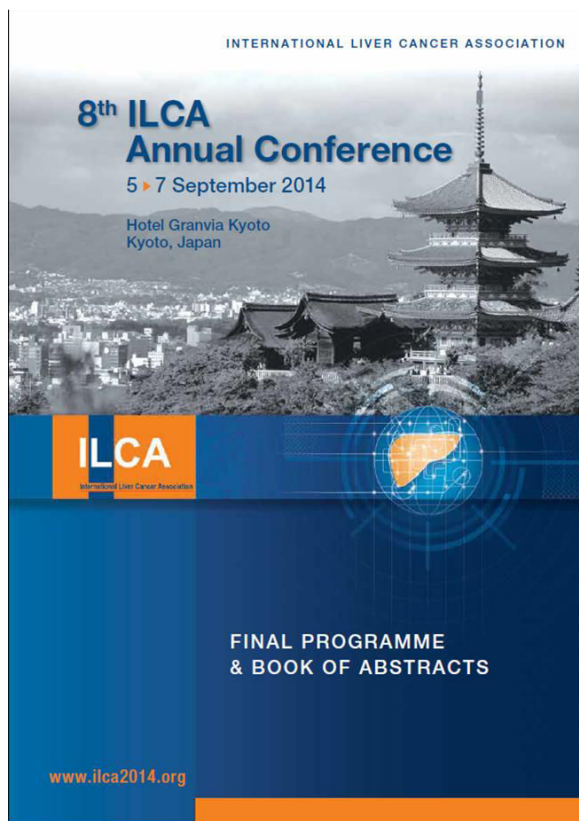
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4. Communications

e-Poster au 8^{ème} congrès annuel de l'International Liver Cancer Association (ILCA) en septembre 2014 à Kyoto, Japon



P-017 FUNCTIONAL CHARACTERIZATION OF T-LINC-1, A TGF-BETA-REGULATED LONG NON CODING RNA IN HUMAN INTRAHEPATIC CHOLANGIOCARCINOMA

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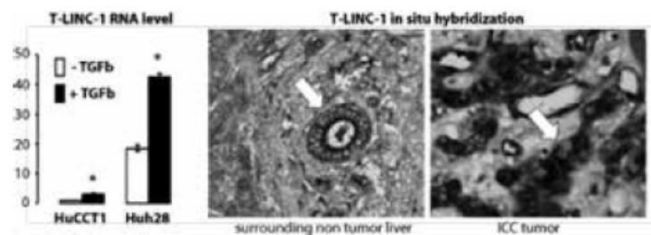
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Introduction: Intrahepatic cholangiocarcinoma (ICC) is the second most common type of primary cancer in the liver after hepatocellular carcinoma (HCC). ICC is an aggressive malignancy with a poor prognosis and limited therapeutic options. The molecular mechanisms contributing to ICC pathogenesis are not well understood. Recently, we demonstrated that the overexpression of TGF-beta in the stroma of ICC was significantly correlated with a poor prognosis, suggesting that TGF-beta contributed to ICC progression. These results prompted us to investigate the role of the TGF-beta and associated target genes, including long non coding RNA, in ICC.

Methods: In vitro experiments were conducted in HuCCT1 and Huh28 ICC cell lines stimulated by TGF-beta. Gene expression profiling was done using Agilent pangenomic microarrays. The expression of relevant target genes was validated at the RNA and protein levels by RT-PCR and Western blot, respectively. Cellular localization of long non coding RNA was determined by in situ hybridization in human ICC. Functional analysis (e.g. proliferation and migration assays) was done following gain and loss of function experiments using expression plasmids and siRNA.

Results: TGF-beta associated gene signatures were established in HuCCT1 and Huh28 cell lines. The deregulation of known and new TGF-beta target genes was validated by RT-PCR, including T-LINC-1, a long non coding RNA strongly up-regulated by TGF-beta. In vitro, T-LINC-1 was found induced by TGF-beta not only in ICC cells but also in HCC cells and hepatic stellate cells. *In vivo*, gene profiling of microdissected ICC demonstrated that T-LINC-1 was up-regulated both in tumor cholangiocytes and in stromal cells. Cell fractionation and in situ hybridization demonstrated that T-LINC-1 was predominantly localized in the cytoplasm and suggested a role in the control of translation, as it was reported for other long non coding RNA (e.g. microRNA precursors or sponges). Interestingly, the predicted secondary structure of T-LINC-1 highlighted a hairpin-like sequence that may serve as a precursor for a microRNA known to target the cell cycle regulator P21. Preliminary results obtained following gain and loss of function demonstrated that T-LINC-1 greatly impacted the expression of P21.

Image:



Conclusion: The study identified the first TGF-beta regulated long non coding RNA which may control the cytostatic effects of TGF-beta in ICC. Thus, deregulation of T-LINC-1 may participate in the oncogenic switch of TGF-beta in cancer cells.

Disclosure of Interest: None Declared

Communication orale aux 75^{ème} journées scientifiques de l'Association Française pour l'Etude du Foie (AFEF) en septembre 2014 à Paris



Identification et caractérisation fonctionnelle de T-linc1, un ARN non codant de longue taille induit par le TGF- β dans le cholangiocarcinome intrahépatique

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Introduction

Le cholangiocarcinome intrahépatique (CCI), seconde tumeur primitive hépatique après le carcinome hépatocellulaire (CHC), est caractérisé par un mauvais pronostic, des options thérapeutiques limitées et une mauvaise connaissance des mécanismes de cancérogenèse. Récemment, nous avons démontré qu'une surexpression de TGF- β dans le stroma du CCI est corrélée à un mauvais pronostic (i.e. survie globale réduite). Dans ce contexte, l'objectif de notre travail est d'étudier le rôle de la voie TGF- β et de ses gènes cibles dans la cancérogenèse du CCI.

