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Aminetou Mint Mohamed

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par

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**ANOMALIES D'ÉPISSAGE DANS LES LEUCEMIES AIGÜES
MYELOBLASTIQUES: ROLE DE WT1 ET DE DEK ET
IMPACT CLINIQUE**

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Résumé

Parmi les nombreuses perturbations participant à l'hétérogénéité bioclinique des leucémies aiguës myéloïdes (LAM), les anomalies d'épissage ont récemment pu être identifiées grâce au développement des techniques de microarray et du séquençage à haut débit. Après une revue bibliographique abordant les possible mécanismes et conséquences des perturbation de l'épissage dans les LAM, nous avons dans un deuxième article, analysé les patrons d'épissage associés à l'expression d'oncogènes connus pour interférer avec le splicéosome, l'ARN et la chromatine, ainsi qu'à la résistance aux principales drogues utilisées dans la prise en charge des LAM. Nos résultats identifient des signatures spécifiques et originales, de nombreux évènements concernant des gènes non dérégulés au plan transcriptionnel. Parmi ces perturbation nous avons, dans une troisième étude, évalué l'impact clinique d'une perturbation d'épissage de l'ARN messager de TET2. Les résultats montrent que l'exclusion de l'exon 2 de TET2 défini un facteur pronostic indépendant chez les malades traités par chimiothérapie intensive, en particulier chez les patients au caryotype normal. La dernière étude aborde l'impact des anomalies d'épissage du transporteur ABCA3 identifiées dans les LAM. In vitro nous trouvons que l'exclusion de l'exon 19 d'ABCA3 favorise la résistance à la daunorubicine et au VP16. Paradoxalement, alors que la résistance liée à cette exclusion d'exon dépasse significativement celle liée à l'expression de la forme sauvage d'ABCA3, l'effet de l'isoforme sans exon 19 sur la rétention cellulaire de daunorubicine apparaît significativement inférieur à celui de l'ABCA3 sauvage. Au plan bioclinique, l'exclusion de l'exon 19 d'ABCA3 réduit les chances de rémission et défini un facteur pronostic indépendant chez les malades traités par chimiothérapie intensive, en particulier chez les patients au caryotype normal.

Abstract

Approximately one-third of expressed genes are misspliced in AML, opening the possibility that additional factors than splicing factor mutations might cause RNA missplicing in these diseases. We performed exon-array analysis and exon-specific PCR (ESPCR) to identify specific landscapes of exon expression that are associated with DEK and WT1 oncogene expression and the resistance of AML cells to AraC, doxorubicin or azacitidine. More than 70% of AEU s identified by exon-array were technically validated through ESPCR. *In vitro*, 1,130 to 5,868 exon events distinguished the 5 conditions from their respective controls while *in vivo* 6,560 and 9,378 events distinguished chemosensitive and chemoresistant AML, respectively, from normal bone marrow. Whatever the cause of this effect, 30 to 80% of mis-spliced mRNAs involved genes unmodified at the whole transcriptional level. These AEU s unmasked new functional pathways that are distinct from those generated by transcriptional deregulation. Having identified aberrant TET2 exon 2 and ABCA3 exon 19 expression in, we tested their prognostic impact In a discovery cohort (n=99), TET2 exon 2 skipping (TET2E2S) was found positively associated with a significant reduction in the cumulative incidence of relapse (CIR). Age, cytogenetics, and TET2E2S were independent prognostic factors for disease-free survival (DFS), and favorable effects on outcomes predominated in cytogenetic normal (CN)-AML and younger patients. Using the same cutoff in a validation cohort of 86 CN-AML patients, TET2E2S^{high} patients exhibited a significantly lower CIR ($p < 10^{-4}$). TET2E2S and FLT3-ITD, but not age or NPM1 mutation status were independent prognostic factors for DFS and event-free survival (EFS), while TET2E2S was the sole prognostic factor that we identified for overall survival (OS). In both the intermediate-1 and favorable ELN genetic categories, TET2E2S remained significantly associated with prolonged survival. Regarding ABCA3, we found that skipping of exon 19 (A3S19) triggered resistance to daunorubicine and VP16 *in vitro*. However such skipping did not significantly modify drug efflux, when compared with wild-type ABCA3. At the clinical level, A3S19 was found negatively correlated with response to IC, OS, DFS, and EFS, independently of age, cytogenetics, FLT3-ITD, and *NPM1* mutation. AS19 improved the European LeukemiaNet Risk Classification of AML whereas it possessed no prognostic impact in patients unfit for IC.

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Liste des abréviations

ADN : Acide désoxyribonucléique

AMPc : Adénosine MonoPhosphate cyclique

Bax : Bcl-2-Associated X protein

Bcl-2 : B-Cell Lymphoma 2

CDK : Cyclin-Dependent Kinases

CTI : chimiothérapie intensive

ELN : European Leukemianet

FADD : Fas-Associated Death Domain

IAP : Inhibitor of Apoptosis Proteins

IL : Interleukine

JAK : Janus Kinase

LAM : leucémie aiguë myéloïde

LAP : leucémie à promyélocytes

NGS : Next generation sequencing (séquençage à haut débit)

OBF : oligonucléotide bifonctionnel

PARP : Poly (ADP-Ribose) Polymérase

PCR : Polymerase Chain Reaction (réaction de polymérisation en chaîne)

qESPCR : RTPCR quantitative spécifique d'exon

RANK : Receptor Activator of NF-kB

RC : rémission complète

SMD : syndrome myélodysplasique

TGF : Transforming Growth Factor

TNF : Tumor Necrosis Factor (facteur de nécrose des tumeurs)

TORC : Transducers Of Regulated CREB activity

TRAIL : TNF-Related Apoptosis-Inducing Ligand

I - INTRODUCTION

I-1. Généralités

Ce travail de thèse s'intéresse aux anomalies d'épissage dans les leucémies aiguës myéloblastiques (LAM). Ce type d'anomalie n'a que récemment été mis à jour grâce aux données du séquençage à haut débit ayant trouvé de nombreuses mutations de gène d'épissage dans les hémopathies malignes¹. En fait, si les mutations des facteurs d'épissage sont moins fréquentes dans les LAM que dans d'autres hémopathies malignes comme la leucémie lymphoïde chronique (LLC) ou les syndromes myélodysplasiques (SMD)¹, de nombreuses anomalies non génétiques acquises par le clone leucémique semblent pouvoir contribuer aux troubles de l'épissage qui concerne environ 30% des gènes exprimés dans les LAM¹.

Les données présentées ici s'articulent autour de 4 travaux correspondant à une revue générale publiée¹, un article original publié², un travail soumis à publication et un article en préparation. Ces travaux ont par ailleurs été présentés sous formes de posters (n=2), d'e-poster (1) et de communication orale aux congrès de l'ASH (2012, 1 poster, 2014, 1 communication orale) et de l'EHA (e-poster en 2013). Ces études se sont intéressées principalement à certains mécanismes potentiellement impliqués dans les anomalies d'épissage des LAM ainsi que sur les conséquences pathogéniques et cliniques de ces anomalies. Pour les premières, nous avons principalement abordé l'effet des expressions anormales de WT1 et de DEK dans les LAM sur l'épissage. Concernant les conséquences pathogéniques nous avons tenté de relier la chimiorésistance des cellules de LAM à certains patrons d'épissage puis détaillés les conséquences fonctionnelles d'un événement d'épissage particulier de l'ARN messager du transporteur ABCA3 sur l'efflux de drogue et la résistance aux molécules de chimiothérapies. Enfin au plan clinique, nous avons détaillé les conséquences d'anomalies d'épissage des messagers des gènes TET2 et ABCA3 sur la réponse à la polychimiothérapie et l'évolution de patients atteint de LAM.

En pratique, après une brève introduction sur les LAM, les transporteur ABC et l'épissage, la présentation comportera les quatre études présentées ci-dessus.

I-2. Leucémies aiguës myéloïdes

I-2.1. Définition

Les LAM sont des hémopathies malignes caractérisées par un blocage de maturation de la lignée myéloïde avec accumulation et prolifération de cellules myéloïdes anormales appelées myéloblastes. Ces cellules envahissent la moelle, le sang et éventuellement d'autres organes hématopoïétiques ou extra-hématopoïétique.

I-2.2. Epidémiologie

La LAM est le type le plus commun de leucémie aiguë chez l'adulte³. L'incidence globale annuelle est de 3,8 cas pour 100 000 aux États-Unis et en Europe, elle augmente avec l'âge, surtout après 50 ans, de telle sorte que l'âge médian au diagnostic est de 60-65 ans.

Les causes restent mal connues. Plusieurs facteurs comme l'environnement, l'exposition aux produits toxiques et les radiations ionisantes peuvent déclencher le processus de leucémogénèse. L'incidence de LAM varie aussi avec la géographie et l'ethnie, ce qui suggère que différents facteurs étiologiques peuvent contribuer au développement de ces hémopathies malignes. Des facteurs génétiques comme la trisomie 21, l'anémie de Fanconi ou le syndrome de Bloom expose au développement des LAM. Certaines hémopathies définissent des états préleucémiques comme les syndromes myéloprolifératifs ou les SMD. L'exposition chronique à des toxiques tels que les pesticides, le benzène, le tabagisme, ainsi que des radiations ionisantes et des agents infectieux favorisent aussi le processus de leucémogénèse. La chimiothérapie anticancéreuse est responsable d'environ 5% des LAM, les principales drogues impliquées correspondent aux alkylants et aux inhibiteurs de topoisomérase II.

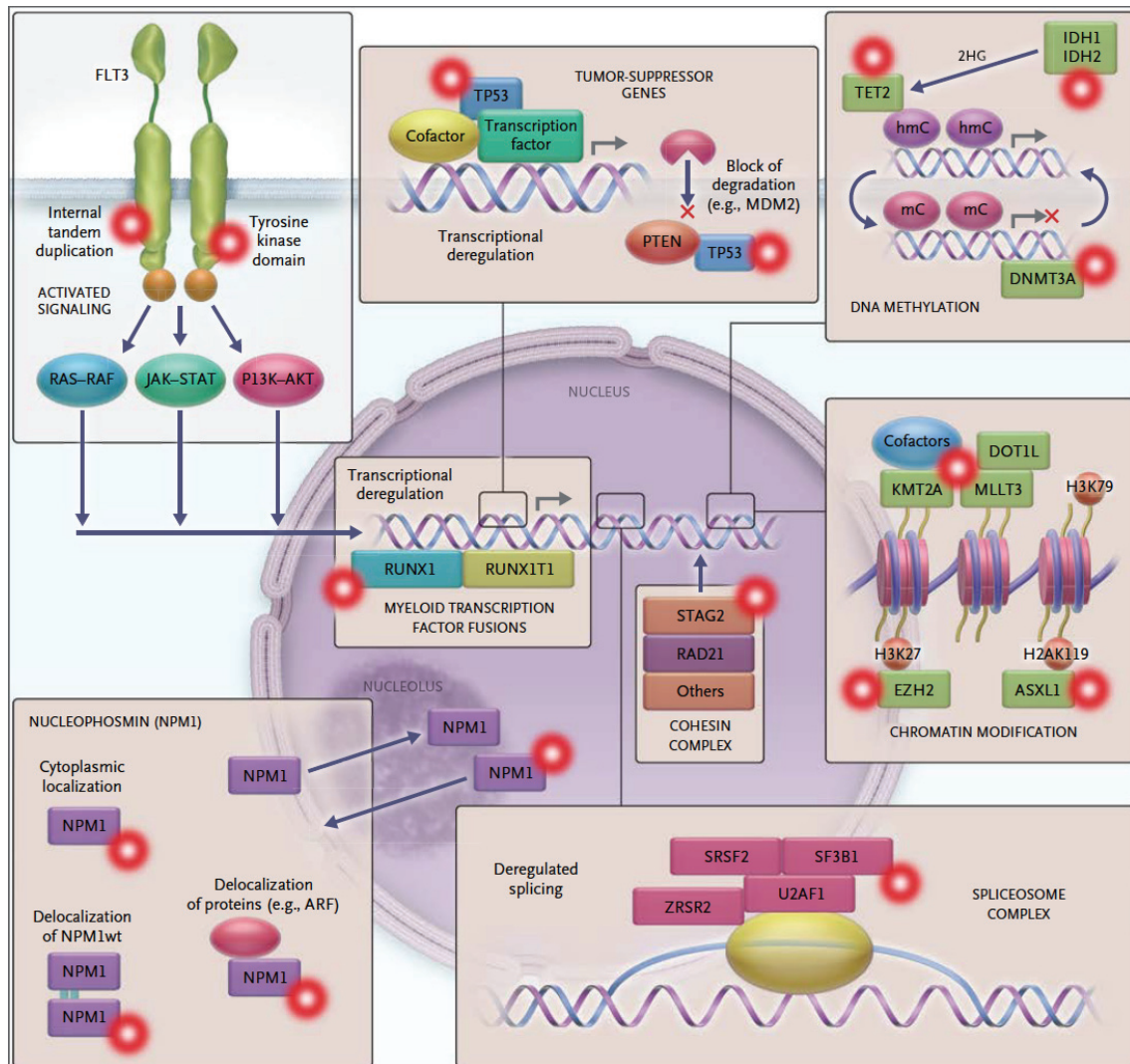


Figure 1. Principales classes de gènes mutés dans les LAM (d'après Döhner *et al.*, 2015³). Dans une revue générale récente, Döhner *et al.* distinguent 8 classes de gènes mutés dans les LAM³. Les mutations des récepteurs à tyrosine kinases de classe III comme FLT3 confèrent un avantage prolifératif via les voies RAF, JAK-STAT et PI3-AKT. Les mutations des gènes de facteurs de transcription myéloïdes comme RUNX1 ou leur réarrangement à travers des translocations comme la t(8;21)(q22;q22);RUNX1-RUNX1T1 dérègle la transcription et altèrent la différenciation. Les mutations de NPM1 entraînent une localisation cytoplasmique anormale de la protéine et de ses partenaires. Les mutations des gènes du spliceosome comme SRSF2, SF3B1, U2AF1, et ZRSR2 altèrent le métabolisme de l'ARN. Les mutations des gènes du complexe cohésine (STAG2 et RAD21) pourraient interférer avec la ségrégation chromosomique et la régulation transcriptionnelle. Les mutations des gènes impliqués dans la régulation épigénétique (ASXL1 et EZH) modifient la chromatine et donc la transcription. La méthylation de l'ADN est modifiée par les mutations de TET2, DNMT3A, IDH1/2. Les mutations de gènes suppresseurs de tumeur comme P53 donnent un avantage prolifératif au clone concerné.

I-2.3. Physiopathologie

La LAM est une tumeur maligne qui se développe au niveau de la moelle osseuse, dans laquelle les cellules leucémiques prolifèrent d'une manière incontrôlée en perturbant l'hématopoïèse normale.

Le clone leucémique est organisé de manière hiérarchique en trois compartiments distincts:

- Les cellules souches leucémiques (CSL) immatures (CD34+ CD38- CD123+), résistantes à la chimiothérapie, capables de s'auto-renouveler ou de s'engager dans un processus anormal de prolifération/maturation.
- Les progéniteurs leucémiques (CFU-L pour *colony forming unit-leukemia*) qui ont perdu les capacités d'autorenouvellement mais qui conservent les propriétés clonogènes et de différenciation limitée.
- Les cellules leucémiques dont la différenciation est limitée à un stade donné de différenciation

La combinaison d'anomalies chromosomiques et géniques acquises représente actuellement la clef de voute physiopathologique des LAM. Elle est par ailleurs associée à des formes cliniques de la maladie et surtout définit des classes pronostiques distinctes de LAM. Les principales anomalies sont schématisées dans la Figure 1 provenant d'une revue générale récente³.

Les anomalies chromosomiques sont très nombreuses. Elles permettent d'expliquer le développement du clone leucémique, possèdent un rôle pronostique et peuvent définir une cible thérapeutique. Par exemple, la translocation t(15 ;17) (q33 ;q32) s'observe dans la leucémie aiguë à promyélocyte, de pronostic favorable. Ces LAM sont pour la plupart guéries sans chimiothérapie, mais grâce à l'utilisation d'agents différenciateurs comme l'acide tout-trans rétinoïques ou le trioxyde d'arsenic. La t(8 ;21) (q22 ;q22) et l'inversion du chromosome 16 représentent aussi deux types d'anomalies associées à un pronostic relativement favorable alors qu'à l'opposé, la del5q, les anomalies du chromosome 7 et les caryotype associant plusieurs anomalies sont associées à une évolution défavorable.

Les principales mutations observées concernent des gènes impliqués dans les voies de signalisation (comme CBL, FLT3, JAK2, RAS), des régulateurs épigénétiques (Dnmt3a, ASXL1, EZH2, IDH1, IDH2, SUZ12, TET2, UTX, WT1) et des facteurs de transcription (CEBPA, ETV6, RUNX1), (Réf 9). La détection de ces anomalies au moment du diagnostic permet d'apprécier le pronostic de la maladie et d'en adapter le traitement. En outre, certaines mutations comme le réarrangement FLT3 représentent des cibles thérapeutiques.

A côté des anomalies cytogénétiques et géniques, les cellules de LAM cumulent des altérations transcriptionnelles, post-transcriptionnelles, protéiques et métaboliques.

I-2.4. Diagnostic, classifications

Les signes conduisant au diagnostic de LAM résultent du développement du clone leucémique et des anomalies de l'hématopoïèse normale. Le premier définit un syndrome tumoral biologique et clinique et le second l'association à différents degrés de cytopénies pouvant conduire à une anémie, des hémorragies et des infections. Le diagnostic de LAM correspond à la mise en évidence d'une blastose médullaire supérieure à 20%. La prise en charge des malades est centrée sur leur traitement. Elle comporte une première étape de classification diagnostique, suivie d'une évaluation pronostique permettant de guider le traitement.

La classification OMS des LAM⁴ (Tableau 1) prend en compte les critères morphologiques et immunologiques, les anomalies cytogénétiques, la présence de signes de dysmyélopoïèse, la nature primitive ou secondaire de la maladie, la présence de certaines mutations comme celles de NPM1, CEBPA et la duplication en tandem de FLT3.

Les principaux facteurs pronostiques des LAM dépendent du patient comme l'état général, l'âge et la présence de comorbidité ; mais aussi du clone leucémique comme la leucocytose, le caryotype et les mutations de certains gènes. À partir de ces éléments, l'ELN a établi une classification pronostique permettant de guider le traitement (Tableau 2).

Tableau 1 : Classification OMS 2008 des LAM (D'après *Döhner, et al., (2010)*⁴)

Leucémie aiguë myéloïde avec anomalies génétiques récurrentes

- LAM avec t(8;21)(q22;q22); RUNX1-RUNX1T1
- LAM avec inv(16)(p13.1q22) ou t(16;16)(p13.1;q22); CBFβ-MYH11
- APL avec t(15;17)(q22;q12); PML-RARA
- LAM avec t(9;11)(p22;q23); MLLT3-MLL
- LAM avec t(6;9)(p23;q34); DEK-NUP214
- LAM avec inv(3)(q21q26.2) ou t(3;3)(q21;q26.2); RPN1-EVI1
- LAM (mégacaryoblastiques) avec t(1;22)(p13;q13); RBM15-MKL1
- Entité provisoire: LAM avec mutation NPM1
- Entité provisoire: LAM avec mutation CEBPA

Leucémie aiguë avec dysmyélopoïèse

Néoplasies myéloïdes iatrogéniques

Autres Leucémie aiguë myéloïde

- Leucémie aiguë indifférenciée
- Leucémie aiguë sans maturation
- Leucémie aiguë avec maturation
- Leucémie aiguë myélomonocytaire
- Leucémie aiguë monoblastique/monocytaire
- Leucémie aiguë érythroïde
- Leucémie aiguë mégacaryoblastiques
- Leucémie aiguë à basophiles
- Panmyélose aiguë avec myélofibrose (myélofibrose aiguë ; myélosclérose aiguë)

Sarcome myéloïde

Proliférations myéloïdes liée au syndrome de Down (trisomie 21)

- Myélopoïèse anormale transitoire
- Leucémie myéloïde associée au syndrome de Down

Néoplasie à cellules dendritiques blastiques plasmacytoïdes

Tableau 2. Classification ELN (European LeukemiaNet) des LAM (d'après *Rollig et al., 2011*⁵).

Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1 inv(16)(p13.1q22) ou t(16;16)(p13.1;q22); CBFβ-MYH11
	Mutation NPM1 sans FLT3-ITD (caryotype normal)
	Mutation (double) CEBP alpha (caryotype normal)
Intermédiaire -I	Mutation NPM1 et FLT3-ITD (caryotype normal)
	NPM1 non muté et FLT3-ITD (caryotype normal)
	NPM1 non muté et FLT3-ITD (caryotype normal)
Intermédiaire-II	t(9;11)(p22;q23); MLLT3-MLL
	Caryotype ni favorable ni défavorable
Défavorable	inv(3)(q21q26.2) ou t(3;3)(q21;q26.2); RPN1-EVI1
	t(6;9)(p23;q34); DEK-NUP214
	t(v;11)(v;q23); réarrangement MLL
	-5 or del(5q); -7; abnl(17p); Caryotype complexe

La duplication en tandem de FLT3 (FLT3-ITD) concerne environ 20% des LAM. Elle se traduit par une activation aberrante et constitutive de la fonction tyrosine kinase FLT3, entraînant l'inhibition de l'apoptose et la prolifération incontrôlée de cellules leucémiques.

CEBPA (*CCAAT/enhancer binding protein alpha*) est un facteur de transcription régulateur essentiel de la granulopoïèse⁶. En 2001, une étude a montré pour la première fois la présence de mutations du gène CEBPA dans les LAM⁷. Ces mutations concernent environ 10 à 15% de LAM. La présence d'une mutation biallélique du gène, concernant habituellement les régions codant les parties N et C terminales de la protéine, sont associées à un pronostic favorable⁸.

Le gène NPM1 (nucleophosmine 1), localisé sur le chromosome 5, code une petite protéine impliquée dans le maintien du centrosome, la biogénèse des ribosome. Elle interagit avec p14-ARF et p53⁹. Les mutations de NPM1 concernent environ 50% des LAM à caryotype normal. Elles sont souvent associées à la duplication de FLT3. En l'absence de FLT3-ITD, elles sont de bon pronostic en terme de réponse au traitement d'induction, de survie sans évènement et de survie globale¹⁰.

Si *CEBPA*, *NPM1* et *FLT3* sont testés en routine au moment du diagnostic de LAM, de nombreuses autres mutations possèdent un impact pronostique important comme les mutations des gènes modificateurs épigénétiques tels que *TET2*, *IDH1*, *IDH2*, *EZH2*, *Dnmt3a* et *ASXL1*³. Ils sont fréquemment mutés chez les patients adultes atteints de LAM, avec une prévalence variant entre 12% à 35% par gène³).

I-2.5. Traitement

Chez l'adulte jeune, le traitement des LAM repose sur la polychimiothérapie suivie ou non d'une allogreffe de cellules souches. Les deux drogues de référence sont une anthracycline, comme la daunorubicine et l'aracytine (AraC). D'autres drogues comme le vépéside peuvent être aussi associées ainsi que des anticorps monoclonaux comme ceux dirigé contre le CD33, régulièrement exprimé par les myéloblastes. Ainsi le traitement des LAM du sujet de moins de 65 ans comporte deux à trois étapes. La première correspond au traitement d'induction de la rémission complète, basé sur une polychimiothérapie aplasante. La rémission est obtenue dans plus de 80% des cas mais, en l'absence de traitement complémentaire, la rechute est quasi constante. La deuxième étape est donc celle du traitement de consolidation de la rémission

complète. Il existe deux principaux types, clairement distincts, de consolidation : la polychimiothérapie et l'allogreffe de cellules souches. Schématiquement la première stratégie s'applique aux LAM ayant un risque modéré de rechute alors que la deuxième est indiquée en cas de forme agressive de la maladie. L'effet antileucémique de l'allogreffe est significativement supérieur à celui des chimiothérapies de consolidation mais, en contrepartie, l'allogreffe reste un traitement lourd, grevé d'un taux de mortalité toxique allant de 5 à 30% selon l'âge et les comorbidités. Le choix entre les deux types de consolidation est donc crucial, il s'effectue au moment du diagnostic. Le principal risque est de sous-traiter certains malades et de les exposer à la rechute où, à l'opposé, de proposer inutilement la greffe et son risque toxique à des malades ayant une maladie relativement peu agressive. Comment apprécier l'agressivité de la maladie au moment du diagnostic ? L'hétérogénéité pronostique des LAM est corrélée à une hétérogénéité biologique et les facteurs pronostiques actuellement utilisés en routine pour guider le choix de la consolidation correspondent, comme nous l'avons vu, aux anomalies cytogénétiques du clone leucémique ainsi qu'aux mutations de certains gènes comme NPM1, CEBPA et FLT3. La classification pronostique de l'ELN (Tableau 2) permet d'apprécier la gravité de la maladie. Ainsi seuls les patients ayant une LAM de risque favorable ne sont pas allogreffés. La troisième étape du traitement est inconstante, elle comporte un traitement d'entretien qui peut être proposés après chimiothérapie ou après allogreffe. Il repose sur des chimiothérapies de faible intensité, l'utilisation d'agents hypométhylants ou sur une immunothérapie qui peut comporter l'infusion de lymphocyte du donneur de cellules souches. Les outils pronostiques ou théranostiques actuels restent insatisfaisants. Par exemple, les nombreux malades au caryotype normal (50-60% des cas) et porteurs de mutation réputées favorable représentent encore un groupe pronostique très hétérogènes.

Le pronostic des LAM du sujet âgé reste particulièrement défavorable avec moins de 5 % de longs survivants. Cette différence s'explique en partie par la plus grande fragilité des patients âgés qui tolèrent mal le traitement intensifs et la greffe. De plus, biologiquement, les LAM du sujet âgé cumulent fréquemment des éléments pronostiques défavorables comme un caryotype complexe ou l'expression de marqueurs de chimiorésistance.

I-3. Chimiorésistance et transporteurs ABC

Le phénomène de résistance multidrogue (MDR) contribue à la résistance des cellules de LAM aux chimiothérapies. Cette résistance est portée par les cellules souches leucémiques de telle sorte qu'elle s'exprime d'avantage au moment des rechutes. Plusieurs mécanismes de résistance aux médicaments ont été identifiés. Parmi eux l'efflux des drogues cytotoxiques provoqué par les transporteurs ABC (*ATP binding cassette transporters*) joue un rôle central dans la résistance multidrogue.

I-3.1. Les ABC transporteurs

Le transport des molécules spécifiques à travers les membranes cellulaires est une fonction essentielle de tous les organismes vivants. Cette fonction est assurée par un grand nombre de transporteurs spécifiques. Les transporteurs membranaires peuvent être soit passifs soit actifs. Les transporteurs passifs permettent de transporter des substances selon leur gradient électrochimique. En revanche, les transporteurs actifs (ou co-transporteurs) autorisent le passage de constituants contre leur gradient de concentration. Parmi ces transporteurs actifs, la famille des transporteurs ABC joue un rôle central dans le phénotype MDR des LAM. Ces transporteurs sont des protéines membranaires capables de transporter une grande variété de substrats à travers les membranes extra et intracellulaires : sucres, acides aminés, ions, peptides, composés hydrophobes et métabolites

Le premier transporteur connu correspond à la P-gp (ABCB1) identifiée en 1976 dans des cellules résistant à la colchicine¹¹. Codée par le gène MDR, P-gp est impliquée dans la résistance multidrogue. À ce jour, il existe plus de 40 transporteurs ABC chez l'homme, divisés en huit sous-familles distinctes sur la base de l'organisation des domaines et d'homologie d'acides aminés¹². Seuls les facteurs impliqués dans la résistance aux drogues seront abordés ici, avec une revue plus détaillée d'ABCA3 qui fait l'objet du travail expérimental de cette thèse.

I-3.2. Structure

Schématiquement, les transporteurs ABC comportent deux domaines nucléaires (NBD, *Nucleotide Binding Domain*) et deux domaines transmembranaires (TMD, *Transmembrane Domain* ou MSD pour *membrane spanning domains*) (Figure 2). Les NBD contiennent plusieurs motifs hautement conservés incluant les séquences Walker A et B, ainsi qu'un domaine « signature » spécifique de cette superfamille. Les NBD se lient ensemble et hydrolysent l'ATP tandis que les TMD participent à la reconnaissance

du substrat et le transportent à travers la membrane lipidique¹³. Un transporteur ABC fonctionnel nécessite la présence de deux unités de base constituées de deux NBD et deux MSD. Ainsi les transporteurs ABC sont classés en transporteurs complets si ils contiennent deux unités en tandem (MSD-NBD-MSD-NMD) ou en demi transporteurs si ils ne possèdent qu'une seule unité (NBD et MSD). Les premiers sont actifs sous forme de monomères alors que les seconds nécessitent une homo ou une hétérodimérisation.

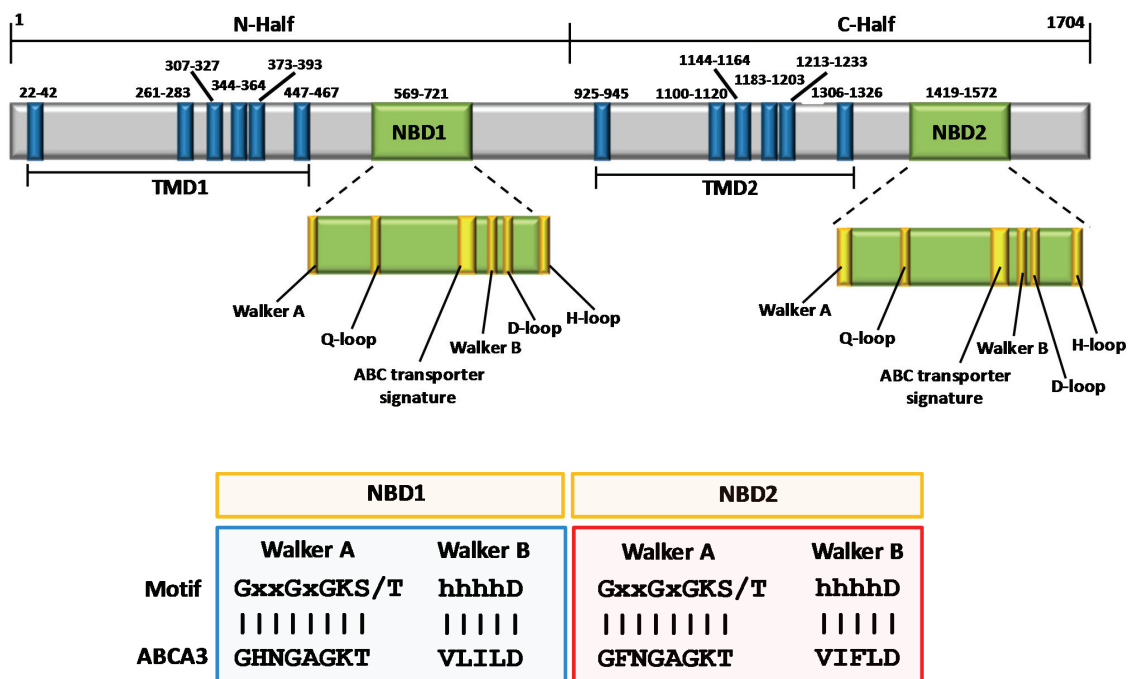


Figure 2. Organisation schématique du transporteur ABCA3 (D'après Paolini *et al.*, 2015¹⁴). Les domaines représentés en bleu correspondent aux hélices alpha. En vert sont représentés les NBD. ABCA3: *adenosine triphosphate (ATP)-binding cassette A3 transporter*; NBD: *nucleotide-binding domains*; TMD: *transmembrane domains*.

I-3.3. Les sous-familles de gènes ABC Transporteurs :

I-3.3.1. La sous-famille ABCB

La sous-famille ABCB est composée de quatre transporteurs entiers et sept demi-transporteurs, c'est la seule sous-famille à avoir deux types de transporteurs chez l'homme. Le transporteur ABCB1 (P-gp) est codé par le gène MDR1 (*multidrug resistance 1*) surexprimé et/ou amplifié dans certaines lignées de cellules tumorales résistantes aux médicaments. *In vitro*, il confère une résistance à plusieurs médicaments utilisés dans le traitement de la leucémie aiguë tels que la doxorubicine, la daunorubicine, la vincristine, la vinblastine, le méthotrexate ou la mitoxantrone¹⁵.

De nombreuses études ont montré que l'expression de la P-gp était un facteur pronostic défavorable pour la réponse au traitement et la survie des LAM de l'adulte¹⁶⁻¹⁸ mais aussi des SMD de haut risque traités par chimiothérapie intensive¹⁹. L'expression de la P-gp est plus fréquente chez le sujet âgé, expliquant ainsi les moins bons résultats de la chimiothérapie intensive chez ces malades.

I-3.3.2. La sous-famille ABCC

Il existe 12 transporteurs entiers de la sous famille ABCC. Ils possèdent des fonctions variées comme le transport d'ions et l'efflux de certains cytotoxiques. *In vitro*, ABCC3 est lié à la résistance à l'étoposide et au méthotrexate²⁰⁻²². Au plan clinique, Steinbach et al ont montré que l'expression d'ABCC3 était associée à une survie brève chez les enfants atteints de LAM²³⁻²⁵. Des résultats discordants ont été publiés au sujet de l'intérêt pronostique de l'expression d'ABCC2, ABCC4 et ABCC5 dans les LAL²⁶. Plusieurs études ont montré que l'expression d'ABCC1 était associée à un mauvais pronostic dans les LAM²⁷.

D'autres membres de la sous-famille ABCC, comme ABCC8 et ABCC9, sont des régulateurs de canaux potassium et n'ont pas de fonction de transport²⁸. ABCC10 et ABCC11 sont des pompes à anions lipophiles et peuvent induire une résistance à la chimiothérapie²⁹. Les gènes *ABCC12* et *ABCC13* codent des formes tronquées de transporteurs³⁰. Enfin, plusieurs transporteurs de la sous-famille ABCC ont été associés à des maladies génétiques humaines comme ABCC2 et ABCC6, ABCC7³¹.

I-3.3.3. La sous-famille ABCD :

Cette sous-famille contient 4 héli-transporteurs aussi connus comme transporteurs péroxisomal. Ils forment des homodimères et des hétérodimères fonctionnels ; 49 protéines distinctes étant produites via 102 transcrits épissés. Chez l'homme, ABCD1 est impliqué dans le transport d'acides gras et d'esters. Des mutations des gènes ABCD sont retrouvées dans le syndrome de Zellweger ainsi que dans l'adrenoleukodystrophie.

I-3.3.4. Les sous-familles ABCE et ABCF

La sous famille d'ABCE est constituée du seul membre ABCE1. Il possède un domaine de liaison de l'ATP mais n'a pas de domaine transmembranaire³².

I-3.3.5. La sous-famille ABCG

Elle est composée de 5 hémi-transporteurs : ABCG, ABCG2, ABCG4, ABCG5, ABCG8, ils fonctionnent soit en homo ou hétérodimères. ABCG2 ou BCRP (pour *Breast Cancer Résistance Protein*) est, avec ABCB1, l'un des transporteurs ABC les plus étudiés. Il est considéré comme l'un des principaux transporteurs responsables de la résistance aux multidrogues³³. Le rôle d'ABCG4 est resté peu connu, mais il semble compléter la fonction d'ABCG1 pour le transport du cholestérol.

I-3.3.6. La sous-famille ABCA

La sous-famille ABCA contient 12 transporteurs entiers impliqués pour la plupart dans le transport de lipides. Certaines protéines ABCA sont composées de plus de 2000 acides aminés³², la protéine ABCA13 (5058 résidus) étant la plus grande protéine ABC connue.

I-3.4. Implication d'ABCA3 dans la résistance des LAM aux chimiothérapies

Des travaux récents d'autres équipes et des résultats obtenus dans notre laboratoire ont porté sur le rôle d'ABCA3 dans la résistance aux drogues. ABCA3 a été cloné à partir de cellules tumorales³⁴. ABCA3 a initialement été trouvé exprimé dans le poumon, au niveau des corps lamellaires où il est impliqué dans les transports lipidiques et le métabolisme du surfactant³⁵. Certaines mutations du gène ABCA3 sont associées à des syndromes de détresse respiratoire du nouveau né ainsi qu'au développement de fibroses pulmonaires. La structure d'ABCA3 est représentée dans la Figure 2 (d'après Paolini *et al.*, 2015)¹⁴.

Yasui *et al.* ont trouvé une amplification et une surexpression du gène *ABCA3* dans des lignées de cellules cancéreuses résistantes aux drogues (camptothécine, cisplatine, étoposide, adriamycine, 1-beta-D-arabinofuranosylcytosine) alors que Hirschmann-Jax *et al.*, ont relié l'expression d'ABCA3 à une résistance à la mitoxantrone³⁶. Une expression d'ABCA3 a ensuite été retrouvée dans des cellules de LAM³⁷ avec un impact pronostic négatif chez l'enfant^{15, 24, 25, 38} et chez l'adulte³⁹.

ABCA3 est localisé au niveau des membranes intracellulaires et ne conférerait pas un efflux « classique » mais plutôt une séquestration intracellulaire et un transport vésiculaire de ses substrats physiologiques ainsi que de certaines drogues comme la daunorubicine. Dans le poumon, ABCA3 est retrouvé exprimé au niveau des corps lamellaires des pneumocytes de type II⁴⁰. Ces corps lamellaires sont des structures

denses, multicouches, sécrétées dans l'espace alvéolaire par exocytose. ABCA3 serait donc impliqué dans le transport transmembranaire de lipides endogènes. Des données identiques ont été obtenues dans les cellules hématopoïétiques et sanguines. Chapuy et al ont montré qu'ABCA3 était exprimé dans le cytoplasme des cellules de LAM³⁹. Dans des cellules HK293, ces auteurs ont trouvé qu'ABCA3 co-localisait avec LAMP1 (*lysosomal-associated membrane protein 1*), un marqueur des membranes lysosomales (et de l'endosome tardif) ainsi qu'avec le colorant vital rouge *Lysotracker*, qui marque la lumière des organelles acides. De façon intéressante, ce travail montrait que l'expression d'ABCA3 augmentait d'un facteur 4 la rétention intracellulaire du *Lysotracker* rouge. Ce phénomène s'accompagnait d'un « gonflement » du lysosome ainsi que d'une augmentation des protéines membranaires lysosomales comme LAMP1 et 2 et de l'activité enzymatique lysosomale³⁹. En microscopie électronique, l'expression d'ABCA3 augmentait le nombre d'organelles de l'endosome tardif incluant les corps multivésiculaires et multilamellaires. Concernant l'effet d'ABCA3 sur les drogues de chimiothérapie, la même étude montrait que l'expression d'ABCA3 redirigeait la DNR du noyau vers les lysosomes, aboutissant à une accumulation cellulaire globale de DNR associée à une diminution de la concentration nucléaire de la drogue. De façon intéressante cette étude montrait aussi qu'ABCA3 était impliqué dans la résistance à un nombre élevé de drogues incluant l'étoposide, l'AraC, la vincristine, la daunorubicine et la mitoxantrone. La même équipe a trouvé qu'ABCA3 favorisait la synthèse d'exosomes à partir de cellules de lignées lymphomateuses B agressives CD20+. Ces exosomes contiennent le CD20 et piègent par ce biais le rituximab, prévenant ainsi l'effet cytotoxique de la drogue⁴¹.

I-4. Epissage alternatif

I-4.1. Généralités

L'expression génique est caractérisée par plusieurs étapes. Chacune de ces étapes est liée à la précédente et peut l'influencer. Ces étapes sont la transcription de l'ARN à partir de l'ADN par l'ARN polymérase II, la maturation, l'export vers le cytoplasme et enfin la traduction. La maturation de l'ARN consiste en plusieurs sous-étapes : Ajout d'une coiffe en 5', exclusion des introns, addition d'une queue poly(A) en 3' suivi du raboutage des différentes parties les une aux autres⁴²⁻⁴⁴.

Les gènes eucaryotes présentent des séquences codantes (exons) et non codantes (introns). L'épissage consiste l'exclusion des séquences introniques et en l'assemblage des séquences exoniques de l'ARN prémessager. L'épissage est dit constitutif lorsqu'un exon est systématiquement inséré dans le transcrit mature. A l'inverse, on parle d'épissage alternatif quand un exon peut être soit inclus soit exclu de l'ARN mature. Plus de 95% des gènes humains subissent un épissage alternatif. Ainsi, à partir d'un seul gène, l'épissage alternatif permet de produire plusieurs ARNm qui peuvent être traduits en protéines aux propriétés et fonctions distinctes (Figure 3). Ce processus contribue à la diversité du protéome et fournit une part d'explication au différentiel entre le nombre de gènes et celui des protéines.