Matériels et Méthodes

Une analyse transcriptomique a été réalisée sur les lignées cellulaires HuCCT1 et Huh28 stimulées par le TGF- β . L'expression des gènes cibles a été validée par RT-PCR et leur localisation cellulaire a été déterminée par hybridation *in situ* sur des coupes de CCI. L'analyse fonctionnelle a été réalisée après modulation de l'expression des gènes cibles par transfection de plasmides et de petits ARN interférents.

Résultats

Les signatures d'expression génique associées au TGF- β ont été établies dans les lignées HuCCT1 et Huh28. La dérégulation de plusieurs gènes cibles (e.g. SMAD7, SNAI1) a été validée par RT-PCR, dont T-linc1, un ARN non codant de longue taille dont la fonction était complètement inconnue. *In vitro*, T-linc1 est induit par le TGF- β dans les lignées de CCI mais également dans les lignées de CHC et les cellules étoilées hépatiques. *In vivo*, l'étude de CCI réséqués montre que T-linc1 est surexprimé dans les cholangiocytes tumoraux et le stroma. L'hybridation *in situ* révèle une prédominance de T-linc1 dans le cytoplasme, suggérant un rôle dans le contrôle de la traduction. De façon intéressante, la structure secondaire prédite de T-linc1 met en évidence une séquence qui pourrait servir de précurseur à un microARN connu pour cibler le régulateur du cycle cellulaire P21. Les résultats obtenus après gain et perte de fonction montrent que la modulation de T-linc1 influence l'expression de P21.

Conclusion

Notre étude est la première à identifier un ARN non codant de longue taille dont l'expression est contrôlée par le TGF- β et qui pourrait contrôler ses effets cytotostatiques dans le CCI. Plus largement, la dérégulation de T-linc1 pourrait participer à orienter la voie du TGF- β vers son versant oncogénique au cours de la progression tumorale.

Présentation à l'Académie Nationale de Chirurgie en 2015 suite à l'obtention du Prix parcours cancérologie 2014 du Master 2 Sciences Chirurgicales



ACADÉMIE NATIONALE
de CHIRURGIE
French Academy of Surgery

« Identification et caractérisation de T-LINC 1, un ARN non codant de longue taille régulé par le TGF β dans le cholangiocarcinome intrahépatique »

Le cholangiocarcinome intrahépatique (CCI) est caractérisé par un mauvais pronostic, des options thérapeutiques limitées et une mauvaise connaissance des mécanismes impliqués dans sa cancérogenèse. Il a été démontré récemment qu'une surexpression de TGF β dans le stroma du CCI est corrélée à un mauvais pronostic, suggérant une implication de la voie TGF β dans la progression du CCI.

L'objectif de notre travail était d'étudier le rôle de la voie TGF β et de ses gènes cibles, dont les longs ARN non codants, dans la cancérogenèse du CCI.

Les signatures d'expression génique associées au TGF β ont été établies dans des lignées cellulaires de CCI (HuCCT1 et Huh28). La dérégulation de plusieurs gènes cibles de la voie TGF β a été validée par RT-PCR, dont T-LINC1, un long ARN non codant dont la fonction était complètement inconnue. *In vitro*, T-LINC1 est induit par le TGF β dans les lignées de CCI. Il a été localisé dans le cytoplasme, mais également dans des corps nucléaires particuliers évocateurs de *paraspeckles*. L'analyse transcriptomique après modulation de T-LINC1 a montré que T-LINC1 pourrait être un régulateur de NEAT1 dans la réponse immunitaire médiée par l'IL8 en agissant au niveau des *paraspeckles*. *In vivo*, l'étude du profil d'expression de CCI humains a montré que T-LINC1 est surexprimé dans les cholangiocytes tumoraux.

5. *Publications de travaux cliniques*

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IV. Discussion

Le CCI est une tumeur peu fréquente dont la cancérogénèse est complexe. Son diagnostic tardif amène peu de patients vers une résection chirurgicale. La quantité de matériel tumoral disponible permettant d'étudier ses altérations moléculaires est donc limitée. Cependant, de multiples variations génomiques et dérégulations de voies de signalisation ont pu être décrites. Ces données sont indispensables au développement de nouvelles thérapies ciblées. Le peu d'efficacité des thérapies ciblant VEGFR, EGFR, PDGFR ou BRAF encourage à orienter la recherche vers d'autres voies ²⁶.

1. *Signature TGFβ dans le CCI*

Le profil d'expression génique précédemment réalisé dans l'équipe avait montré une surexpression du TGFβ dans le stroma tumoral de CCI humains réséqués corrélée à un mauvais pronostic ¹⁸. Une surexpression de TGFβ avait également déjà été montrée dans une signature spécifique du stroma du CCI ²⁴. Cependant, il n'existait pas de signature transcriptomique spécifique de l'impact du TGFβ sur les cellules de CCI. La validation de notre signature est montrée par la présence de gènes cibles connus de la voie TGFβ. L'analyse par enrichissement d'ensemble de gènes (GSEA) conforte sa spécificité avec l'association à des signatures liées au TGFβ, aux protéines SMAD2/3 et à la TEM.

Nous avons validé une activation endogène de la voie TGFβ dans la lignée cellulaire Huh28 concordant avec son phénotype mésenchymateux. Peu de lignées cellulaires de CCI existent (moins de 100 : d'origine humaine ou murine) en comparaison par exemple au CHC (plus de 200) (source Cellosaurus, <https://web.expasy.org/cellosaurus/>). Cette lignée pourrait permettre de tester des inhibiteurs de la voie TGFβ (i.e. efficacité et marqueurs de réponse).

De nouveaux gènes cibles du TGFβ codants et non-codants ont été identifiés. Les deux gènes KRT 14 et 17 codants pour des kératines de type I, protéines constituant les filaments intermédiaires du cytosquelette des cellules épithéliales, font partie des nouveaux gènes codants induits par le TGFβ dans notre signature. KRT14 et 17 sont exprimés dans les cancers épidermoïdes dont les cancers oraux ^{112,113}. KRT17 est également exprimé dans le

cholangiocarcinome, et les cancers du sein et de l'estomac où il est un marqueur pronostique ¹¹⁴⁻¹¹⁶. Dans le cancer oral, la vimentine, qui est exprimée au cours de la TEM, régule la différenciation tumorale. Les niveaux d'expression de la vimentine et de la KRT14 sont corrélés et un niveau d'expression élevé vimentine-KRT14 est corrélé au pronostic ¹¹³. La présence de KRT14 et 17 augmentés dans d'autres cancers valide notre signature.

2. *ARNInc et CCI*

Parmi les gènes non-codants, trois ARNInc intergéniques sont présents dans notre signature TGF β : LINC00340, LINC00312 et LINC00313. Ceux sont les premiers ARNInc régulés par le TGF β identifiés dans le CCA. LINC00312 est surexprimé dans plusieurs cancers, notamment pulmonaire où il agit comme régulateur du TGF β via sa liaison au facteur de transcription YBX1 ¹¹⁷. Dans les cancers de la thyroïde et de la vessie, LINC00312 agit par l'intermédiaire du microARN miR-197-3p ^{118,119}. LINC00313, qui a été moins étudié, est également surexprimé dans les cancers du poumon et papillaires de la thyroïde ^{120,121}. Il a été récemment associé à un mauvais pronostic du CCI dans une analyse de réseaux d'ARNInc agissant comme éponge à miARN à partir de la base TCGA ⁸⁷.

Notre travail s'est intéressé particulièrement à LINC00340 devant la présence de 2 sondes de microarrays surexprimées par le TGF β . LINC00340 ou CASC15 (cancer susceptibility candidate 15) est un ARNInc intergénique situé sur le chromosome 6p22. L'étude de CASC15 est rendue complexe par ses nombreuses isoformes. CASC15 comprend 12 exons et au moins 8 isoformes sont prédites actuellement (source www.ensembl.org). Un ARNInc anti-sens NBAT1/CASC14 est également décrit sur le locus.

Dans le mélanome, Lessard et al ont mis en évidence plusieurs isoformes ¹²². L'expression globale des différentes isoformes de CASC15 était corrélée à la progression et prédictive de la récurrence et de la survie. L'inhibition globale de CASC15 était associée à un *switch* phénotypique entre prolifération et invasion évocateur de la TEM, mais une régulation par le TGF β n'avait pas été montrée. CASC15 aurait un rôle pro-oncogénique dans le mélanome en agissant au niveau épigénétique sur EZH2 ¹²³.

Deux isoformes ont été particulièrement étudiées dans le neuroblastome par Russell et al.¹²⁴. L'isoforme courte CASC15-S a été identifiée comme isoforme fonctionnelle associée à un effet suppresseur de tumeur. La faible expression de CASC15-S est corrélée à un phénotype de neuroblastome agressif. Son inhibition augmente la prolifération et la migration cellulaire, alors que l'inhibition de l'isoforme longue n'a pas d'impact sur la viabilité cellulaire. Le rôle suppresseur de tumeur de CASC15 dans le neuroblastome a depuis été confirmé dans des études plus récentes pour les 2 isoformes et 3 *single nucleotide polymorphisms* (SNP)^{125,126}. CASC15 et CASC14 agissent de façon synergique pour contrôler la progression du neuroblastome¹²⁵.

Au vu de ces études contradictoires, il semble que CASC15 ait plusieurs fonctions dépendantes des organes et du stade tumoral. Au cours de nos travaux, nous avons renommé CASC15 par TLINC pour *TGFβ induced long intergenic non coding RNA*. Nous nous sommes intéressés aux deux isoformes courte et longue déjà connues dans le neuroblastome. L'isoforme courte a été mise en évidence dans la lignée HuH28 associée à un phénotype mésenchymateux et donc une action plutôt « tardive » du TGFβ. L'isoforme longue a été mise en évidence dans la lignée HuCCT1 associée à un phénotype épithélial et donc une action plutôt « précoce » du TGFβ. Ces résultats suggèrent des fonctions différentes de TLINC par le biais de plusieurs isoformes selon le contexte cellulaire en réponse à l'activation de la voie TGFβ.