I-4.2. Mécanismes

I-4.2.1. Reconnaissance des sites d'épissage

L'épissage est le processus d'élimination des exons ou des introns à partir d'un ARNm. L'exclusion des exons ou des introns nécessite la reconnaissance d'un ensemble de séquences caractéristiques appelé sites d'épissage (Figure 3).

Au niveau des introns, il existe des séquences conservées appelées consensus d'épissage, situées à la jonction avec les exons et qui servent à l'exclusion des introns ou raboutages des exons.

Ces sites sont appelés :

- Le site 5' ou site donneur. Correspondant à la jonction exon-intron, il est défini par 8 nucléotides AG/GURAGU, représenté souvent par GU.
- Le site 3' ou site accepteur. Il est défini par 2 séquences, correspondant à la jonction intron/exon représenté par AG.

- Le site de branchement, situé dans l'intron et responsable de la première réaction de transestérification, représenté par le nucléotide A et en amont du site accepteur⁴²⁻⁴⁴

La reconnaissance des sites d'épissages est réalisée par un complexe protéique : le spliceosome. L'épissage commence par la reconnaissance du site d'épissage en 5' et 3' de l'intron (ou l'exon) via des protéines et de snRNP U1. Les étapes de reconnaissances et de sélection des sites d'épissage sont influencées par des séquences régulatrices, localisées dans les exons aussi bien que dans les introns de l'ARN pré messager. Ces séquences peuvent agir positivement ou négativement sur l'utilisation du site de l'épissage⁴²⁻⁴⁴. Les éléments cis-régulateurs sont importants pour favoriser l'identification des sites d'épissage et pour choisir les paires de sites d'épissage correctes. Ils permettent aussi de faciliter la reconnaissance des sites d'épissage réels tout en évitant la reconnaissance de nombreux sites cryptiques. Ces séquences peuvent être activatrices (*enhancer*) ou inhibitrices (*silencer*) et spécifiques des exons (ESE/ESS pour *Exonic Splicing Enhancer/Silencer*) ; ou des introns (ISE/ISS). Les séquences activatrices se lient avec les protéines RS, via leur motif de reconnaissance à l'ARN, pour l'assemblage du spliceosome. Alors que les séquences inhibitrices souvent liées par la famille des ribonucléoprotéines (hnRNP).

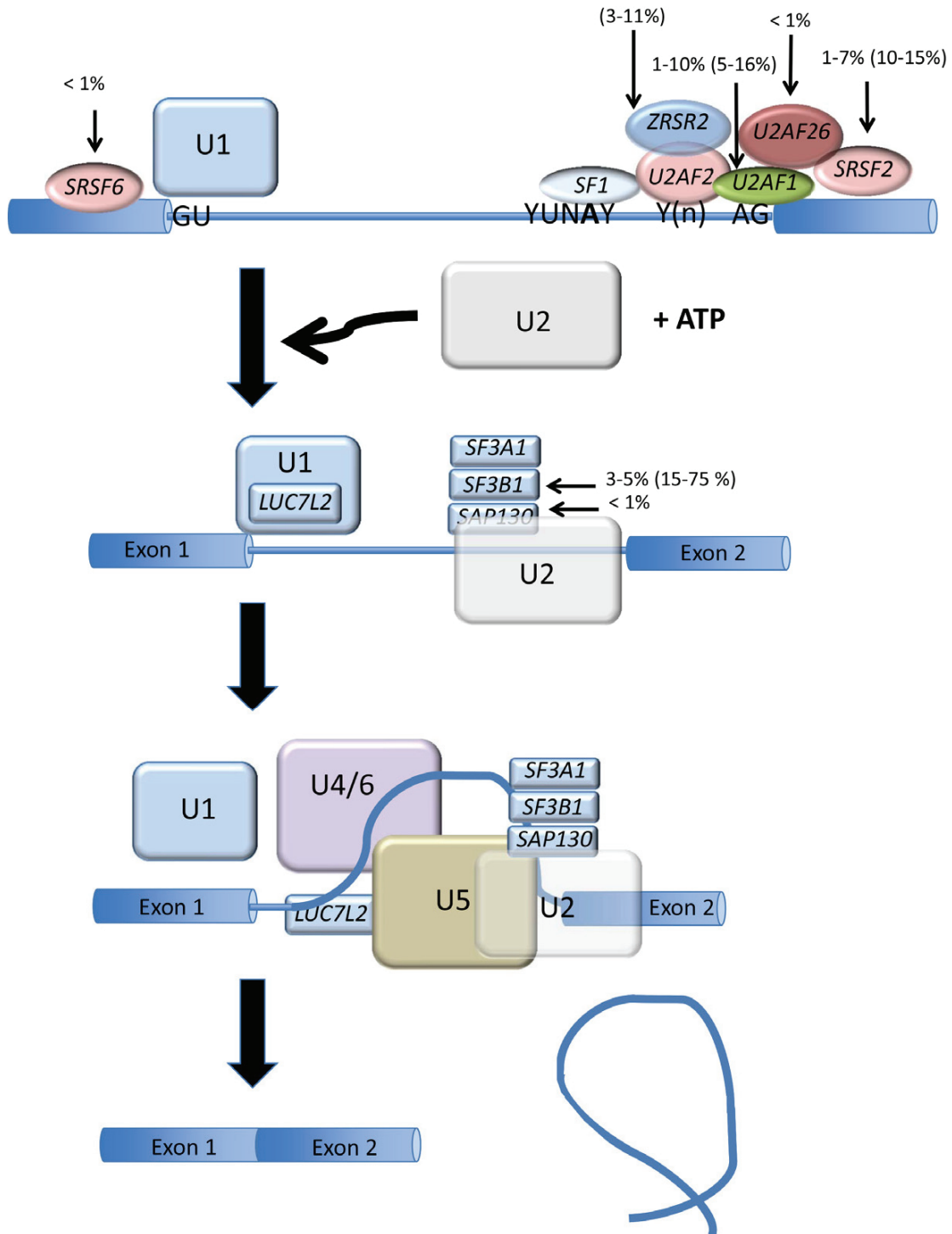


Figure 3. Mutations des gènes du splicéosome dans les LAM et les SMD (d'après Mint-Mohamed *et al.*, 2014¹). La figure représente les deux principales étapes de la réaction d'épissage. Les différentes étapes sont catalysées par le splicéosome qui reconnaît les sites 5' et 3' d'épissage de même que le site de branchement (*branch site*). Le site 5' est reconnu par le U1 snRNP (U1 snRNA-protein particle) et le site de

branchement par le U2 snRNP complexé à des protéines au niveau du site 3'. Un complexe formé de 3 snRNP, U4, U5 et U6, intègre ensuite le spliceosome où des réarrangements permettent finalement de catalyser l'excision d'intron. Les principaux sites de mutations des gènes de facteur d'épissage sont représentés pour les LAM et les SMD.

Le spliceosome est composé de particules ribonucléoprotéiques (snRNPs, small nuclear ribonucleoproteins) et de facteurs protéiques non snRNP. En général l'épissage est assuré par le spliceosome dépendant de U2, qui est le spliceosome majeur chez les eucaryotes^{1, 42-44}. Le spliceosome se compose de 5 snRNPs (U1, U2, U4/U6 et U5 snRNP) constituées d'un petit ARN (snRNA) auquel s'associent les protéines. Il existe deux familles de protéines : les protéines Sm qui interagissent avec snRNAs U1-U5 au niveau de site ARN Sm et les protéines qui s'associent spécifiquement à un snRNA (comme les protéines U1A, U1C et SF). Le spliceosome est composé par d'autres protéines que les snRNP telles les protéines U2AF65/35, SF1 et SR.

L'assemblage du spliceosome (Figure 3) consiste en plusieurs étapes. Dans un premier temps et au niveau de l'extrémité 5', la molécule pré-ARNm s'associe avec les protéines snRNP U1 formant le complexe E. La snRNP U2 se lie ensuite au site de branchement afin de former le complexe A. Le recrutement du complexe tri-snRNP U4/U5-U6 vient former le complexe B. Suite aux réarrangements provoquant la dissociation des U1 et U4 snRNP, le complexe B acquiert une structure catalytiquement active (B*). Le spliceosome effectue ensuite la première réaction de trans-estérification, générant le complexe C. Par la suite, le complexe C subit plusieurs réarrangements afin d'effectuer la seconde réaction. Après ces deux réactions, le spliceosome se désassemble et libère l'ARN messager.

Les protéines SR sont caractérisées par leur domaine RS (*Arginine-Serine containing domain*). Elles participent au recrutement de la snRNP U1 au niveau de site 5' d'épissage. Ces protéines sont aussi impliquées dans l'assemblage du spliceosome en facilitant l'incorporation du complexe U4/U5/U6. Au niveau de site de branchement, l'assemblage de spliceosome implique la fixation de la protéine SF1 / BBP (*splicing factor 1/branch binding protein*) ainsi que le facteur de U2AF au niveau de la séquence polypyrimidine. La protéine SF /BBP interagit avec la sous-unité 65 kDa de U2AF (U2AF65), alors que la sous unité 35 kDa de U2AF (U2AF35) fixe sur les nucléotides AG du site 3' d'épissage.

I-4.2.2. Réaction d'épissage :

La réaction d'épissage consiste en la ligature de deux exons et la libération de l'intron. Cette réaction se déroule en deux étapes de transestérification. Lors de la première étape, le phosphate en 5' du premier exon subit une attaque nucléophile par le groupe 2' hydroxyle au niveau de l'adénosine du site de branchement. Cette réaction génère une liaison phosphodiester entre le site de branchement et la partie 5' terminal de l'intron. Ensuite, l'hydrolyse en 3' de l'exon attaque le phosphate en 5' du deuxième exon ce qui conduit à la formation d'une nouvelle liaison entre les 2 exons et à la libération de l'intron sous forme de lasso^{1, 42-44} (Figure 3).

Les profils d'utilisation des sites d'épissage donnent lieu à plusieurs types d'épissage (Figure 4):

1. Promoteurs alternatifs: L'initiation de la transcription au niveau de promoteurs différents, dits alternatifs, donne lieu à différents ARN messagers (Figure 4, A).
2. Sites Poly-A alternatifs: L'épissage peut également conduire à l'utilisation de sites de polyadénylation différents.
3. Sites 5'd'épissage alternatifs: Par l'utilisation de différents sites 5' d'épissage, les exons peuvent être raccourcis ou rallongés en taille.
4. Sites 3'd'épissage alternatif: l'utilisation de sites 3' d'épissage alternatifs peut aussi jouer sur la taille d'un exon.
5. Exon cassette: C'est le cas le plus fréquemment rencontré. Quand un exon est bordé par des sites 5' et 3' alternatifs, on l'appelle exon cassette. Il peut être inclus ou exclu de l'ARNm.
6. Exons mutuellement exclusifs: Dans certains cas, un exon ne peut être inclus en même temps qu'un autre.
7. Rétention d'introns: l'intron peut ne pas être éliminé du transcrit mature. Dans ce cas, il fait partie de l'ARN messager qui est transporté au cytoplasme pour la traduction.

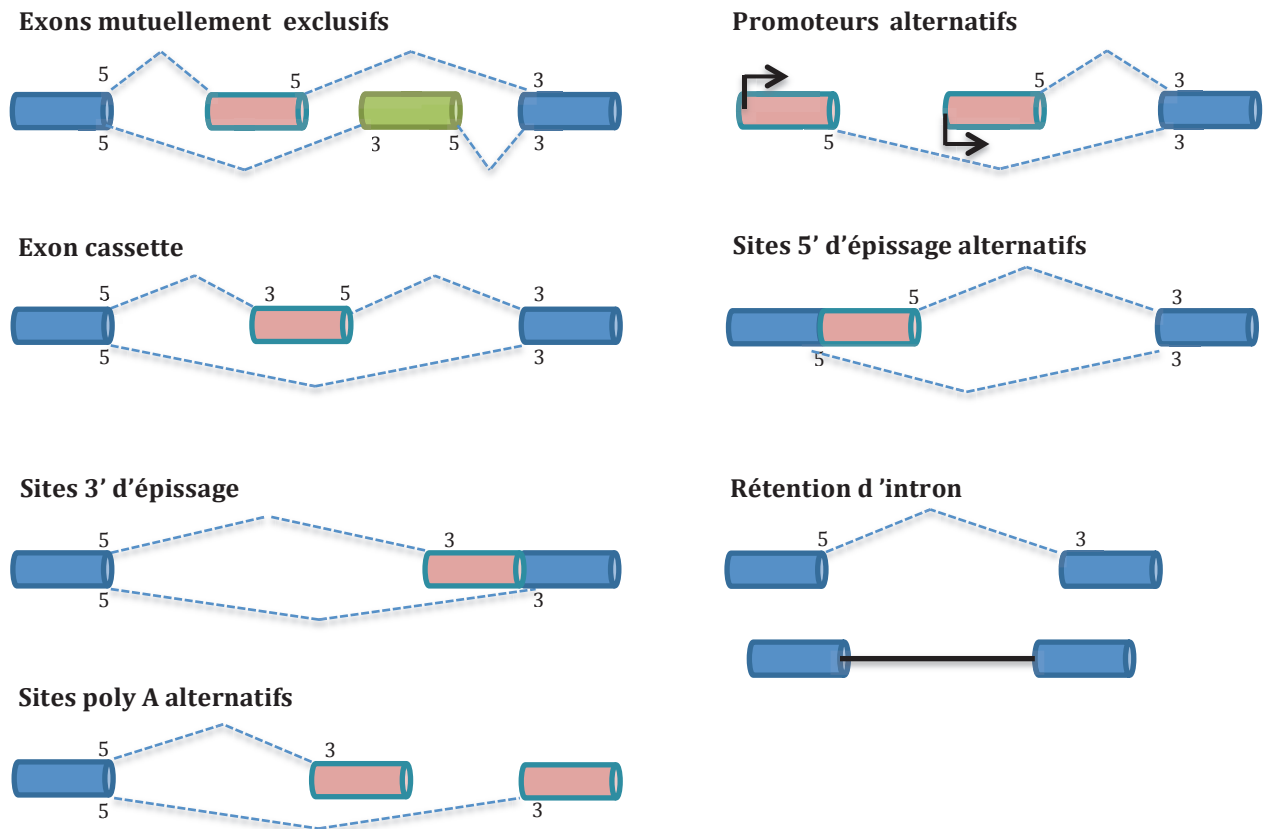


Figure 4. Principaux patrons d'épissage alternatif.

1-4.2.3. Épissage alternatif et cancer

Comme le détaillera le premier article de cette thèse, une dérégulation du programme d'épissage caractérise la cellule maligne⁴⁵. Les mutations acquises des gènes de facteurs d'épissage représentent les premières anomalies ayant conduit à explorer le patron d'épissage dans les cellules leucémiques. En fait si une mutation donnée peut conduire à une dérégulation spécifique du patron d'épissage, comme dans le cas de SF3B1⁴⁶. la myriade d'anomalies cellulaires caractérisant la cellule maligne influence aussi l'épissage à de nombreux niveaux de telle sorte que les patrons spécifiques observés dans les hémopathies malignes et les tumeurs solides dépendent vraisemblablement de causes multiples. Leur identification apparaît importante puis qu'elle contribue à expliquer l'initiation et la croissance tumorale, aide au diagnostic et à l'évaluation théranostique et constitue une nouvelle classe de cibles thérapeutiques.

I-5. Mécanismes à l'origine des anomalies d'épissage dans les leucémies aiguës myéloïdes

I-5.1. Introduction

Dans le début des années 2010, de nombreuses études ont rapporté une fréquence élevée de mutations des gènes de facteur d'épissage dans les hémopathies malignes¹. Parallèlement, les techniques de microarray spécifiques de l'épissage et le séquençage à haut débit de l'ARN ont permis de mettre à jour des dérégulations des patrons d'épissages dans ces affections. Ainsi, environ un tiers des gènes exprimés sont anormalement épissés dans les LAM. Certaines mutations de gènes du spliceosome retrouvées chez les patients définissent des patrons spécifiques d'épissage *in vitro* et même *in vivo*. Cependant, la complexité de la réaction d'épissage où interviennent plusieurs centaines de facteurs suggère que d'autres facteurs spécifiques de la cellule leucémique contribuent au patron d'épissage des LAM où la fréquence des mutations des gènes d'épissage est d'ailleurs significativement plus faible que dans les autres hémopathies myéloïdes comme les SMD. Le but de ce travail bibliographique était d'associer certaines caractéristiques cellulaires des LAM avec l'épissage. Les anomalies épigénétiques et la surexpression de WT1 caractérisent la cellule leucémique et plusieurs études permettent de suggérer qu'elles contribuent à déréguler l'épissage de la cellule de LAM.

I-5.2. Article publié:

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How mRNA is misspliced in acute myelogenous leukemia (AML)?

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ABSTRACT

Approximately one-third of expressed genes are misspliced in AML, opening the possibility that additional factors than splicing factor mutations might cause RNA missplicing in these diseases. AML cells harbor a constellation of epigenetic modifications and regularly express large amounts of WT1 transcripts. Histone acetylation/methylation and DNA CpG methylation favor either exon skipping or inclusion, mainly through interfering with RNA Pol II-mediated elongation. This can result either from the binding of various factors on Pol II or alternatively from the recruitment of DNA binding factors that create roadblocks to Pol II-induced elongation. WT1 exhibits pleiotropic effects on mRNA splicing, which mainly result from the binding properties of WT1 via its zinc fingers domains to DNA, RNA, and proteins. Through the repression of the kinase SRPK1, WT1 modifies the splicing of VEGF, which plays important roles in hematopoiesis and angiogenesis. At the protein level, WT1 interacts with the splicing factors U2AF2, WTAP, and RPM4. Therefore, AML cells appear to have acquired numerous properties known to interfere with mRNA splicing. The challenge is now to elucidate these links in order to trigger mRNA splicing at the therapeutic level.

INTRODUCTION

Protein synthesis is a finely regulated process that begins with DNA replication, followed by transcription, and concluding with translation of the protein. Post-transcriptional modifications occur mainly between the transcription and translation processes, and ensure integrity and generate the diversity that characterizes the final protein products. Among post-transcriptional modifications, RNA splicing involves the removal of noncoding sequences (introns) from the primary transcript, or pre-mRNA (Figure 1). RNA splicing is orchestrated by small nuclear ribonucleoproteins (snRNPs), small nuclear RNAs, and protein factors that form spliceosomes. This process greatly expands the coding capacity of complex genomes, as it can generate protein products with distinct and even opposite properties from a single gene locus [1, 2]. It is therefore not surprising that dysfunction of alternative splicing (AS) can lead to various diseases, including cancers. Accordingly, abnormal AS has been demonstrated to contribute to many aspects of tumor

initiation and addiction, including the control of cell proliferation and programmed cell death, metabolism of cancer cells, angiogenesis, metastasis, response to treatment and clinical outcome [3].