L'étude fonctionnelle a montré une tendance à l'augmentation de la migration cellulaire après surexpression de TLINC. L'analyse transcriptomique a montré une surexpression de nombreux gènes impliqués dans la réponse inflammatoire, dont IL8, après surexpression de TLINC dans la lignée HUCCT1. Ces résultats ont été confirmés par la GSEA. La surexpression d'IL8 dans le groupe de tumeurs présentant une expression forte de TLINC confirme ce rôle dans la réponse inflammatoire. Ces résultats permettent d'émettre l'hypothèse d'un rôle de TLINC dans le remodelage d'un microenvironnement inflammatoire impliqué dans la cancérogénèse.

La présence de TLINC dans les cholangiocytes tumoraux et dans le stroma tumoral confirme un rôle à différents niveaux transcriptionnel et post-transcriptionnel. L'analyse transcriptomique de la lignée HuCCT1 après surexpression de TLINC a notamment montré l'ARNInc NEAT1 parmi les gènes surexprimés. NEAT1 est essentiel à la formation de structures nucléaires particulières, les *paraspeckles*¹²⁷. NEAT1 régule l'expression d'IL8 au cours de la réponse immunitaire aux virus en se liant à un répresseur d'IL8 dans le noyau¹²⁸. Ces résultats supposent que TLINC pourrait réguler l'expression d'IL8 en se liant à NEAT1 dans le noyau où il aurait également une action au niveau épigénétique. NEAT1 est déjà connu pour être co-régulé avec MALAT1, un autre ARNInc dans des cellules d'adénocarcinome du sein humain¹²⁹. NEAT1 et MALAT1 sont tous les 2 surexprimés dans le cholangiocarcinome humain et représentent les ARNInc les plus mutés dans les tumeurs hépatiques¹³⁰. De multiples mécanismes d'action pour un même ARNInc ont déjà été montrés pour plusieurs ARNInc dans le CCI. Par exemple, SPRY4-IT1 a un rôle dans la régulation épigénétique mais agit également dans le cytoplasme comme éponge pour miR-101-3p¹³¹.

CASC15 a été identifié dans plusieurs autres cancers mais sans étude des différentes isoformes. Dans le cancer gastrique, l'expression de CASC15 est corrélée à un mauvais pronostic. In vitro, la surexpression de CASC15 induit la TEM. CASC15 agit à plusieurs niveaux, dans le noyau en diminuant l'expression de *CDKN1A* par une régulation épigénétique au niveau de EZH2, et dans le cytoplasme comme éponge à miR-33a-5p¹³². Dans le CHC, CASC15 a un rôle pro-oncogénique liée à la TEM. Son expression est corrélée à un mauvais pronostic¹³³. CASC15 est également surexprimé dans le cancer du côlon. Il régule la progression tumorale par l'intermédiaire de miR-4310 et la voie Wnt/ β caténine¹³⁴. Ce lien avec la TEM dans plusieurs cancers renforcent nos résultats montrant la régulation de CASC15 par le TGF β .

L'identification croissante de nouveaux ARN non-codants montre que la régulation de l'expression des gènes est plus complexe que celle imaginée auparavant. De multiples réseaux moléculaires comprenant des ARNInc, des miARN et des ARNcirc interviennent

dans la régulation des voies de signalisation. Ceci complique la compréhension des mécanismes de cancérogénèse mais ouvre également de nouvelles possibilités thérapeutiques.

Perspectives : L'étude de LINC00312 et LINC00313, les deux ARNinc régulés par le TGFβ dans notre signature, vont être étudiés dans l'équipe. Des travaux préliminaires ont clairement permis de valider l'induction de LINC00312 et LINC00313 par le TGFβ (voie canonique SMAD dépendante) (figure 20).

Expression de LINC00312 et LINC00313 après inhibition de SMAD3 et SMAD4 +/-TGFβ dans les cellules HUCCT1

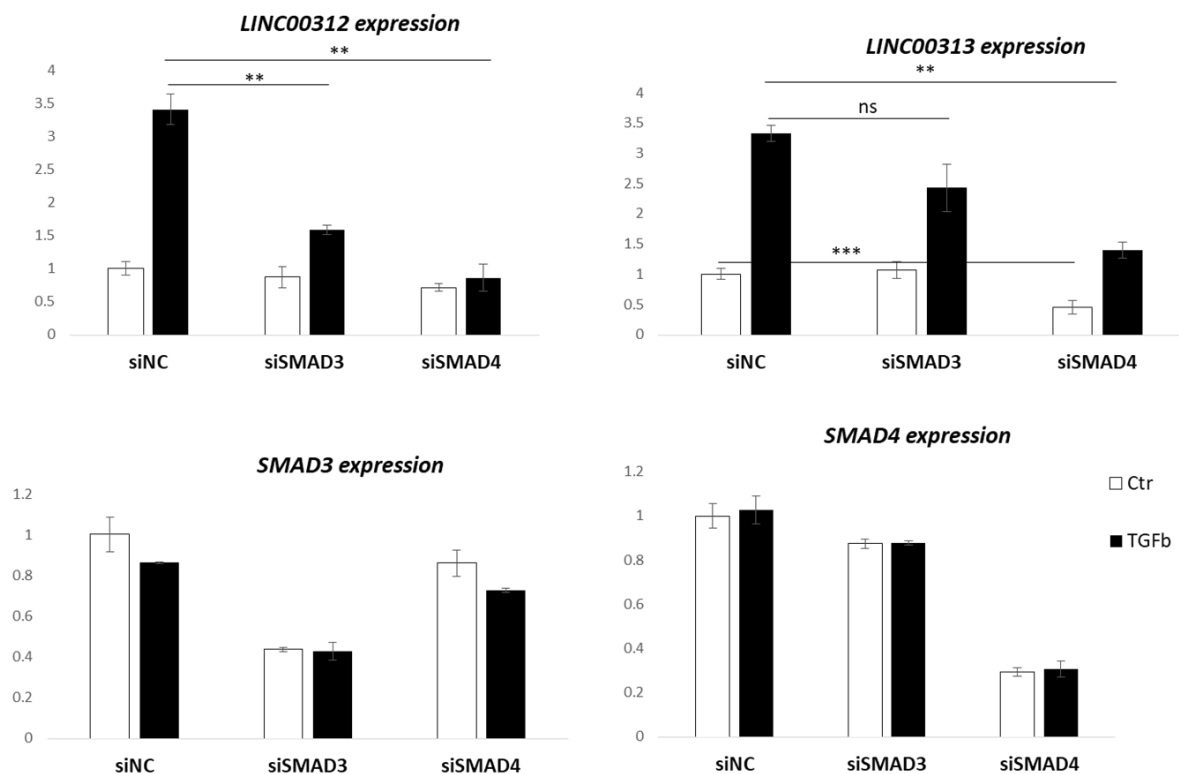


Figure 20 : Niveaux d'expression de LINC00312 et LINC00313 par qPCR après inhibition de SMAD3 et SMAD4 par siRNA, sans et avec stimulation par le TGFβ1 (1ng/ml) pendant 16 heures dans les cellules HUCCT1.

Le panel du haut montre une induction de l'expression de LINC00312 et LINC00313 par le TGFβ. L'expression de LINC00312 est significativement moins importante après inhibition de SMAD3 et SMAD4 et donc dépendante de la voie canonique. L'expression de LINC00313 est significativement moins importante après inhibition de SMAD3 sans et avec stimulation par le TGFβ et donc également dépendante de la voie canonique.

Le panel du bas montre les contrôles de l'inhibition significative de SMAD3 et SMAD4 par leurs siRNA respectifs.

3. *ARN non codants biomarqueurs*

Nos résultats ont montré que TLINC/CASC15 est un potentiel biomarqueur diagnostique dans les tissus de CCI humains. Les 2 isoformes de TLINC/CASC15 étaient surexprimées dans le tissu tumoral par rapport au tissu adjacent non tumoral. Cependant, l'expression des 2 isoformes était corrélée et ne montrait pas de corrélation au pronostic. La plupart des études publiées sur CASC15 ne prennent pas en compte les différentes isoformes. Cependant, la surexpression globale de CASC15 a été constatée in vivo dans le tissu tumoral de plusieurs cancers. Dans la majorité des cancers, cette expression a été associée à un caractère pro oncogénique et un mauvais pronostic. Dans le neuroblastome en revanche, son expression a été associée à un effet suppresseur de tumeur. CASC15 représente donc un potentiel biomarqueur diagnostique et pronostique dans les tissus tumoraux.

Dans le but d'identifier une signature d'ARNInc prédictive de la survie, nous avons comparé les profils d'expression génique de tumeurs d'une cohorte de patients. Parmi les 9 ARNInc significativement associés à la survie sans récurrence et globale, 5 étaient surexprimés dans les CCI de bon pronostic et 4 étaient surexprimés dans les CCI de mauvais pronostic dont l'ARNInc ANRIL. Nous avons focalisé notre analyse sur ANRIL (ou CDKN2B-AS1) car il a déjà été identifié comme biomarqueur pronostique dans d'autres cancers (CHC, cancer du rein et de l'utérus). Cependant, le biomarqueur idéal est non invasif et donc détectable dans le sang, les urines ou la salive. Des miARN détectables dans le sérum ou la bile ont déjà été bien étudiés dans le CCI soit individuellement ou en panel ^{85,135,136}. En revanche, peu d'études se sont intéressées à la détection d'ARNInc dans le sérum, le plasma ou la bile. Le dosage dans le plasma d'ARNInc connus pour être dérégulés dans le cholangiocarcinome hilaire a montré que les ARNInc Prostate Cancer Associated Transcript 1 (PCAT1), MALAT1 et CPS1 Intronic Transcript 1 (CPS1-IT1) étaient de potentiels biomarqueurs diagnostiques sériques ¹³⁷. Il n'existe actuellement pas de donnée sur la recherche d'ARNInc dans le plasma ou le sérum dans le CCI.

Des ARNInc intacts ont pu être détectés dans des exosomes extraits de la bile de patients présentant un cholangiocarcinome ¹³⁸. Comme dans le plasma, une caractéristique des exosomes présents dans la bile est leur stabilité due à leur protection par une double couche lipidique. Dans l'étude de Ge et al., la combinaison de 2 ARNInc (ENST00000588480.1 et ENST00000517758.1) a été validée pour sa capacité diagnostique meilleure que le CA 19-9. La faible expression des 2 ARNInc était corrélée à un meilleur pronostic ¹³⁸.