Acute myelogenous leukemia (AML) represents a heterogeneous spectrum of myeloid malignancies that harbor a constellation of chromosomal abnormalities, gene mutations, and epigenetic modifications. These genetic abnormalities have enabled understanding of the biology of the disease, helped generate the main diagnostic and prognostic tools, and represent key therapeutic targets. Recent reports have shown a significant deregulation of AS in AML, with approximately one-third of expressed genes being abnormally spliced in AML compared to normal CD34⁺ bone marrow cells [4]. Several reports have evidenced somatic mutations of splicing factors in AML, yet their frequency appears significantly lower than that observed in other myeloid malignancies such as in the myelodysplastic syndromes (MDS). In addition to spliceosome gene mutations, what other processes of

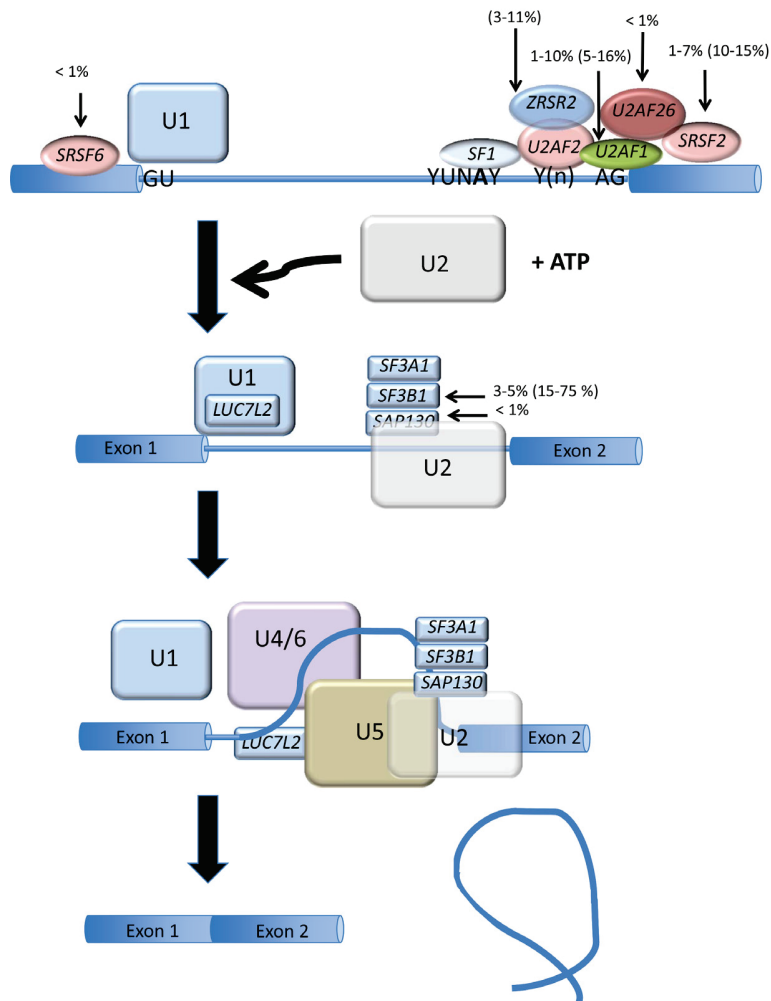


Figure 1: Splicing factor gene mutation in AML and MDS. The two main steps of mRNA splicing are represented. The steps are catalyzed by spliceosomes and specified by three RNA sequence elements called the 5' splice site, the 3' splice site and the branch site (YUNAY). The 5' splice site is recognized by the U1 snRNP-protein particle (snRNP) while the branch site is recognized by the U2 snRNP complexed with proteins at the 3' splice site. In a later step of the spliceosome formation pathway, a tri-snRNP complex composed of U4, U5 and U6 snRNPs joins the spliceosome. Further rearrangements of the spliceosome lead to catalysis of the splicing reaction and the production of the spliced product mRNA and the excised intron. The main splicing factor gene mutation is represented for AML and MDS (in parenthesis).

factors can influence and alter AS in AML? Here we have concisely reviewed some of the main cellular processes known to influence AS, including those that are deregulated in AML such as deregulated transcription, epigenetic changes (histone modifications and CpG methylation), and WT1 gene overexpression.

Abnormal mRNA splicing in AML and spliceosome gene mutations

Although abnormal RNA splicing has been previously demonstrated for several genes in AML, the connection between hematological malignancies and RNA splicing has recently emerged from studies based on next generation sequencing (NGS). Mutations in spliceosomal

genes, in particular splicing factor 3 subunit b1 (SF3b1), were first identified in myelodysplastic syndromes (MDS) [5], myeloproliferative neoplasms (MPN) [6], MDS/MPN [7], and chronic lymphocytic leukemia (CLL) [8], as well as other hematological disorders [9]. Mutations in other spliceosomal genes such as U2 small nuclear RNA auxiliary factor 1 (U2AF1) [10] and serine arginine-rich splicing factor 2 (SRSF2) were subsequently identified [11]. The mutational landscape of the spliceosome is now available for hematological malignancies [12]. These findings have opened new avenues for understanding the underlying biology and for therapeutic intervention in this setting. Overall, the proportion of cases carrying spliceosome mutations ranges from <1–90% in MDS, MDS/MPN and secondary AML compared to <1–10.5%

in de novo AML [5, 6] (Figure 1). In addition to this mutational spectrum, exon-array technologies and RNA sequencing (NGS) have enabled the assessment of the pattern of RNA splicing in hematological diseases. Certain spliceosome mutations have been found associated with missplicing in specific genes and might therefore help explain some aspects of the deregulated pattern of AS in AML. For example, in MDS, MPN, MDS/MPN, and secondary AML (sAML), U2AF1 (U2AF35) mutations, which are observed in 1–10% of de novo AML, have been found associated with abnormal splicing of genes involved in cell cycle progression and RNA processing that are somatically mutated or deleted in various cancers [13]. Yoshida *et al.* compared the effect of U2AF1 mutation on gene expression and splicing in HeLa cells and TF-1 myeloid cells [5]. The authors found that the S34F mutation quantitatively inhibited AS and triggered a significant enrichment of genes in nonsense-mediated mRNA decay (NMD), suggesting that the mutant U2AF35 triggered abnormal RNA splicing in HeLa and TF-1 cells, leading to the generation of unspliced RNA species carrying premature stop codons that induced NMD activity.

The development of natural compounds and synthetic analogues that target SFs is in progress [14]. The rationale for use of these spliceosome inhibitors in leukemia and MDS is that the majority of the splicing mutations found in hematological malignancies are heterozygous and considered gain of function or change of function/neomorphic mutations [5, 15]. Accordingly, it may be that treatment-triggered homozygous inactivation of spliceosomal genes may be more toxic to the heterozygous mutant cells than normal cells, and thus result in preferential killing of the mutant cells.

Recently Adamia *et al.* showed that approximately 29% of expressed genes are differentially and recurrently spliced in AML patients compared to normal bone marrow donors [4]. Alternative exon usages (AEUs) were found to involve oncogenes, tumor suppressor proteins, splicing factors and heterogeneous-nuclear-ribonucleoproteins, and proteins involved in apoptosis, cell proliferation, and spliceosome assembly [4]. *In silico*, these deregulations trigger important pathways involved in leukemogenesis. To date, their consequences on the physiopathology, diagnosis, response to treatment and disease outcome in AML remain unknown. In addition, little is understood regarding the causes and mechanisms that underlie abnormal AS in AML. Given such a proportion of abnormally spliced genes in AML, it could be proposed that additional factors other than spliceosomal mutations, which account for $\leq 10\%$ of AML cases (Figure 1), would be involved in the deregulation of RNA splicing in AML. AS is influenced not only by signaling pathways that target the splicing machinery but also by transcription factors and chromatin structure. Accordingly, in addition to spliceosome gene mutations, numerous factors might influence RNA splicing in

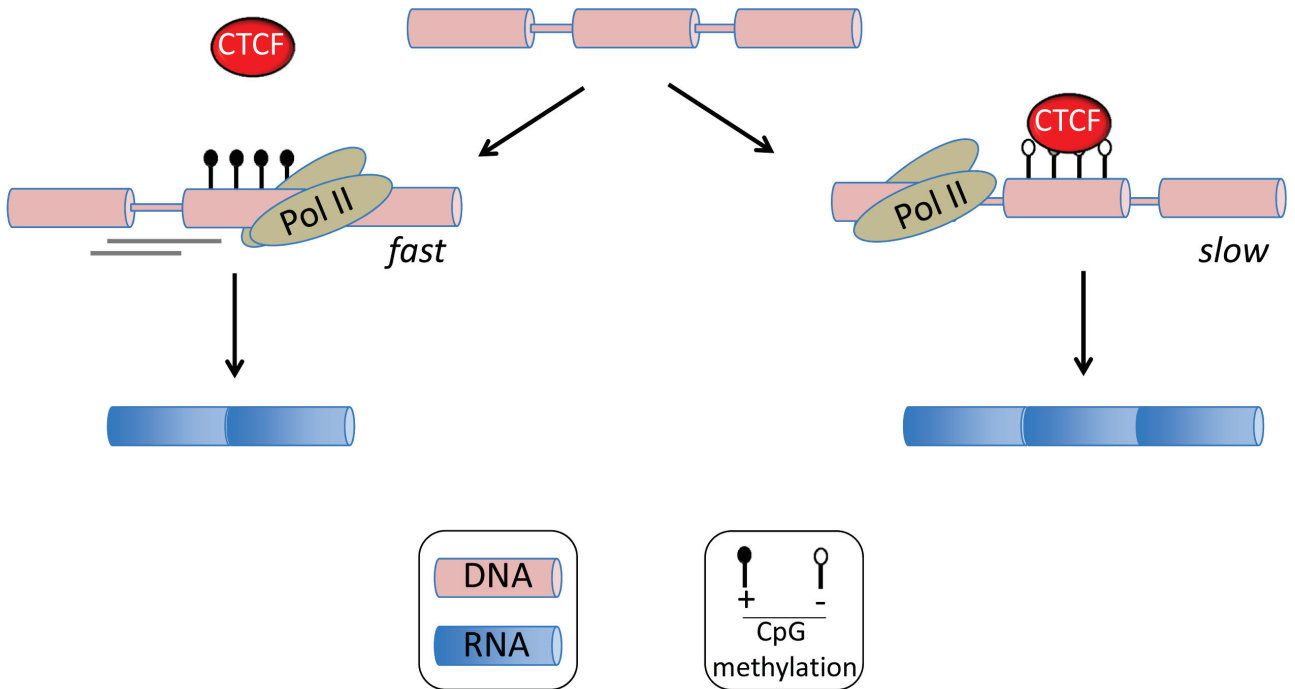
the context of AML. These include transcriptional deregulation, protein-protein interaction and epigenetic modifications.

Epigenetics in alternative mRNA splicing

In addition to RNA sequence elements and their associated splicing factors, chromatin structure and histone modifications have been found to interfere with AS regulation (reviewed in ref [16]). This became apparent when mRNA splicing was found to be closely coupled with DNA transcription in mammalian cells [17]. In fact, transcription and splicing influence one another. For example, the transcription machinery can recruit several splicing factors such as SRSF3 that binds to the carboxy-terminal domain of RNA polymerase II (Pol II) [18]. Alternatively, some factors interacting with DNA may create roadblocks to Pol II-induced elongation [19]. Such pausing during elongation has been evidenced with the DNA-binding protein CCCTC-binding factor (CTCF) that promotes inclusion of an alternative exon 5 in CD45 through a roadblock effect [20] (Figure 2A).

The link between chromatin structure and splicing was first observed through the demonstration that the more compact chromatin structure of a replicated reporter plasmid acted as a barrier to Pol II-mediated elongation and resulted in higher exon inclusion [19]. Nucleosome positioning, histone modification and DNA methylation have been found to interfere with AS. Nucleosomes are preferentially positioned at exons and are thereby presupposed to create transient pauses to Pol II-mediated elongation, providing extra time for the recognition of 3' splice sites by splicing factors [21]. Histone post-translational modifications represent a main regulator of AS (reviewed in ref [16, 19]) and acts through two distinct mechanisms. First, as illustrated in Figure 2B, some histone modifications, such as acetylation of histone 3 at Lys9 (H3K9ac), increase Pol II-mediated elongation and thereby favor exon skipping. Schor *et al.* demonstrated this for the neural cell adhesion molecule (NCAM) exon 18 upon neuron depolarization [19, 22]. Alternatively, the same study showed that neuron differentiation promoted inclusion of exon 18 in NCAM through H3K9 methylation (H3K9me), causing a reduction in Pol II-mediated elongation [19, 22]. Second, histone modifications can trigger the recruitment of factors influencing splicing, such as in the case of H3K36me3 at the fibroblast growth factor receptor 2 (FGFR2) locus that recruits the negative splicing factor PTB through the adaptor protein MRG15, resulting in exclusion of an alternative exon [23]. Abnormal DNA methylation represents a key pathogenic pathway in AML, yet its pathogenicity has mainly been linked to the global transcriptional deregulation of key genes involved in

A



B

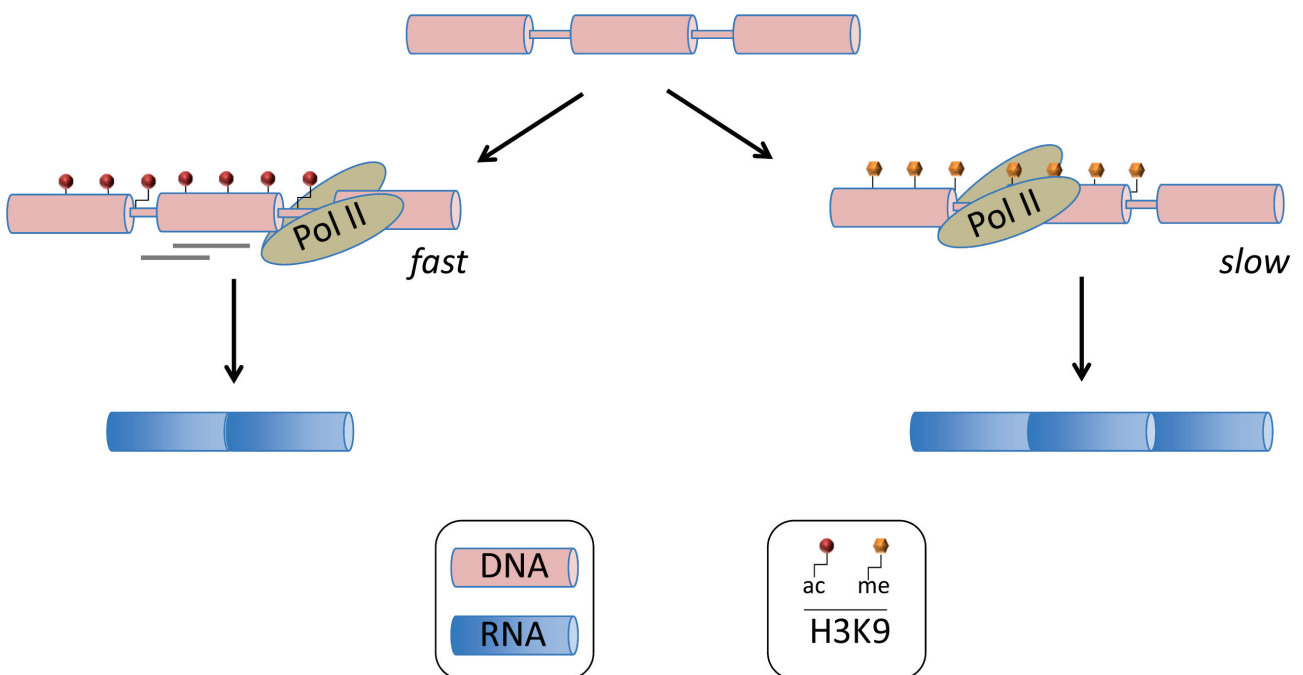


Figure 2: Epigenetic alterations in alternative mRNA splicing. (A) DNA CpG methylation influences mRNA splicing. The figure illustrates the role of differential CpG methylation in the recruitment of cellular factors that create a roadblock to Pol-II-mediated elongation, resulting in stalling of transcription and favoring exon inclusion. This has been demonstrated by Shukla *et al.* with CTCF that binds unmethylated CG-rich DNA sequences located downstream of an alternative exon [20]. (B) Histone post-translational modifications influence mRNA splicing. Acetylation of histone 3 at Lys9 has been demonstrated by Schor *et al.* to increase Pol II-mediated elongation and thereby favoring exon skipping.

tumor development [24]. However recent studies have suggested that CpG methylation and transcriptional silencing are not synonymous [25]. Figure 2A gives a schematic example of how CpG differential methylation might influence AS [20]. Indeed, in the above example of CD45 splicing, Shukla *et al.* showed that DNA methylation of the intronic site prevented CTCF binding, releasing Pol II and thereby facilitating the skipping of exon 5 [20]. Epigenetic modifications are the hallmark of AML and several recent studies have highlighted the misregulation of DNA and histone methylation in this disease [25]. For example the role of abnormal DNA methylation in leukemia has recently been reinforced by the discovery of DNMT3A [26] and TET2 mutations [27] in about 20% of AML. DNMT3A is an active DNA methyltransferase [25]. Its gene mutations are regularly heterozygous and are predicted to disrupt the catalytic activity of the enzyme. TET2 converts 5mC into several oxidative intermediates, including 5-hydroxymethylcytosine (5hmC), which are likely involved in the process of active DNA demethylation. Similarly to DNMT3a substitutions in AML, patient-associated TET2 mutations are largely loss-of-function mutations that consequently result in decreased 5hmC levels and a reciprocal increase in 5mC. Regarding the deregulation of histone methylation, several histone lysine methyltransferases (KMT) have been found mutated in cancers. For example inactivation of EZH2 by loss or mutation are present in MDS and, to a lesser extent in AML [25]. EZH2 is the catalytic component of the PRC2 complex, which is primarily responsible for the methylation of H3K27. ASXL1 is an additional epigenetic modifier found mutated in AML. ASXL1 mutations result in loss of polycomb repressive complex 2 (PRC2)-mediated histone H3K27 trimethylation. Mutations in ASXL1 exon 12 are present in 5% - 30% of de novo AML where its frequency is 5 times higher in older patients [28], whereas some studies suggest a higher prevalence in secondary AML. Given the aforementioned link between missplicing and epigenetic changes, it will be therefore an important task to assess whether or not; missplicing is involved in the functional consequences of the mutation of epigenetic modifiers such as TET2, EZH2 and ASXL1 in AML.

WT1 possesses pleiotropic effects on the splicing machinery

WT1 expression in AML

The Wilms' tumor gene (WT1) was among the first tumor suppressor genes to be cloned [29]. Originally named for its role in the pediatric kidney malignancy, Wilms' tumor, it has since been implicated in many other cancers including hematologic malignancies. In AML *ex vivo*, reduction of WT1 expression levels leads

to decrease of proliferation and apoptosis of leukemic cells [30], indicating that WT1 acts as an oncogene in these diseases. Although the diagnostic level of WT1 expression does not seem to possess a significant prognostic impact, its decreasing level upon induction of chemotherapy has been found to correlate with subsequent favorable outcome. WT1 mutations occur in about 10% of AML with normal karyotype, whereas their prognostic impact remains unclear. In addition to its prognostic usefulness, WT1 has become a broadly used marker for minimal residual diseases and a promising therapeutic target for anti-sense molecules, antibodies, and vaccine strategies [31].

More than 30 different WT1 isoforms are generated by a combination of alternative RNA splicing, the usage of different start codons and RNA editing [32]. The most widely studied isoforms are the inclusion or exclusion of exon 5 and an alternative splice donor site in exon 9, which encodes three amino acids, KTS (Figure 3). Thus, WT1 can be exon 5+ or exon 5- and KTS+ or KTS-, and all four isoforms are expressed in several tissues [33]. Interestingly, some of these isoforms exhibit specific biological properties, and their expressions depend on the age of patients and disease phenotype (i.e. MDS vs. AML) [34, 35].

At the molecular level, WT1 is a zinc-finger DNA-binding protein that can act as a transcriptional activator or repressor depending on the cellular or chromosomal context [36] (Figure 3). As a zinc finger transcription factor, WT1 can bind DNA, RNA, and protein, affecting the flow of eukaryotic genetic information from transcription to translation. WT1 isoforms possess distinct biochemical properties. The -KTS isoform colocalizes in the nucleus with regions of active transcription and binds DNA, RNA, and protein, whereas the +KTS isoform is capable of binding to RNA and proteins and colocalizes with splicing machinery in nuclear speckles [37].

Direct and indirect WT1-RNA interactions

The WT1-RNA interaction was first suggested by Larsson *et al.* who found that the +KTS WT1 isoform colocalizes with small nuclear RNA-protein particles (snRPs) in COS7 cells {Larsson, 1995 #148}. The mouse WT1 +KTS counterpart was further observed to accumulate on nascent transcripts when transfected in *Xenopus* oocytes [38], while Ladomery *et al.* [39] and Morrison *et al.* [40] identified the presence of WT1 in messenger ribonucleoprotein particles.

Yeast two-hybrid screens identified the splice factors U2AF2 (U2AF65) [41], WTAP [42], and RBM4 [42] as WT1-interacting proteins (Figure 4A). U2 auxiliary factor 2 (U2AF2/65) interacts with the zinc finger domain of both + and -KTS WT1 isoforms via a serine-arginine domain within its N terminus. WT1 associated protein

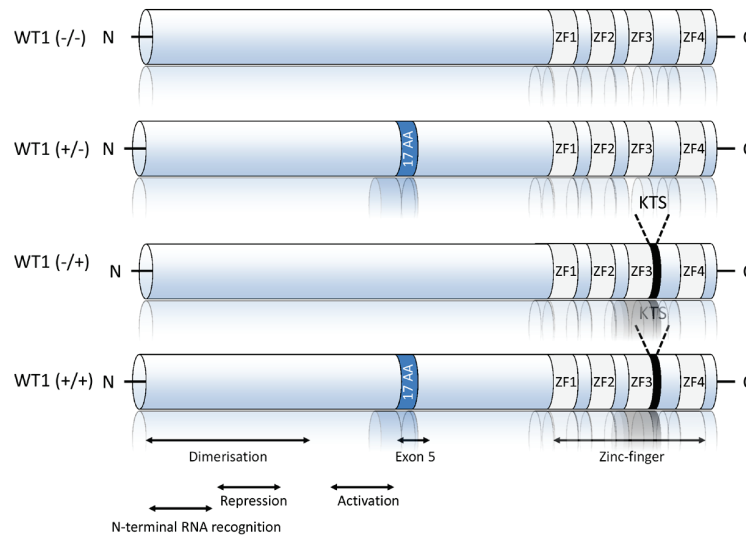


Figure 3: Schematic representation of the four main Wilms tumor suppressor gene (WT1) isoforms. The best studied splice variants of WT1 include mammalian-specific 17 amino acids encoded by exon 5 and three amino acids (KTS) at the terminal end of zinc finger three, encoded by an alternative splice donor site in exon 9. These generate the four main WT1 isoforms, with molecular masses of 52–54 kDa. The N-terminal domain includes dimerization, transcriptional activation, RNA recognition [41, 66, 67] and repression domains. DNA and RNA binding activities are both mediated by the zinc-finger domain.

(WTAP) binds the +KTS but not -KTS WT1 isoform (Figure 2A), and is involved in 3' splice site selection [41]. Interestingly WTAP has been recently found to play an important role in abnormal proliferation and arrested differentiation of leukemia cells [44]. RNA-binding motif protein 4 (RBM4) is a splice factor that promotes skeletal muscle-specific exons [45] and is involved in stem-cell differentiation in the central nervous system [46] and the pancreas [47]. It is also involved in embryonic development in *Drosophila* and translational control in mouse and human [48, 49]. Markus *et al.* identified RBM4 as a binding partner of WT1 through yeast two-hybrid assays [43 d]. In contrast to U2AF65 and similar to WTAP, RBM4 is specific to the +KTS WT1 isoform [43] (Figure 2A). Mechanistically, minigene experiments have shown that WT1 counteracted the splicing effect of RBM4. Morrison *et al.* found that WT1 cofractionated and co-immunoprecipitated with the splice factor PSF in nuclear extracts prepared from mouse mesonephric fetal kidney M15 cells [50].

Besides its interaction with splice factors, WT1 interacts with RNA processing through RNA binding via its zinc finger domain [38, 51]. Accordingly WT1 is not restricted to the nucleus but shuttles between the nucleus and cytoplasm, where it is involved in the regulation of mRNA export, localization, and translation. *In vitro* binding assays have shown that a hairpin loop is critically required for RNA binding by WT1 zinc fingers [38]. Interestingly Morrison *et al.* found through immunoprecipitation coupled with PCR differential-display that WT1 interacted with the RNA of p54^{nrb}, which encodes a splice factor related to PSF [50].

Functional and pathogenic consequences of WT1-RNA interactions

The above-summarized interactions between WT1 and the splicing machinery are presupposed to alter the alternative exon usage that characterizes AML cells [4]. Two recent studies have addressed the consequences of WT1-RNA interactions and unmasked the effects of WT1 mutants on the splicing of the vascular endothelial growth factor (VEGF) and its consequences on angiogenesis, hematopoiesis, and tumor development [52, 53].

Angiogenesis is a key pathogenic mechanism in cancer and leukemia [54]. It is positively and negatively regulated by VEGF₁₆₅ and VEGF_{165b} splice isoforms, respectively [55]. VEGF splicing is controlled at least in part by serine-arginine-rich proteins (SRSFs), which include SRSF1, also called ASF, SF2, SF2/ASF, or ASF/SF2 [56]. SRSF1 promotes the expression of the angiogenic VEGF-A₁₆₅ isoform via the selection of a specific 3' proximal splicing site (3' PSS) whereas the anti-angiogenic VEGF-A_{165b} is expressed through the selection of a 3' distal splicing site (3' DSS, Figure 4B). Its nuclear localization is brought about by phosphorylation by a number of splicing factors, including SRPK1 [57], and Amin *et al.* demonstrated that WT1 isoforms lacking the KTS domain bind to the SRPK1 promoter and repress expression through a specific WT1 binding site [52] (Figure 4B). This repression results in SRSF1 hypophosphorylation that results in the selection of the 3' DSS, thereby inhibiting angiogenesis through the expression of the VEGF-A_{165b} isoform. In contrast, certain WT1 mutants carrying a substitution in the zinc-finger domain were unable to

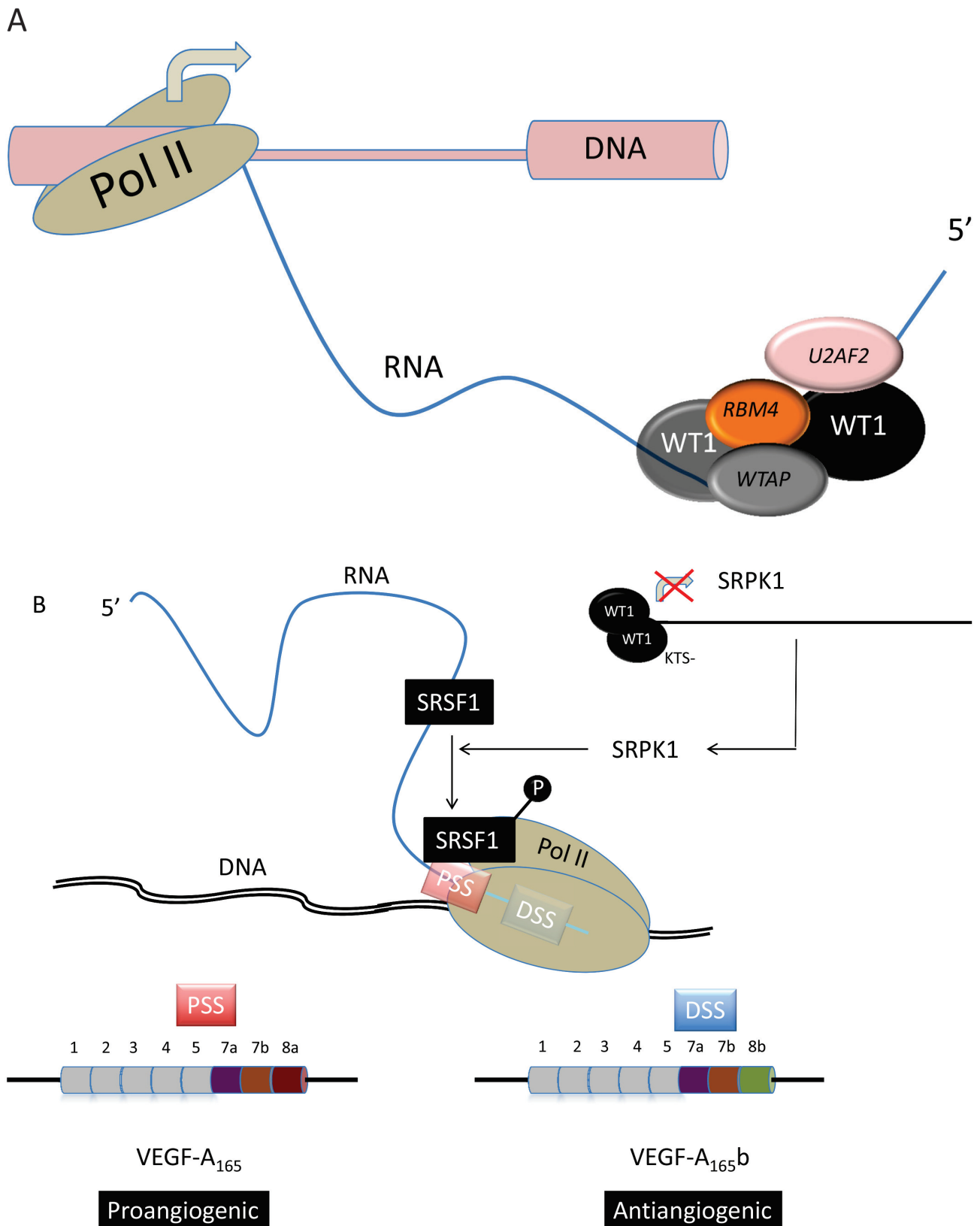


Figure 4: WT1 in alternative mRNA splicing. (A) WT1 interacts with U2AF2, RBM4, and WTAP splicing factors. (B) -KTS (Figure 3) WT1 represses SRPK1 transcription, leading to SRSF1 hypophosphorylation and thereby for the selection of a 3' proximal splicing site (PSS) during VEGF-A synthesis. This PSS usage results in the expression of the VEGF-A₁₆₅ pro-angiogenic factor [52, 57].

repress SRPK1 expression, leading to a SRPK1-mediated phosphorylation of SRSF1 that triggers the expression of the pro-angiogenic VEGF-A₁₆₅ isoform [52].

In addition to their differential roles in angiogenesis, VEGF isoforms have been found to possess distinct effect on hematopoiesis. Cunningham *et al.* showed that WT1-deficient mouse embryonic stem cells exhibit reduced hematopoietic potential [53]. This diminished hematopoiesis was caused by a VEGF-A-dependent apoptosis of hematopoietic progenitor cells associated with a shift in VEGF-A isoforms toward VEGF-A 120. Interestingly, high levels of VEGF-121 (the human counterpart of murine VEGF-A120) have recently been identified as an independent prognostic factor associated with poor survival in AML [58].

Conclusion and perspective

In addition to acquired and/or selected somatic mutations, AS offers AML cells a rapid, dynamic, and reversible means to deal with their environment, which depends on numerous factors influencing tumor development, maintenance and recurrence. These influencing factors include the bone marrow stroma, immune system and treatments. Given the huge excess of

abnormally spliced genes in AML [4], it is reasonable to propose that alteration of splicing regulation participates in the phenotypic plasticity of AML cells. Accordingly, validating the *in silico* evidenced pathways deregulated in AML upon RNA missplicing will allow better assessment of the pathogenic implications of AS in this disease [4]. The example of S34F U2AF1 mutation, which has been demonstrated to trigger a specific pattern of AS [5], suggests that specific patterns of AS possessing specific functional consequences might be related to specific defects in the splicing process.

Numerous questions remain to be addressed (Figure 5). For example, with the present reviewed data in mind, it becomes important to determine whether and how histone modifications, DNA methylation and WT1 expression modify AS and participate in the AML AS landscape. The main biological prognostic factors in AML have consisted of cytogenetic, gene mutations and transcriptional deregulations. Some patterns of AS have been found to correlate with tumor aggressiveness in solid tumors [58] while in AML, the expression of certain isoforms, such as for WT1 itself, TP53 [60], HOXA9 [61], BAALC [62], VEGF [58], or BCL-X [63], have been found to exhibit distinct effects on disease outcome. This encourages the investigation of the

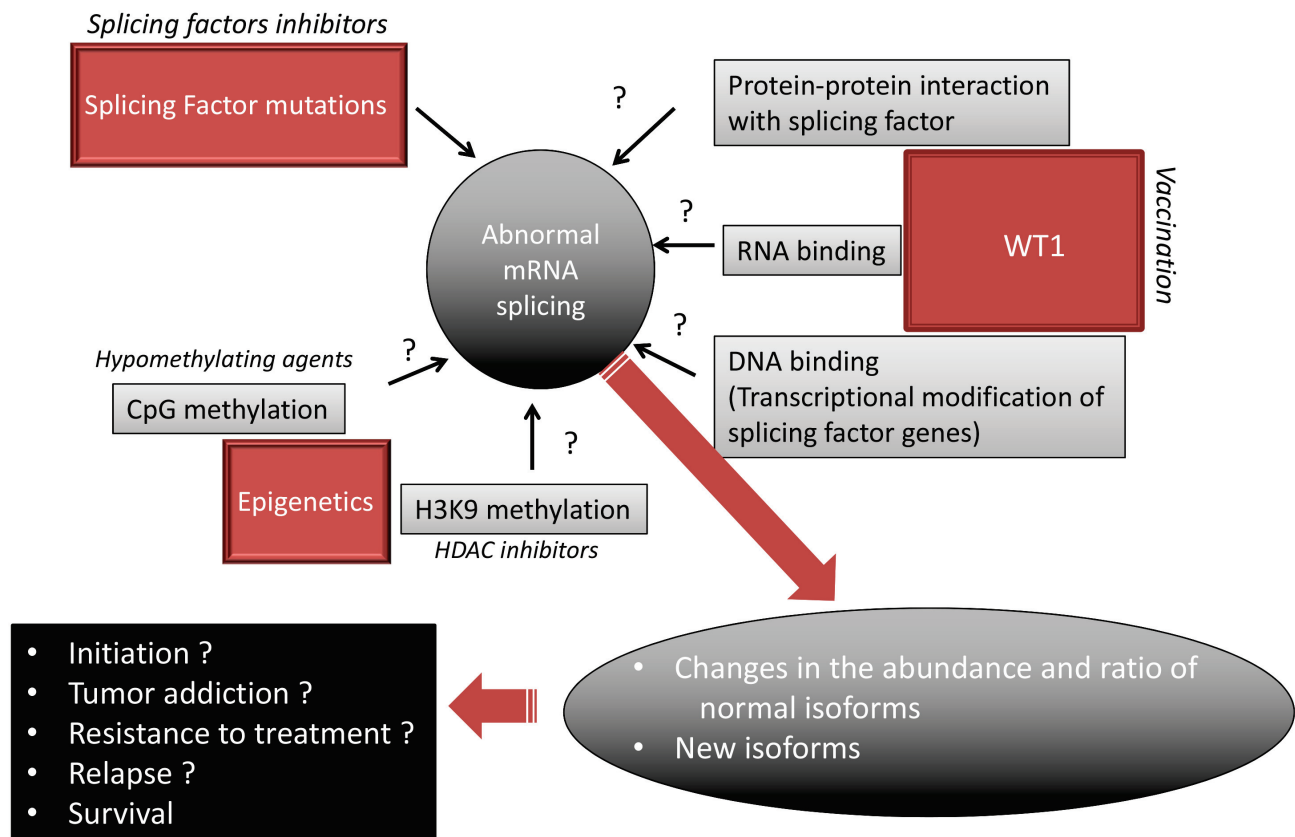


Figure 5: Proposed model of mRNA missplicing in AML. Hypothetical mechanisms are denoted by a question mark. A targeted treatment is represented for each proposed mechanism.

prognostic implication of the newly evidenced myriad of AML-associated alternative exon events [4]. Histone deacetylase inhibitors, DNA hypomethylating agents, and WT1 vaccination are currently used in many AML patients. Given that epigenetic and WT1 interfere with AS, it is possible that the antileukemic effect of these drugs relies at least in part on modification of AS. A recent meta analysis of published cancer vaccine trials has shown that objective clinical responses (including stable disease) are observed in 64% of evaluable WT1-vaccinated patients with hematological malignancies while immunogenicity of WT1-based cancer vaccines was demonstrated by the detection of a specific immunological response in 68% of cases [64]. Some drugs directly target the spliceosome, such as spliceostatin that targets SF3B1, have been found to possess antitumor effect *ex vivo* [14]. Whether these specific splicing factor inhibitors act in non-mutated AML remains to be assessed. In addition to these approaches, steric-blocking oligonucleotides have been designed to specifically redirect alternative splicing, repair defective RNA, and restore protein production. Although these product were designed to treat genetic disorders such as Duchenne muscular dystrophy [65], it will be interesting to develop such strategy to target the specific splicing defects observed in AML.

Conflict of Interest

The authors declare no conflict of interest

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I-5.3. Conclusion, perspectives

La Figure 5 résume les données de la littérature appliquées aux anomalies d'épissage des LAM. En plus des mutations des gènes d'épissages, de nombreuses perturbations connues de la cellule leucémique semblent pouvoir concourir aux dérégulations d'épissages. Sachant qu'environ 30% des gènes exprimés sont anormalement épissés dans les LAM (ref), l'impact de ces dérégulations sur l'initiation et le maintient du phénotype tumoral, sur la résistance au traitement et le risque de rechute restent à identifier.

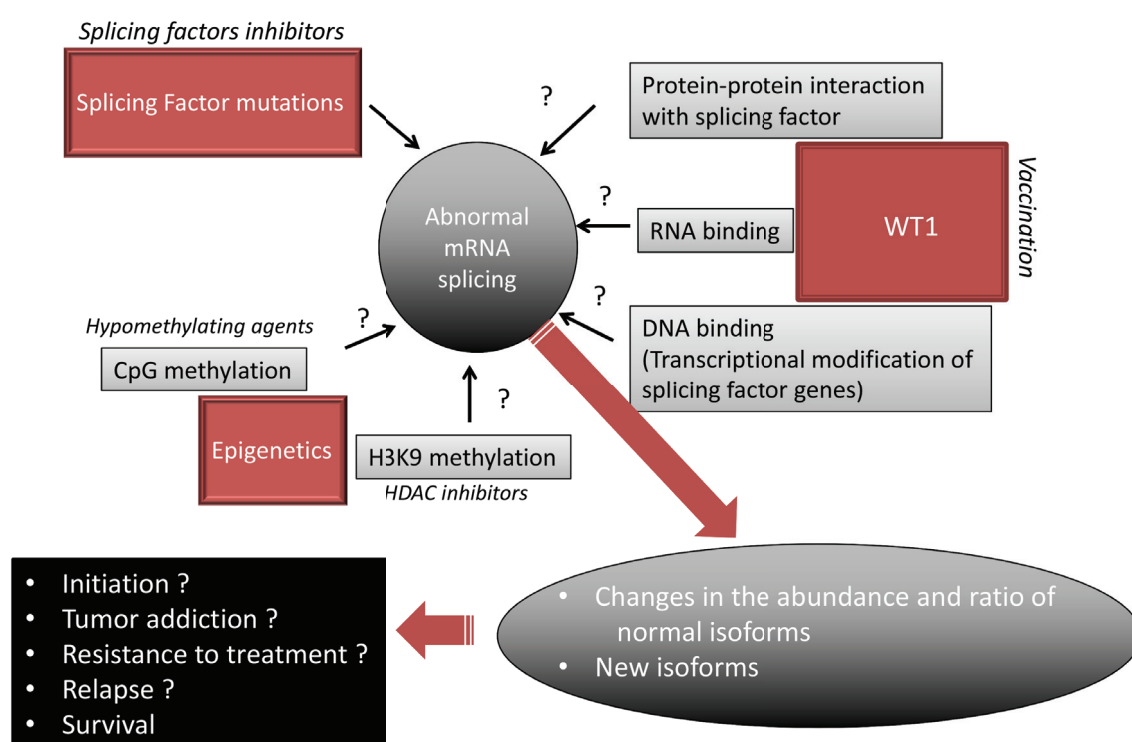


Figure 5. Mécanismes et conséquences possibles des anomalies d'épissage dans les LAM (d'après Mint-Mohamed *et al.*, 2014¹).

Au plan mécanistique, dans ce travail, nous avons choisi d'aborder l'influence de WT1 mais aussi de DEK dans les modifications d'épissages des LAM. Nous avons par ailleurs voulu savoir si la résistance aux drogues était associée à un patron d'épissage particulier dont la détection permettrait d'apprécier le génie évolutif de la maladie au moment du diagnostic et dont le ciblage pourrait améliorer le traitement par CTI.