Les ARNcirc sont une autre forme d'ARN non-codants abondants avec des propriétés de stabilité et de spécificité d'organe intéressantes en tant que biomarqueur ^{73,139}. Comme les ARNInc, les ARNcirc peuvent être excrétés dans des exosomes et constituer des biomarqueurs d'autant plus stables. Le développement d'outils de bioinformatique et de séquençage ARN ont permis de prédire de nombreux ARNcirc. Une isoforme circulaire d'ANRIL associée au risque d'athérosclérose, a été identifiée dans des lignées cellulaires de cancer ¹⁴⁰. Hsa_circ_0001649 et circRNA Cdr1as ont été associés à la progression du CCI ^{141,142}. En 2013, Salzman et al. avait prédit au moins une isoforme circulaire de CASC15 dérivée de l'exon 7 dans la lignée cellulaire HeLa ¹⁴³. Au moins 3 ARNcirc liés à CASC15 ont aussi été mises en évidence dans le carcinome basocellulaire ¹⁴⁴. A l'aide d'amorces spécifiques (convergentes et divergentes) et de traitements à la RNase R (dégradation spécifique des ARN linéaires), nous avons ainsi confirmé l'existence de plusieurs isoformes circulaires prédites de TLINC dans les lignées cellulaires de CCI traitées avec le TGFβ et les tissus de CCI. L'analyse par PCR des tissus de CCI de notre cohorte a montré que l'amplification des isoformes circulaires n'était pas identique dans toutes les tumeurs. La variabilité entre les tumeurs pourrait permettre d'utiliser ces ARNcirc comme biomarqueurs pronostiques.

Perspectives : Nos travaux n'ont pas permis d'identifier un ensemble exhaustif d'ARNcirc, ni d'évaluer précisément leur expression mais seulement de détecter l'existence de certains d'entre eux dans le CCI. Pour les identifier à large échelle, un profil d'expression des ARNcirc dans les lignées de CCI avec et sans TGFβ a récemment été réalisé dans l'équipe.

La combinaison au profil d'expression de microARN va permettre d'identifier les ARNcirc potentiels éponges à microARN (figure 21). Des résultats préliminaires ont déjà été obtenus.

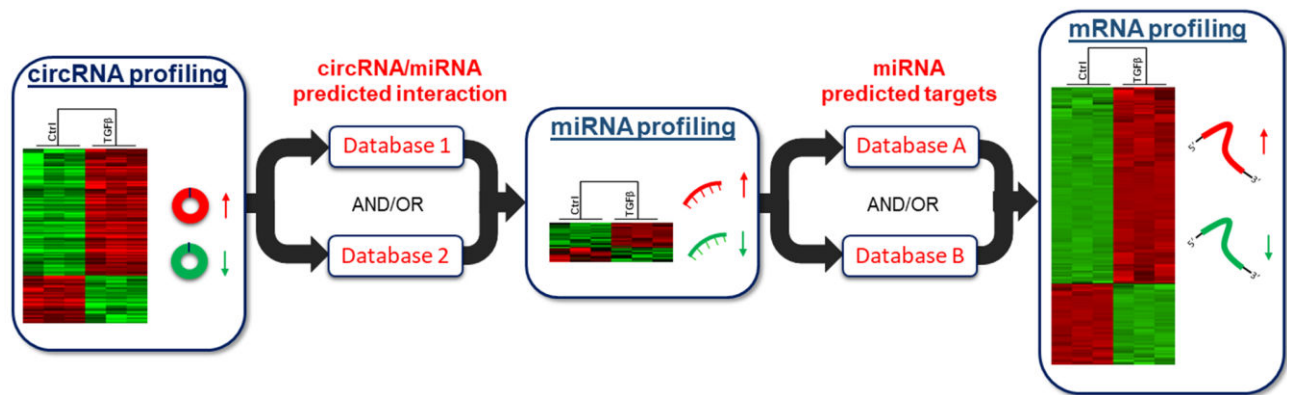


Figure 21 : Schéma du déroulement de l'analyse intégrée du profil d'expression des ARNcirc régulés par le TGFβ, puis du profil d'expression des microARN prédits interagissant avec ces ARNcirc et du profil d'expression de leurs ARNm cibles prédits.

4. Applications thérapeutiques

1. Voie TGFβ

La voie TGFβ est une cible déjà étudiée pour de nouvelles thérapies en cancérologie hépatique. L'action pharmacologique peut se situer au niveau du ligand avec des oligonucleotides antisens, de l'interaction entre ligand et récepteur avec des anticorps monoclonaux ou du signal intracellulaire avec des inhibiteurs des récepteurs kinase ¹⁴⁵. Le galunisertib est un des inhibiteurs du TGFBR1. Il a permis de diminuer la prolifération cellulaire sur des coupes de tumeurs fraîches de CHC en potentialisant les effets du sorafenib ¹⁴⁶. L'utilisation du galunisertib sur des cultures primaires de CCI a eu pour effet une diminution de la migration cellulaire ¹⁴⁷. Nous l'avons utilisé dans nos travaux en raison de son usage déjà possible en pratique clinique chez l'homme. Des essais cliniques sont recensés pour des patients porteurs de CHC, cancer pancréatique, cancer colorectal, mais pas encore dans le CCI. Le M7824 est un anticorps monoclonal conçu pour bloquer les voies de signalisation couramment utilisées par les cellules cancéreuses pour contrer le système immunitaire. Il agit contre la protéine programmed death-ligand 1 (PD-L1) liée au domaine

extracellulaire du récepteur TGFβ 2. Un essai de phase II utilisant cet anticorps en monothérapie chez des patients avec des cholangiocarcinomes localement avancés en échec de chimiothérapie aux sels de platine doit démarrer cette année (ClinicalTrials.gov NCT03833661).