II – RESULTATS

II-1. Anomalies d'épissage associées à l'expression de DEK ou WT1 et à la chimiorésistance dans les leucémies aiguës myéloïdes

II-1.1. Introduction

L'objectif de ce second travail était d'évaluer d'une part les effets des expressions de WT1 et de DEK sur l'épissage des cellules de LAM et de rechercher si la résistance aux principales drogues utilisées dans le traitement des LAM pouvait définir une signature en termes de maturation des mRNA. Concernant l'effet des deux oncogènes sur l'épissage, le rationnel liant WT1 au spliceosome a été détaillé dans le précédent article. DEK a été initialement identifié comme partenaire de CAN dans la translocation t(6;9) (p23;q34) observée dans une minorité de LAM de mauvais pronostic. DEK joue un rôle important dans la granulopoïèse et l'oncogenèse, son inhibition ayant un effet antitumoral. Impliquée dans le remodelage de la chromatine, DEK joue aussi un rôle dans l'épissage. Comme WT1, DEK interagit avec U2AF35/U2AF65 pour réguler la reconnaissance du site accepteur d'épissage par le spliceosome.

La contribution de l'épissage au polymorphisme d'expression des gènes pose la question de son impact dans la résistance au traitement des LAM. Une relation entre épissage et agressivité tumorale a clairement été mise à jour dans certaines tumeurs solides alors qu'aucune étude systématique ne s'est adressée aux leucémies. Nous avons donc recherché si une résistance à l'AraC, à la daunorubicine ou à l'azacitidine, trois drogues régulièrement utilisées dans la prise en charge des LAM, pouvait s'associer à une signature en terme d'épissage.

Pour aborder ces questions nous avons tout d'abord analysé les patrons d'épissages dans des lignées de cellules leucémiques. Les résultats obtenus *in vitro* ont ensuite été validés *in vivo*, dans des échantillons provenant de patients adultes prélevés au moment du diagnostic de LAM. Au plan technique, les patrons d'épissage ont tout d'abord été analysés à l'aide de puces exon. Dans une seconde étape, nous avons déterminé la spécificité de cette approche à l'aide de PCR spécifiques d'exon. Enfin, des RTPCR quantitatives spécifiques d'exon ont été mises au point pour quantifier certains événements *in vitro* et *in vivo*.

II-1.2. Article :

Mint Mohamed, A., Balsat, M., Thenoz, M., Koering, C., Payen-Gay, L., Cheok, M., Mortada, H., Auboeuf, D., Pinatel, C., El-Hamri, M., Dumontet, C., Cros, E., PascaleFlandrin, G., Nibourel, O., Preudhomme, C., Michallet, M., Thomas, X., Nicolini, F., Solly, F., Guyotat, D., Campos, L., Wattel, E., Mortreux, F., 2015. Oncogene- and drug resistance-associated alternative exon usage in acute myeloid leukemia (AML). *Oncotarget*.

Oncogene- and drug resistance-associated alternative exon usage in acute myeloid leukemia (AML)

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ABSTRACT

In addition to spliceosome gene mutations, oncogene expression and drug resistance in AML might influence exon expression. We performed exon-array analysis and exon-specific PCR (ESPCR) to identify specific landscapes of exon expression that are associated with DEK and WT1 oncogene expression and the resistance of AML cells to AraC, doxorubicin or azacitidine. Data were obtained for these five conditions through exon-array analysis of 17 cell lines and 24 patient samples and were extended through qESPCR of samples from 152 additional AML cases. More than 70% of AEU identified by exon-array were technically validated through ESPCR. *In vitro*, 1,130 to 5,868 exon events distinguished the 5 conditions from their respective controls while *in vivo* 6,560 and 9,378 events distinguished chemosensitive and chemoresistant AML, respectively, from normal bone marrow. Whatever the cause of this effect, 30 to 80% of mis-spliced mRNAs involved genes unmodified at the whole transcriptional level. These AEU unmasked new functional pathways that are distinct from those generated by transcriptional deregulation. These results also identified new putative pathways that could help increase the understanding of the effects mediated by DEK or WT1, which may allow the targeting of these pathways to prevent resistance of AML cells to chemotherapeutic agents.

INTRODUCTION

Acute myelogenous leukemia (AML) represents a heterogeneous spectrum of myeloid malignancies that harbor a constellation of chromosomal abnormalities and gene mutations as well as transcriptional, proteomic, metabolomic and epigenetic modifications. These abnormalities have enabled better understanding of disease mechanisms and generated diagnostic and prognostic tools that rely on key therapeutic targets. Intensive anthracycline and cytarabine (AraC)-based combination chemotherapy has been the backbone of acute myeloid leukemia (AML) treatment for nearly forty years [1]. In patients for whom such intensive treatment is unsuitable, hypomethylating agents can prolong survival [2]. However, for both of these therapeutic approaches resistance can be an issue. For example, the expression and activity of the ATP Binding Cassette B1 (ABC-B1; MDR1/Pgp) gene is significantly correlated with the response to intensive chemotherapy (IC) and disease outcome [3].

Beyond transcriptional modifications, recent reports from exon-array analyses have shown a significant deregulation of splicing in cancers, with approximately one-third of expressed genes being abnormally spliced in AML compared to normal CD34⁺ bone marrow cells [4]. These splicing events can be caused by numerous factors such as spliceosome mutations [5], histone acetylation/methylation, DNA CpG methylation and oncogene expression [6]. WT1 and DEK are two oncogenes that are regularly expressed in AML cells and both interact with splicing machinery. WT1 physiologically influences alternative splicing (AS) through interactions with WTAP, RBM4 and the U2AF1/U2AF2 heterodimer, which is involved in splice acceptor site recognition by the splicing machinery [7–9]. In addition, WT1 mediates transcriptional repression of the splicing factor kinase SRPK1 to alter mRNA splicing [10]. In line with this evidence, Cunningham et al. recently showed that WT1 regulates murine hematopoiesis via altered VEGF splicing [11]. DEK was first discovered after the identification of the translocation t(6;9) (p23;q34) in a subset of AML patients [12], and further studies showed a critical role for DEK in normal granulopoiesis and in tumor development [13]. DEK is involved in chromatin remodeling and may also influence mRNA splicing. In fact, like WT1, DEK interacts with U2AF35/U2AF65 to regulate splice acceptor site recognition by the splicing machinery [14].

In AML, some spliceosome gene mutations are associated with a specific pattern of AS [15], whereas in solid tumors certain isoform switches associated with tumor development are independent of somatic mutations [16]. Specific patterns of AEU events have been associated with tumor aggressiveness and response to treatment [17, 18], while certain hematological malignancies harbor a specific pattern of spliceosome

mutations such as refractory with ring sideroblasts and thrombocytosis with SF3B1 mutation [19], as well as AML with trisomy 13 and SRSF2 mutation [20]. Thus, accounting for AS patterns in cancer is becoming an important challenge because specific AS events may represent useful biomarkers while the development of spliceosome- [21] and AS-specific [22, 23] therapeutic tools are in progress. Here we investigated whether the expression of certain oncogenes and the resistance of AML cells to anti-cancer drugs might be associated with specific exon expression patterns. We used exon array analysis confirmed by exon-specific PCR (ESPCR) to evaluate AML cells expressing various amounts of either DEK or WT1 and AML cells that show sensitivity or resistance to AraC, doxorubicin (DXR) or azacitidine (AZA). Data gathered from cell lines were confirmed in patient-derived samples in which exon-arrays confirmed by exon-specific RT-PCR permitted the identification of specific exon expression patterns that are putatively involved in disease phenotypes and responses to treatment.

RESULTS

Oncogene-associated exon expression profile in AML cells

WT1 and DEK are frequently overexpressed in AML and can interfere with spliceosome activity [7–11, 14]. We hypothesized that the expression of these two oncogenes might influence exon expression patterns in AML cells. We thus used Affymetrix HTA2 exon arrays to examine the exon expression profiles of three AML cell lines (MOLM13, Kasumi-1 and KG1) that had normal or knocked-down levels of WT1 or DEK gene expression. shRNA-WT1 significantly reduced the level of WT1 mRNA and protein expression in the three cell lines (Figure S1A) and similar results were obtained with DEK (data not shown). Microarray data were cross-compared between the cell lines and only statistically significant modifications ($p < 0.05$) were selected. Computational analyses of exon arrays and annotation of exon events were carried out as previously described [24] and as detailed in the Methods section. Figure 1A shows the distribution of quantitative and qualitative gene modifications in cells stably knocked down for either WT1 or DEK expression as compared to cells treated with control PLKO vector. For WT1, 1, 573 AEU events were identified across 1, 200 genes, of which 495 (41%) were altered at the whole gene expression level (Figure 1A). For DEK, 1, 130 AEU events were identified among 934 genes, with 188 (20%) altered at the level of whole gene expression (Figure 1A). The distribution of alternative splicing events was significantly different between DEK and WT1 expression ($p = 0.008$, Pearson's Chi-squared test, Figure 1B). Notably, the proportions of alternative last exon ($p = 0.009$), acceptor ($p < 10^{-4}$), intron-retention

($p = 0.004$) and promoter ($p = 0.021$) were significantly different between the two cell categories. Cells knocked down for either DEK or WT1 were found to share 99 exon events with the same regulation that were harbored by 83 genes (Supplementary Table 1). Among these genes, 24 and 36 were found to be transcriptionally deregulated by DEK and WT1, respectively (Supplementary Figure 2), including 20 genes that were transcriptionally modified

by both oncogenes. These 20 genes displayed the same deregulation with either WT1 or DEK expression.

Gene ontology (GO) analysis was performed to gain insight into the functional significance of either full gene or exon expression profiles that distinguish PLKO- from shRNA-infected cells. As shown in Figure 1C, D, large subsets of enriched genes were related to pathways that are known to be affected in

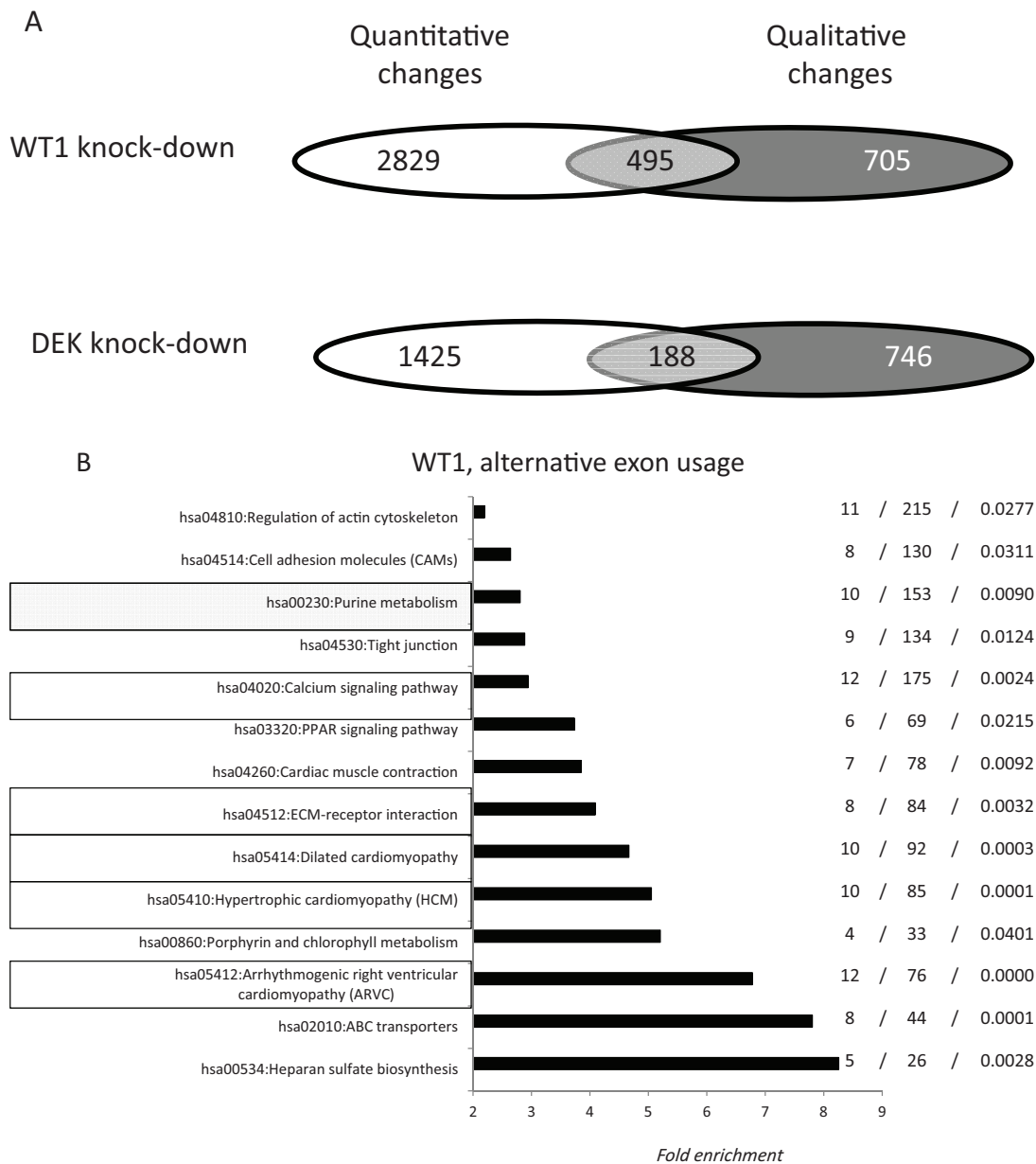


Figure 1: Distribution of alternative exon usages in AML cell lines after WT1 and DEK expression. A. Distribution of quantitative and qualitative gene modifications in cells expressing either high or low levels of WT1 or DEK. Total RNA was analyzed using the exon microarray platform GeneChip HTA2 (Affymetrix). Microarray data were cross-compared between cell lines and only statistically significant modifications ($p < 0.05$) that were shared by at least 2 of the 3 cell lines (MOLM13, Kasumi-1 and KG1) were selected. For the two cell categories (WT1 high versus WT1 low, and DEK high versus DEK low), Venn diagrams show the distribution of genes modified at the level of whole gene expression (white), alternative exon usage (dark grey) or both (light grey).

(Continued)

AML cells. DEK and WT1 displayed common and specific functional pathways with respect to both AS and whole gene expression. Six functional pathways, which represented 37.5% and 43% of DEK- and WT1-associated exon pathways, respectively, were found to be shared by the two cell categories (Figure 1C, 1D). Fourteen of the 16 pathways (87.5%) generated by AS in cells knocked down for DEK were distinct from those induced by whole transcriptional changes (Figure 1B). The proportion was 13/14 (93%) in cells knocked down for WT1 (Figure 1C). Thus, AS analysis unmasked numerous functional pathways that were undetectable through whole gene expression analysis. To validate exon array-predicted exon usage at the technical level, ESPCR was carried out for 65 mRNAs. Of these 65 array-predicted exon usages, 46 (71%) were validated by ESPCR (Figure 1D, Table 1; Supplementary

Figure 3). Together these results suggest that in AML DEK and WT1 oncogenes trigger distinct landscapes of AEU events that have putative implications for disease development and response to chemotherapy.

Exon expression profiles in chemoresistant AML cells

The combination of AraC and doxorubicin represents the backbone of AML induction chemotherapy (IC), while AZA is an effective alternative for treating AML in elderly patients. Thus, the sensitivity of K562, K562/AraC and K562-R7 cells to AraC and doxorubicin was assessed with a trypan blue dye exclusion assay (Supplementary Figure 4). Azacitidine sensitivity of the SKM1 cell preparations used here was previously verified with an MTT assay [25]. In AraC-resistant

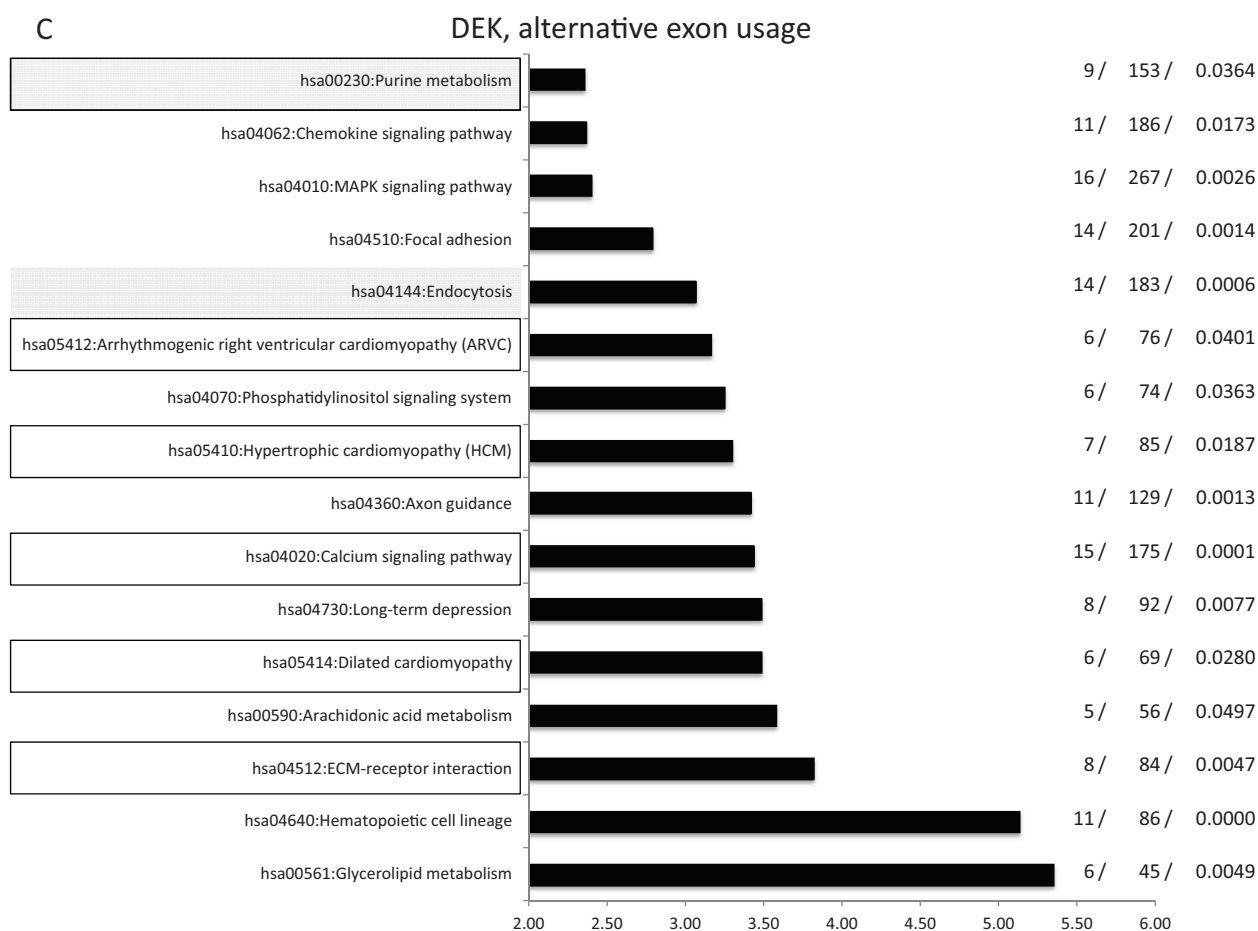


Figure 1: (Continued) B-C. Pathway enrichment analysis. For ontology analysis, gene lists were analyzed using DAVID software (KEGG pathways). The complete set of genes featured in the microarrays was used as the reference background. The three numbers on the right represent the number of deregulated mRNAs, the overall number of genes within the pathway and the *p* value, respectively. Data are presented for genes that were qualitatively modified following WT1 (D) and DEK (E) expression. Black rectangles represent exon-associated pathways shared by WT1- and DEK-expressing cells, while for each cell category the filled gray rectangles indicate pathways generated by both quantitative and qualitative changes in gene expression.

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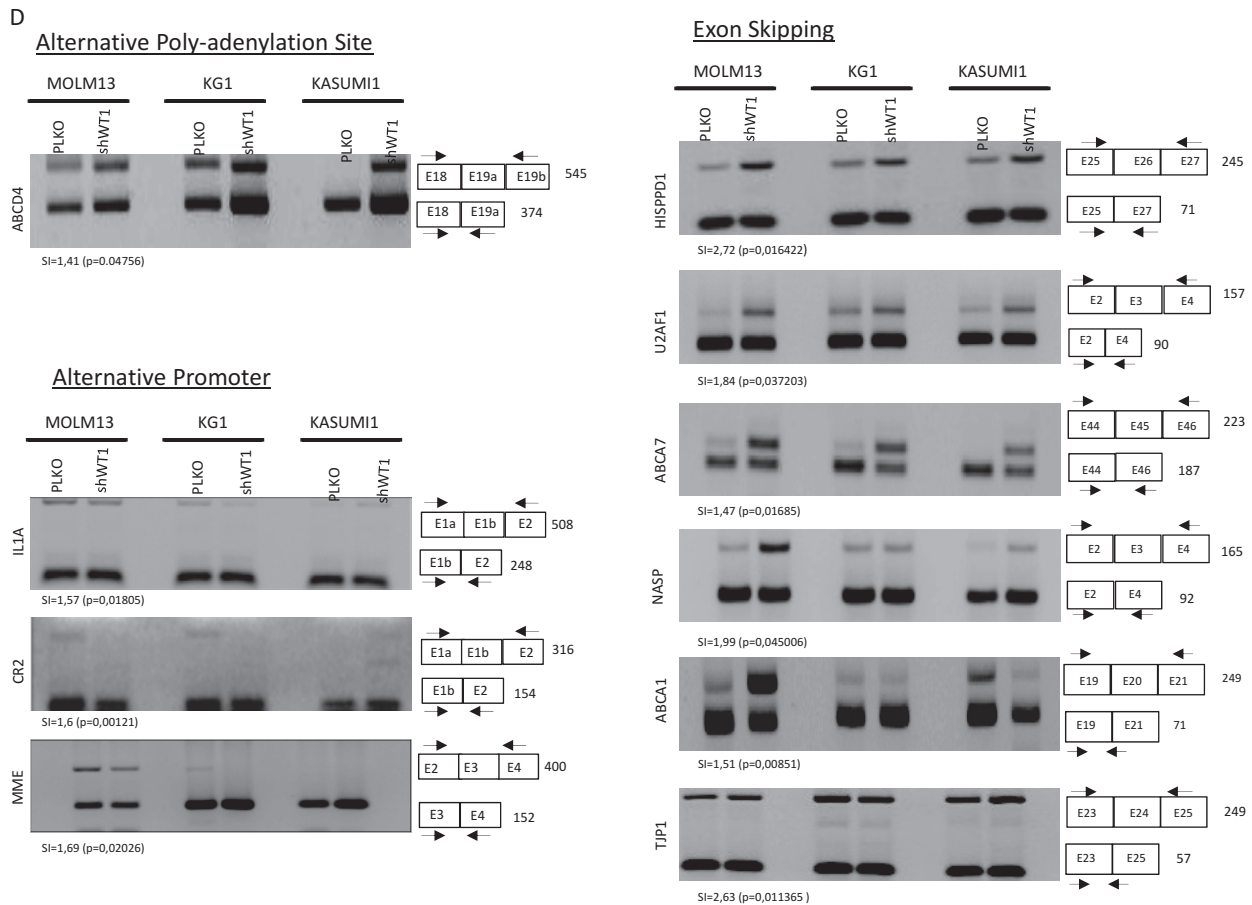


Figure 1: (Continued) D. Validation of microarray-predicted exon events. Exon-specific RT-PCR assays were performed with RNA samples derived from WT1+ and WT1- MOLM13, Kaumi-1 and KG1 cells. Numbers indicate the expected size (bp) of the PCR products. SI (splicing index) and *p* values are indicated for each exon event. SI ≥ 1.2 was considered a significant change in exon expression and was used for comparisons.

Table 1: Validation of microarray-predicted exon events in AML cell line

	Frequency
Exon Skipping	42/57
Alternative Promoter	3/5
Alternative PolyA	1/3
Total	46/65

K562/AraC cells, 5, 868 AEU events (Figure 2A) were identified for over 2, 836 genes, of which 1, 928 (68%) were altered at the level of whole gene expression (Figure 2A). The values were 4, 966, 2, 513 and 1, 673 (67%), for K562-R7 cells that are resistant to doxorubicin (Figure 2A) and 4, 093, 2, 124 and 1, 089 (51%) for SKM1 cells that are resistant to AZA. The distribution of AEU events shared by the three cell lines is represented in Supplementary Tables 2–4. K562/AraC and K562-R7

cells were found to share 1, 035 exon events that had the same regulation and were harbored by 574 genes. For AraC- and AZA- resistant cells and DXR- and AZA-resistant cells the values were 106 and 59 respectively. GO analysis identified numerous functional pathways that are associated with resistance to AraC, DXR or AZA (Figure 2B-2D). Seventeen of the 20 pathways (85%) generated by AS in AraC-resistant cells were distinct from those affected by overall transcriptional changes

(Figure 2C). The proportion was 13/16 (81%) in DXR-resistant cells (Figure 2D) and 33/35 (91%) in AZA-resistant cells (Figure 2E). Thirteen functional pathways, which represented 65% and 32% of AraC- and AZA-associated exon pathways, respectively, were found to

be shared by the two cell categories (Figure 2) while both AraC- (30%) and DXR- (37.5%) resistant cells and DXR- (37.5%) and AZA- (17%) resistant cells showed a value of 6. The three resistant cell lines were found to share five pathways (hsa04660:T cell receptor signaling

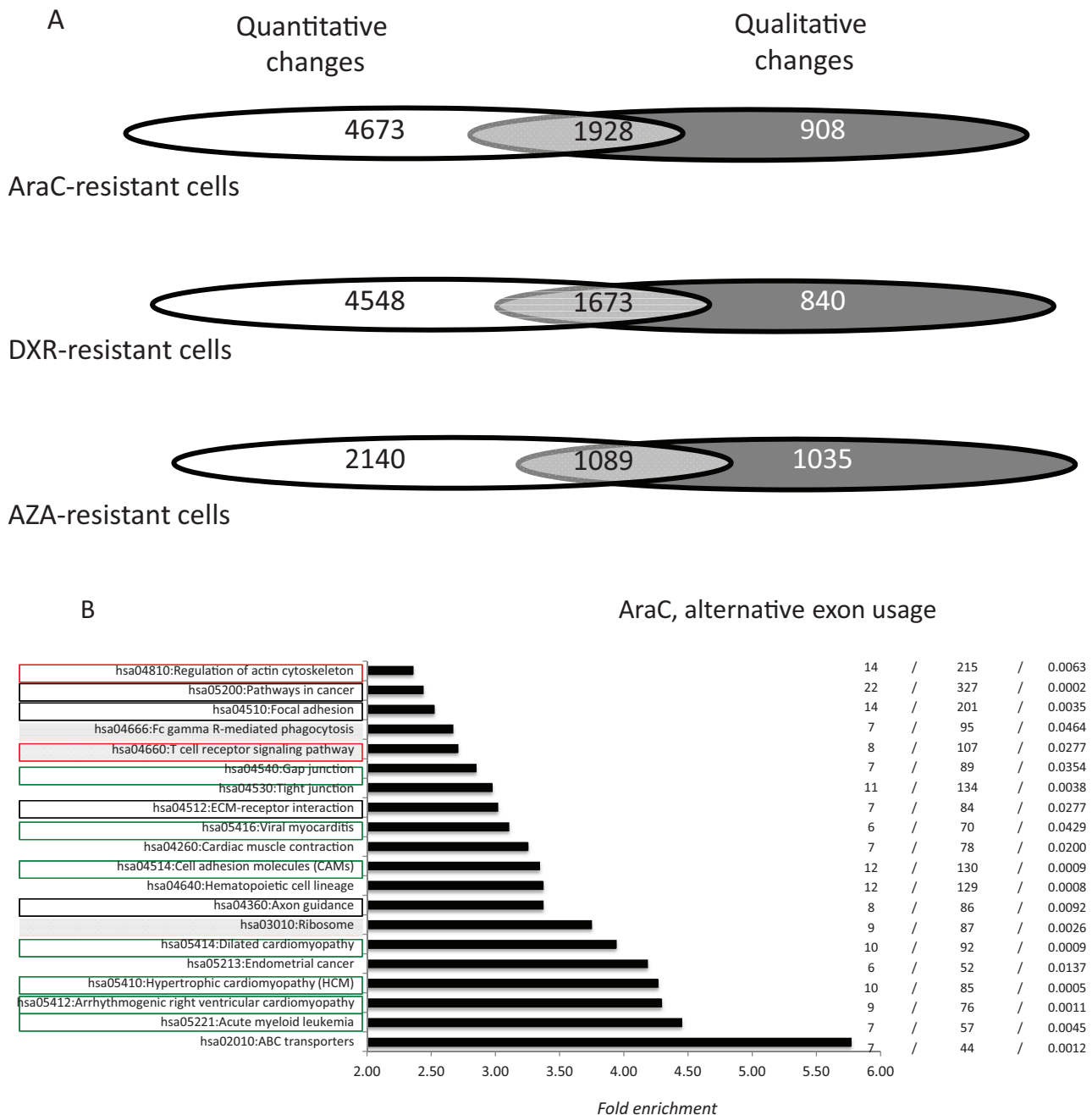


Figure 2: Distribution of alternative exon usages in AML cells that are resistant to anti-cancer drugs. A. Distribution of quantitative and qualitative gene modifications in AraC-, DXR- and AZA-resistant cells. Total RNA was analyzed using the exon microarray platform GeneChip HTA2 (Affymetrix). Microarrays were performed in triplicate for the six cell lines and only statistically significant modifications ($p < 0.05$) were selected. For the three cell categories (resistant to AraC, DXR and AZA), Venn diagrams show the distribution of genes that were modified at the level of whole gene expression (white), alternative exon usage (dark grey) or both (light grey).

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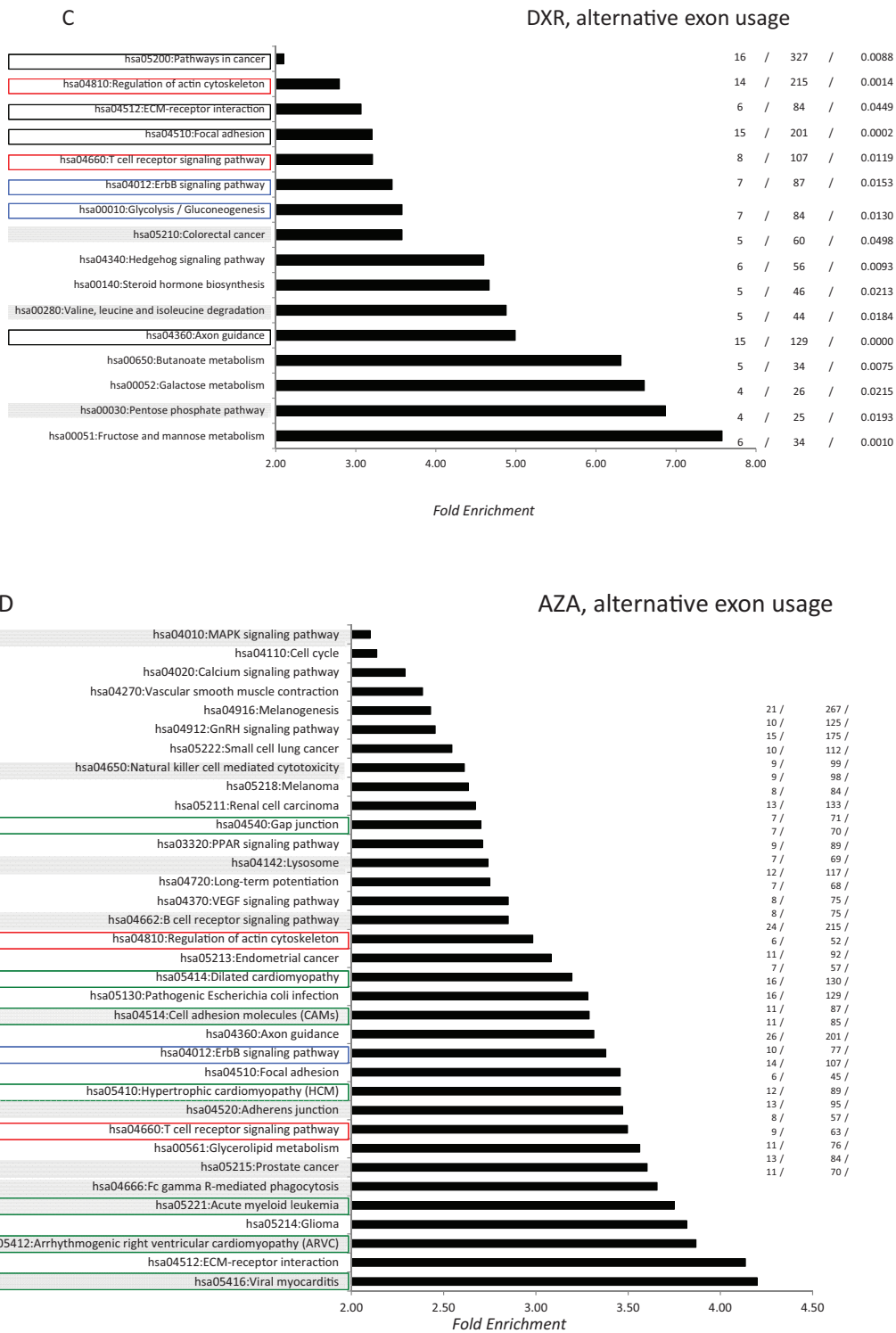


Figure 2: (Continued) B-D. Pathway enrichment analysis (see legend for Figure 1C-1D) of genes that were qualitatively modified in cells that are resistant to AraC (C), DXR (D) and AZA (E) Pathways shared by AraC- and DXR-resistant cells, AraC-, dXR- and AZA-resistant cells, DXR- and AZA-resistant cells and AraC- and AZA-resistant cells are represented by black, red, blue and green rectangles, respectively. For each resistant cell type, filled gray rectangles represent those pathways that were associated with both quantitative and qualitative transcriptional changes.

pathway, hsa04510:Focal adhesion, hsa04360:Axon guidance, hsa04810:Regulation of actin cytoskeleton, and hsa04512:ECM-receptor interaction) that represented 25%, 31% and 14% of AraC-, DXR- and AZA-associated pathways, respectively.

Exon-array analysis of fresh bone marrow samples derived from patients with chemo-sensitive versus -resistant AML

Exon array analysis was performed with 24 samples derived from 17 patients treated with AraC-anthracycline-based IC (protocol ALFA 07-01 [26]). Patients were distributed in two groups (PG1 and 2) and samples were divided into three groups (SG1-3) (Table 2). PG1 corresponded to patients who achieved a prolonged complete remission (CR) (> 4 years) and who were in persistent CR at the time of their last follow-up. PG2 corresponded to patients with primary resistant disease. SG1 and SG2 corresponded to samples harvested from patients in the PG1 group at the time of diagnosis and CR, respectively. SG3 corresponded to the diagnosis samples from PG2 cases. SG2 samples served as negative controls and their exon expression was compared with the SG1 and SG3 samples. For SG1 samples, 6, 560 AEU events were identified across 2, 911 genes, of which 2, 233 (77%) were altered at the level of whole gene expression (Figure 3A). The values were 9, 378, 3, 738 and 2, 973 (79%) for SG3 samples, which corresponded to AML with induction failure (Figure 3A). With the exception of exon/ALE events that were significantly more frequent in SG3 samples (267/6, 560 versus 451/9, 378, $p = 0.027$, Fisher exact test), the distribution of AEU types did not significantly differ between the two AML types (Figure 3B). Figure 3 (3C-3E) represents the distribution of the functional pathways generated by AEU and transcriptional events in samples derived from the SG1 and SG3 groups. For SG1, 15 of the 19 pathways (79%) generated by AS were distinct from those generated by whole transcriptional changes (Supplementary Figure 5A). For SG3 the values were 13 and 16 (81%) (Supplementary Figure 5B). SG1 and SG3 shared nine exon pathways, or 47% and 56% of the SG1 and SG3-associated pathways (Figure 3C-D). We next evaluated the distribution of oncogene- and drug-associated AEU and pathways between samples from the SG1 and SG3 groups. Thirty-three (0.5%), 38 (0.6%), 77 (1.2%), 77 (1, 2%) and 216 (3.3%) exon events were shared at the gene, exon position, and regulation levels

between shDEK cells, shWT1 cells, AraC-, DXR- and AZA-resistant cells and fresh AML samples from SG1, respectively (Supplementary Tables 5-9). The values were 55 (0.6%), 39 (0.4%), 153 (1.6%), 120 (1.3%), and 323 (3.4%) for SG3 and these differences were not statistically significant. Despite the absence of quantitative differences in the frequency of drug-associated exon events between SG1 and SG3, there was a distinct qualitative event distribution between the two groups (Supplementary Tables 7-9). Hence, none of the AraC- or DXR-associated events identified in the diagnosis samples were shared by SG1 and SG3 (Supplementary Tables 7 and 8). In contrast, 88 AZA-resistance-specific exon events were shared between the SG1 and SG3 samples. For AraC-associated exon events, GO analysis identified no functional pathway in samples from the SG1 group, while several functional pathways involved in cancer, leukemia and drug resistance were identified in SG3 samples (Figure 3E). For DXR-associated exon events, GO analysis identified three pathways in SG1 samples (hsa04110: Cell cycle, hsa00052: Galactose metabolism, and hsa04144: Endocytosis) and two pathways in SG3 samples (hsa04810: Regulation of actin cytoskeleton and hsa05200: Pathways in cancer). For AZA-associated exon-events GO identified 7 and 19 pathways in SG1 and SG3 samples, respectively (Figure 3E). We next investigated whether the mRNA of genes that are frequently mutated in AML are mis-spliced in these cell lines and patient-derived samples. We randomly selected 40 genes that are frequently mutated in AML (Supplementary Table 10) [27, 28] and found that 13 of these genes (32.5%) were mis-spliced in at least one leukemic condition. As a control, we randomly selected from the NCBI database (<http://www.ncbi.nlm.nih.gov/geo/?term=acute+myeloid+leukemia>) 40 mRNAs that are expressed in AML cells. Five of these genes (12.5%) were mis-spliced in at least one leukemic condition ($p = 0.03$, Pearson's Chi-squared test).