Le TGFβ est impliqué dans la TEM qui est impliquée dans de nombreux mécanismes de résistance aux traitements. La TEM a été identifiée *in vitro* comme un mécanisme de résistance à la gemcitabine et au cisplatine utilisés en combinaison dans le traitement du CCI ¹⁴⁸. Une diminution de la sensibilité au 5-fluorouracile liée aux caractéristiques mésenchymateuses qui accompagnent la TEM induite par le TGFβ dans la lignée de CCI TFK-1 a également été observée ¹⁴⁹. L'acquisition de propriétés de cellules souches cancéreuses induites par la TEM dans le CCI est un des effets qui contribue à la résistance aux chimiothérapies ²⁰. Des thérapies ciblant de TGFβ pourraient donc être utilisées en combinaison de chimiothérapie systémique pour améliorer leur efficacité.

Cependant, le TGFβ a des propriétés suppressives de tumeur à un stade tumoral précoce mais des effets tumorigéniques à un stade avancé. Mu et al. ont montré que le TGFβ épithélial n'induisait pas la fibrose et protégerait du développement du cholangiocarcinome ⁷¹. Les thérapies ciblant le TGFβ trop tôt dans le développement tumoral pourraient donc être délétères. Le moment d'utilisation de ces thérapies doit donc être bien évalué ⁴⁹. La sélection des patients potentiels répondeurs est nécessaire. Les ARN non codants induits par le TGFβ pourraient être des biomarqueurs prédictifs de réponse aux thérapies ciblant le TGFβ.

Perspectives : L'effet du galunisertib sur tumeurs fraîches de CCI va être étudié dans l'équipe. Un modèle d'étude *in ovo* permettant d'implanter des tumeurs fraîches au niveau de la membrane chorio-amniotique d'œufs de poule est en cours de mise au point.

II. ARN non codants

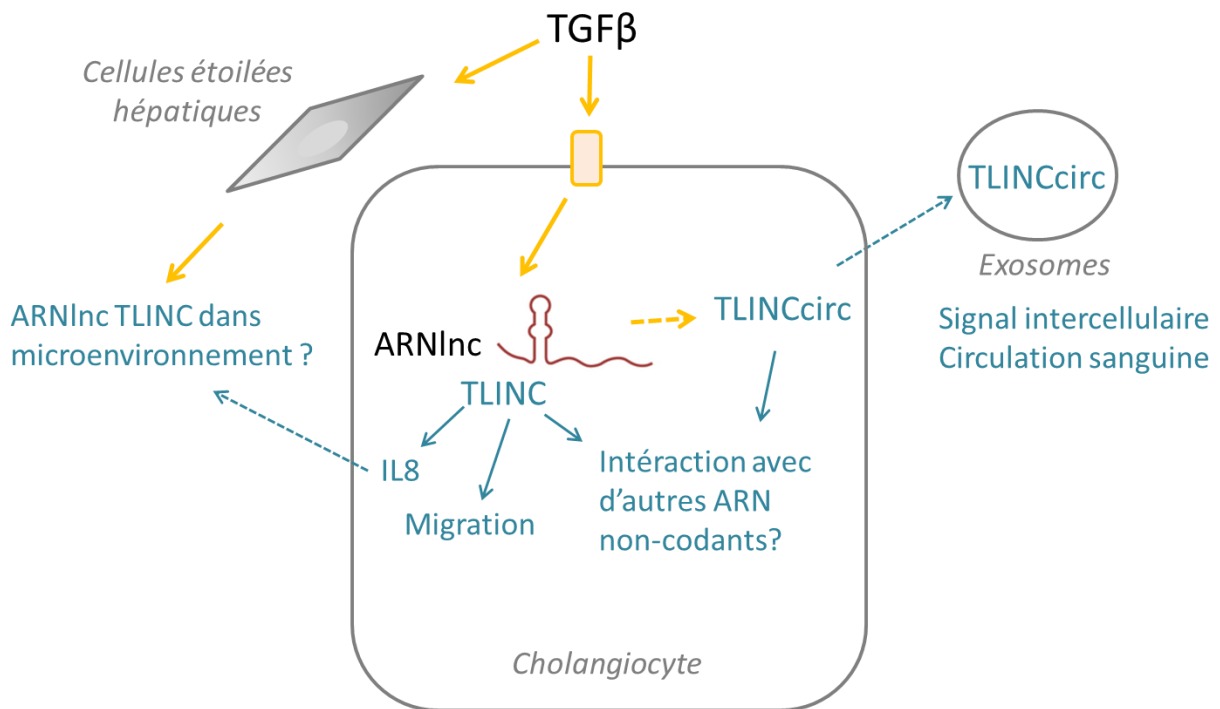
Les ARN non codants sont de potentielles cibles thérapeutiques du CCI en inhibant ou en renforçant leur action. Le microARN miR-195 placé dans des vésicules extracellulaires délivrées aux cellules de CCI chez le rat a permis une diminution de la taille tumorale et une prolongation de la survie ¹⁵⁰.

Il a été montré que CASC15/TLINC participe à la résistance au traitement du cancer du côlon ¹⁵¹. CASC15 est surexprimé dans les tissus et les cellules de cancer colique résistants à l'oxaliplatine. Les patients avec un haut niveau d'expression de CASC15 ont un mauvais pronostic. CASC15 agit comme éponge au microARN miR-145 pour lever la répression de l'expression d'ATP Binding Cassette Subfamily C Member 1 . La diminution de CASC 15 resensibilise les cellules à l'oxaliplatine et représente donc une potentielle cible thérapeutique dans les cancers du côlon résistants à l'oxaliplatine.

Les ARNInc peuvent également représenter des outils thérapeutiques. Un ARNInc artificiel a été créé pour cibler plusieurs miARN surexprimés dans les cellules de CHC résistantes au sorafenib. Après infection des cellules par l'ARNInc artificiel, une diminution de la prolifération et une augmentation de l'apoptose renforçant les effets du Sorafenib ont été observées ¹⁵².

V. Conclusion

La voie TGF β est une des voies de signalisation impliquées dans la cancérogénèse du CCI. Nos travaux ont permis d'identifier de nouveaux gènes cibles de la voie TGF β *in vitro* et de déterminer la pertinence clinique de leur dérégulation *in vivo* dans le CCI. Parmi ces nouveaux gènes, plusieurs ARNinc ont été mis en évidence dont TLINC/CASC15 qui exercerait un rôle dans le remodelage d'un microenvironnement inflammatoire impliqué dans la cancérogénèse du CCI. La surexpression de TLINC a également une tendance à augmenter la migration cellulaire. Les ARNinc sont de potentiels biomarqueurs diagnostiques et/ou pronostiques du CCI tel que l'ARNinc ANRIL. Les ARNcirc, une autre catégorie d'ARN non-codants, pourraient être aussi des biomarqueurs du CCI intéressants car très stables et détectables de façon non invasive dans le sérum. Enfin, les ARN non-codants pourraient constituer de potentielles cibles thérapeutiques en tant que médiateurs de la voie TGF β . Les interactions complexes entre les différents ARN non-codants constituent encore un large domaine de recherche pour de nouvelles thérapies.



VI. Références

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Titre : Etude de la voie TGF β dans le cholangiocarcinome intrahépatique

Mots clés : cholangiocarcinome, voie de signalisation TGF β , ARN non-codant

Résumé : Le cholangiocarcinome intra hépatique (CCI) est une tumeur hépatique primitive développée aux dépens des canaux biliaires. Son pronostic est mauvais avec les traitements actuels qui augmentent peu la survie des patients. Sa cancérogénèse est complexe impliquant de nombreuses voies de signalisation dont la voie TGF β . L'hypothèse du projet est l'implication des ARN longs non-codants (ARNlnc) comme médiateurs de la voie TGF β dans le développement du CCI.

Les objectifs de notre travail étaient d'identifier des ARNlnc régulés par le TGF β et potentiels biomarqueurs diagnostiques ou pronostiques.

Nous avons identifié une signature transcriptomique spécifique du TGF β après stimulation de lignées cellulaires de CCI. Parmi les nouveaux gènes cibles, plusieurs ARNlnc ont été identifiés dont *CASC15* renommé *TLINC* pour *TGF β -induced long intergenic non-coding RNA*.

TLINC aurait un rôle dans le remodelage du microenvironnement impliqué dans la cancérogénèse du CCI notamment par la régulation de l'IL8. Ce rôle pourrait s'exercer par l'interaction avec d'autres ARNlnc déjà identifiés dans le CCI e.g. *NEAT1*. *TLINC* est surexprimé dans les tumeurs humaines de CCI et pourrait constituer un biomarqueur diagnostique. Des isoformes circulaires de *TLINC* mises en évidence dans les tumeurs pourraient être détectables dans le sérum et constituer des biomarqueurs non invasifs.

L'analyse transcriptomique d'une cohorte de patients divisée en 2 sous-groupes de pronostic différent a identifié une signature d'ARNlnc prédictive de la survie. L'ARNlnc *ANRIL*, déjà connu dans d'autres cancers, est un des ARNlnc qui pourrait constituer un biomarqueur pronostique.

Title : TGF β signaling pathway in intrahepatic cholangiocarcinoma

Keywords : cholangiocarcinoma, TGF β pathway, non-coding RNA

Abstract: Intrahepatic cholangiocarcinoma (ICC) is a primary liver tumor developed from bile ducts. ICC prognosis is poor with current treatments that slightly increase patient survival. ICC carcinogenesis is complex and involves multiple signaling pathways including TGF β pathway. Our hypothesis relies on the involvement of long non-coding RNA (lncRNA) as mediators of TGF β pathway in the development of ICC.

The aim of the study was to identify and to characterize TGF β regulated lncRNA as ICC potential diagnostic or prognostic biomarkers.

We identified a specific transcriptomic signature after stimulation of ICC cell lines with TGF β . Among the novel TGF β target genes, several lncRNAs were identified including *CASC15* renamed *TLINC* standing for *TGF β -induced long intergenic non-coding RNA*.

TLINC may play a role in the remodeling of an inflammatory microenvironment involved in ICC carcinogenesis, including the regulation of IL8. This role could be exerted by the interaction with other lncRNAs already identified in the ICC e.g. *NEAT1*. *TLINC* is overexpressed in human ICC tumors and may represent a relevant diagnostic biomarker. Circular isoforms of *TLINC* found in tumors may be detectable in serum and be noninvasive biomarkers.

Transcriptomic analysis of tumors from a cohort of patients divided into 2 prognostic groups identified a lncRNAs signature predictive for survival. lncRNA *ANRIL*, already known to be upregulated in other cancers, is one of the lncRNAs that could be a prognostic biomarker in ICC.