Quantitative exon-specific PCR analysis of *ex-vivo* identified AEUs in cells derived from AML patients

The above results identified either common or specific AEUs in AML cells displaying specific biological or clinical leukemic features. Of note, numerous AEUs corresponding to previously unknown putative disease markers and therapeutic targets were identified in the absence of whole gene transcriptional changes. Moreover,

Table 2: Distribution of the 24 AML samples analyzed by exon-array

Patients Group	n	Outcome	Samples Group	Disease stage at sampling
1	7	CR* > 4 years	1	Diagnosis
			2	CR
2	10	Primary failure	3	Diagnosis

*CR, complete remission

over 70% of the AEU events identified through exon-array analysis were confirmed by exon-specific PCR, although whether the interplays between exon expression and cellular phenotype identified *in vitro* are also present

in an *in vivo* setting awaits confirmation. To begin to address this question and extend technical validations to *in vivo* and functional levels, five AEU events that were identified through exon-microarrays, including



B AML, chemosensitive disease (SG1), alternative exon usage

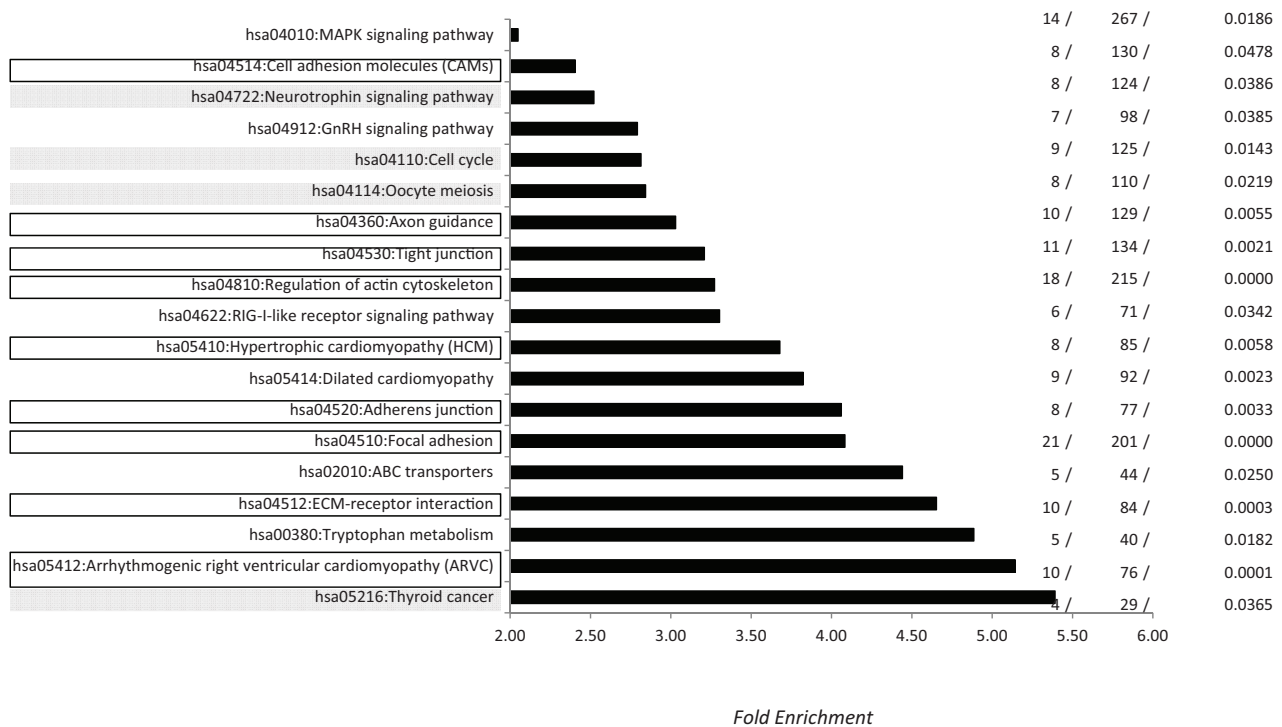
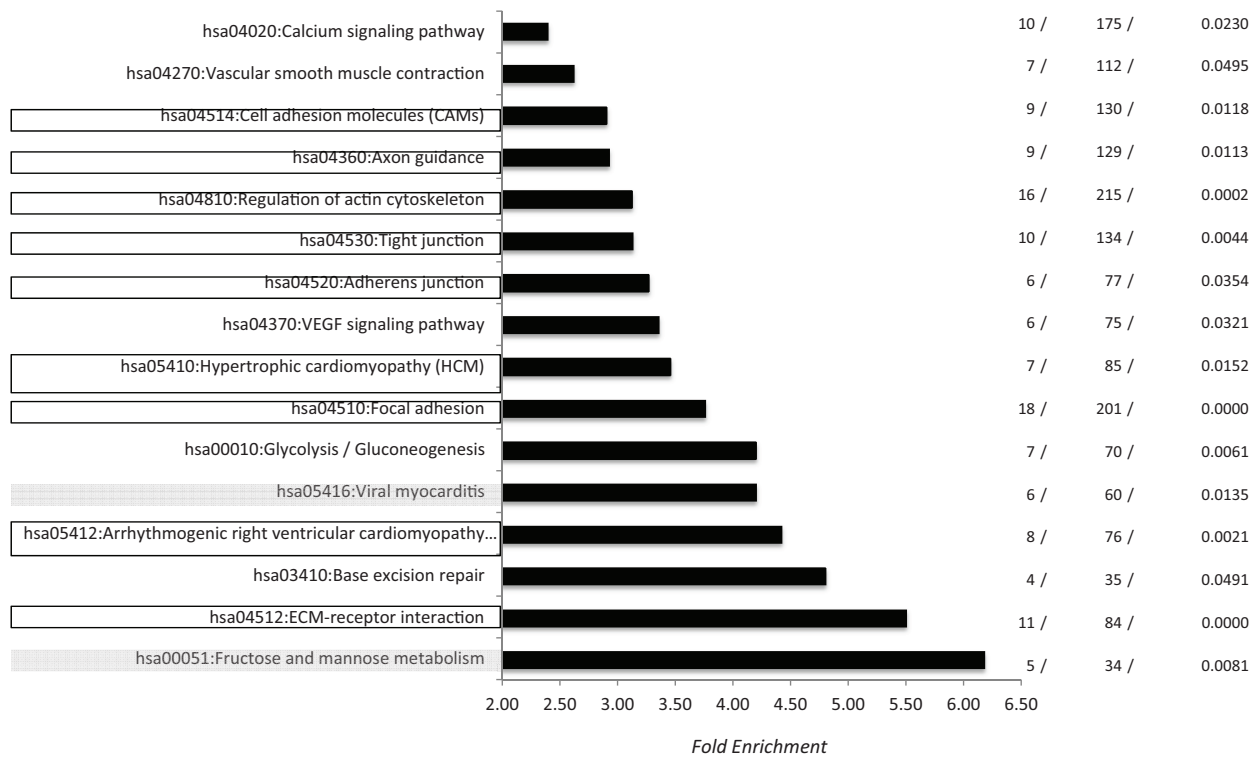


Figure 3: Distribution of alternative exon usages in fresh AML samples derived from patients with chemosensitive (SG1) and chemoresistant (SG3) disease. A. Distribution of quantitative and qualitative gene modifications in fresh AML diagnosis bone marrow samples derived from patients with chemosensitive (SG1, $n = 7$) and chemoresistant (SG3, $n = 10$) disease. Microarray data from each sample group were cross-compared with those of 10 control bone marrow samples (SG2) as detailed in the Methods section (Table 2). For the two sample categories, Venn diagrams show the distribution of genes modified at the whole gene expression level (white), alternative exon usage (dark grey) or both (light grey). B-C. Pathway enrichment analysis (see legend for Figure 1D-1G) of genes that are qualitatively modified in cells from SG1 (C) and SG3 (D) groups. Black rectangles represent exon-associated pathways shared by SG1 and SG3 samples, while for each sample category filled gray rectangles identify pathways generated by both quantitative and quantitative changes in gene expression.

(Continued)

C AML, chemoresistant disease (SG3), alternative exon usage



D AML, resistant disease (SG3), alternative exon usage/Arac-resistant cells

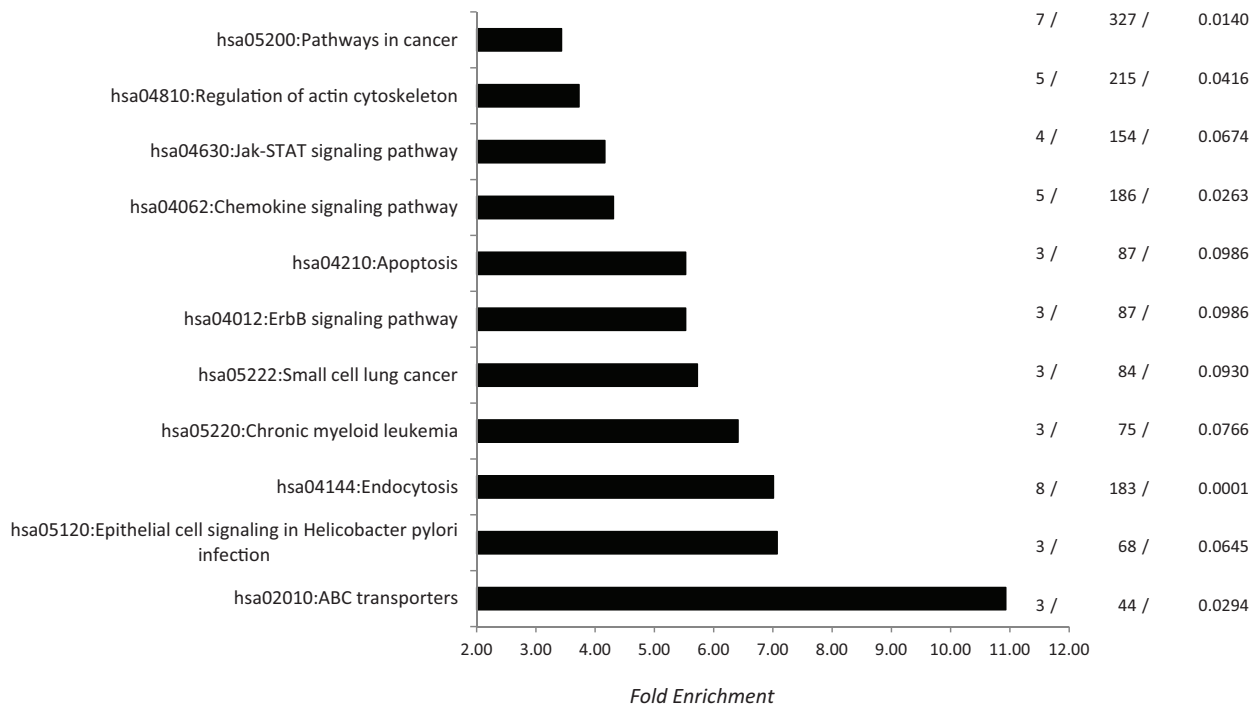
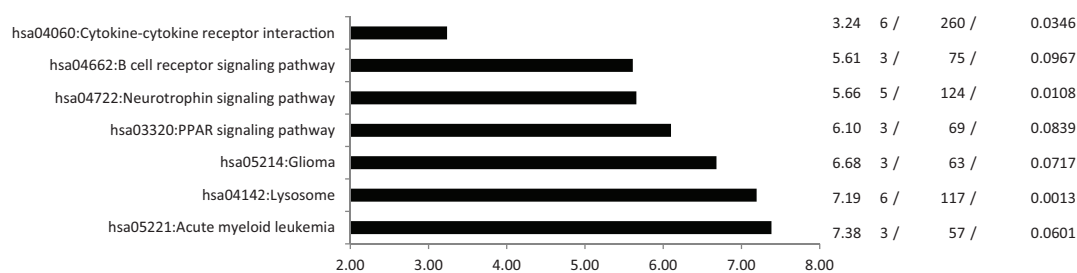


Figure 3: (Continued) D. Pathway analysis of genes found to be qualitatively modified in both AraC-resistant cells and SG1-derived samples.

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E AML, chemosensitive disease (SG1) , alternative exon usage/AZA-resistant cells



AML, resistant disease (SG3) , alternative exon usage/AZA-resistant cells

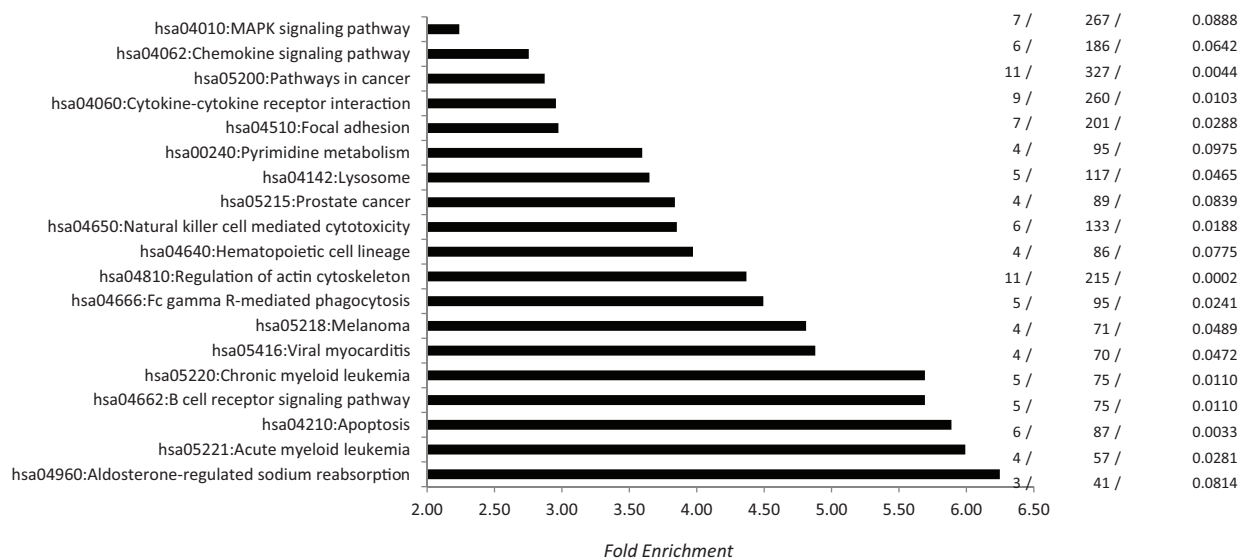


Figure 3: (Continued) E. Pathway analysis for genes that were qualitatively modified in both AZA-resistant cells and SG1- (top) or SG3- (bottom) derived samples.

mRNA from the TET2 gene and four ATP transporters, were analyzed in detail in AML cell lines as well as in bone marrow samples derived from 152 AML and 37 normal bone marrow samples. TET2 is a dioxygenase that catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine and promotes DNA demethylation [29]. TET2 somatic mutations occur in 7–23% of AMLs and 25% of myelodysplastic syndromes (MDS), and are distributed across the entire coding sequence without clustering into obvious hot spots [30]. These mutations decrease TET2 enzymatic activity by producing truncations or affecting its catalytic activity [29]. Little is known about TET2 exon expression patterns in AML, although exon-array analyses performed here and confirmed by ESPCR and qESPCR identified an AEU event involving TET2 exon 2 in AraC-resistant K562/AraC cells and in SG1 samples that were derived from patients who achieved a prolonged CR with IC. Two sets of qESPCR primers were designed to amplify the two alternative splicing events identified in TET2 mRNA by FASTERDB (Figure 4A). In addition, whole TET2 gene expression was quantified through RT-PCR amplification

of TET2 exon 10 sequences. K562/AraC cells carried no significant change at the whole TET2 transcription level while exon-array analysis showed no significant changes in mRNA splicing for the remaining TET2 gene exons in AraC-resistant versus -sensitive cells (Figure 4 and not shown). *In vivo*, the level of TET2 exon 2 expression was significantly lower in the 152 AML cases tested compared to the 37 control bone marrow samples derived from healthy donors (Figure 4B, $p < 10^{-4}$, Mann-Whitney test) and this change occurred in the absence of significant alterations in TET2 exon 10 expression (Figure 4B). These results confirm that the skipping of TET2 exon 2 identified through exon-arrays is a bona fide AEU event in patient-derived samples. Meanwhile, exon-array experiments confirmed by ESPCR identified AEU events in 13 ATP-binding cassette (ABC) transporter gene mRNAs in DEK- and WT1-expressing cells, as well as in AraC-, DXR-, AZA-resistant cells and SG1 and SG3 patient samples. We designed specific PCR primer pairs in order to amplify 4 AEU events that exon microarrays and ESPCR identified in the mRNA of four ABC transporter genes, namely ABC-A2, -A3, -A5 and -C3 upon WT1

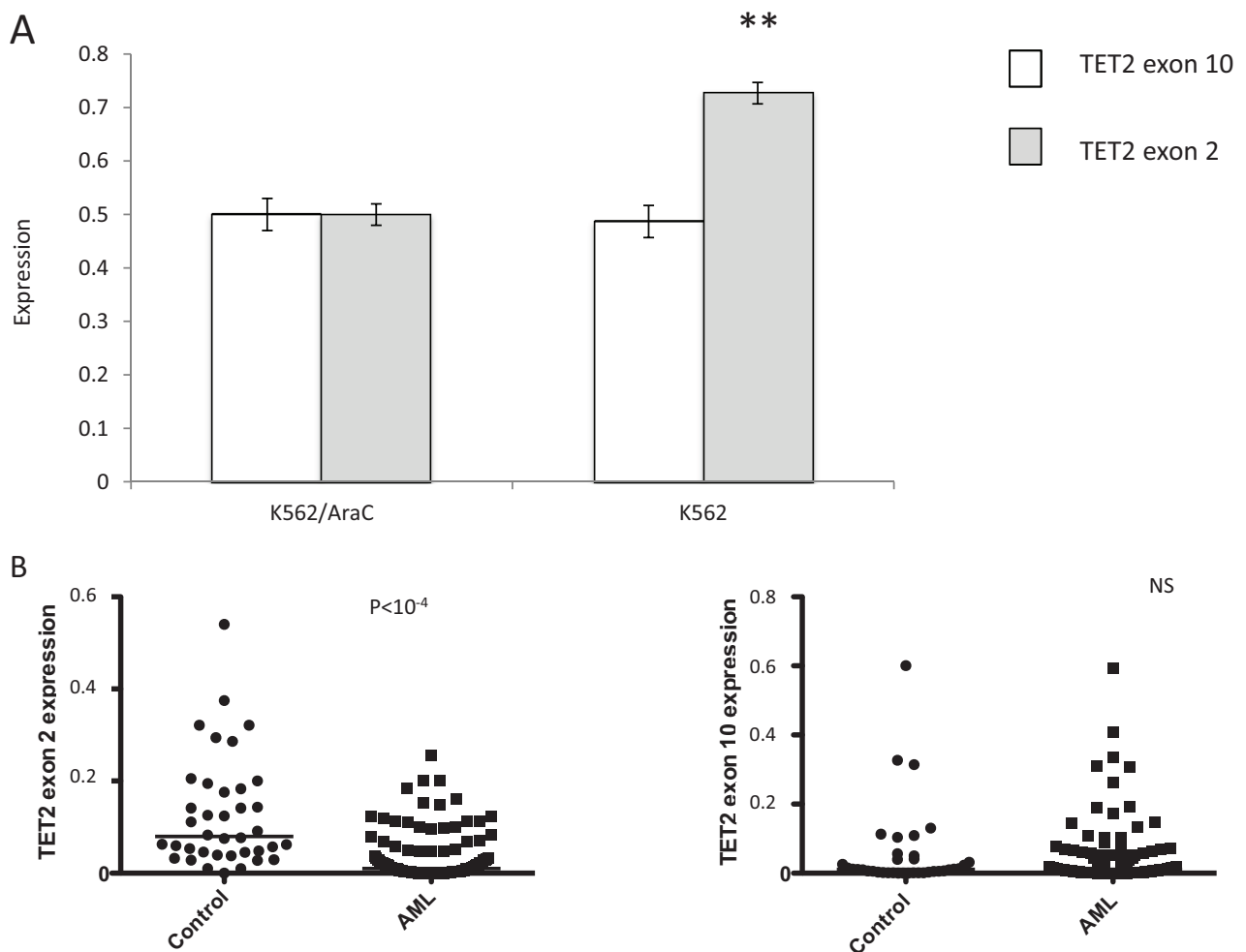


Figure 4: Quantitative exon-specific RTPCR (qESPCR) analysis of TET2 exon 2 expression *in vitro* and *in vivo*. A. TET2 exon 2 skipping in K562/AraC cells and B. AML diagnosis cells and control cells. qESPCR shows a decreased level of TET2 exon 2 expression in AraC-resistant K562/AraC cells whereas whole TET2 gene expression, as measured through qRTPCR analysis of the TET2 exon 10 sequence, is unchanged between the two cell categories. Meanwhile, qESPCR detected significantly lower levels of TET2 exon 2 expression in fresh AML cells (152 samples) compared to control cells (37 samples), while qRTPCR showed that whole TET2 gene expression was unchanged (right).

expression in cell lines (Supplementary Figure 1). WT1 expression was quantified in the six cell lines and also in 152 AML bone marrow samples and 37 marrow samples from healthy donors (Supplementary Figure 7). As with TET2 AS analysis, for each ABC transporter mRNA two sets of qESPCR primers were designed to amplify the two alternative splicing events identified by FASTERDB (Figure 5). This assay provided an estimated relative expression level for two isoforms for each of the four ABC transporters. Microarray and qESPCR analyses gave consistent results with the three cell lines for ABC-A3 (Figure 5A) and ABC-A5 (not shown), whereas Kasumi-1 cells showed a distinct isoform expression pattern compared to KG1 and MOLM13 for ABC-A2 (Figure 5B) and ABC-C3 (not shown). For ABC-A3, qESPCR confirmed the microarray analysis and showed a significant exclusion of ABC-A3 exon 19 in K562/AraC

cells (Supplementary Figure 8). qESPCR was carried out with bone marrow samples derived from 152 AML patients and 37 healthy bone marrow donors. For each exon event, non-parametric bivariate correlation analyses determined that there was a statistically significant correlation between WT1 expression and the distribution of the four ABC transporter isoforms in AML but not in the control samples (Figure 5). The correlation between WT1 and ABC-A3 and -A5 isoform expression was in parallel with that from fresh AML samples and the three cell lines (Figure 5A). For ABC-A2 isoforms (Figure 5B), a correlation was present between AML and Kasumi-1 cells that was opposite that for AML and MOLM13 or KG1 cells. For ABC-C3 (not shown), expression levels in AML and MOLM13 or KG1 were similar but the opposite was true for AML and Kasumi-1 cells. The two TET2 isoforms identified in SG1 samples

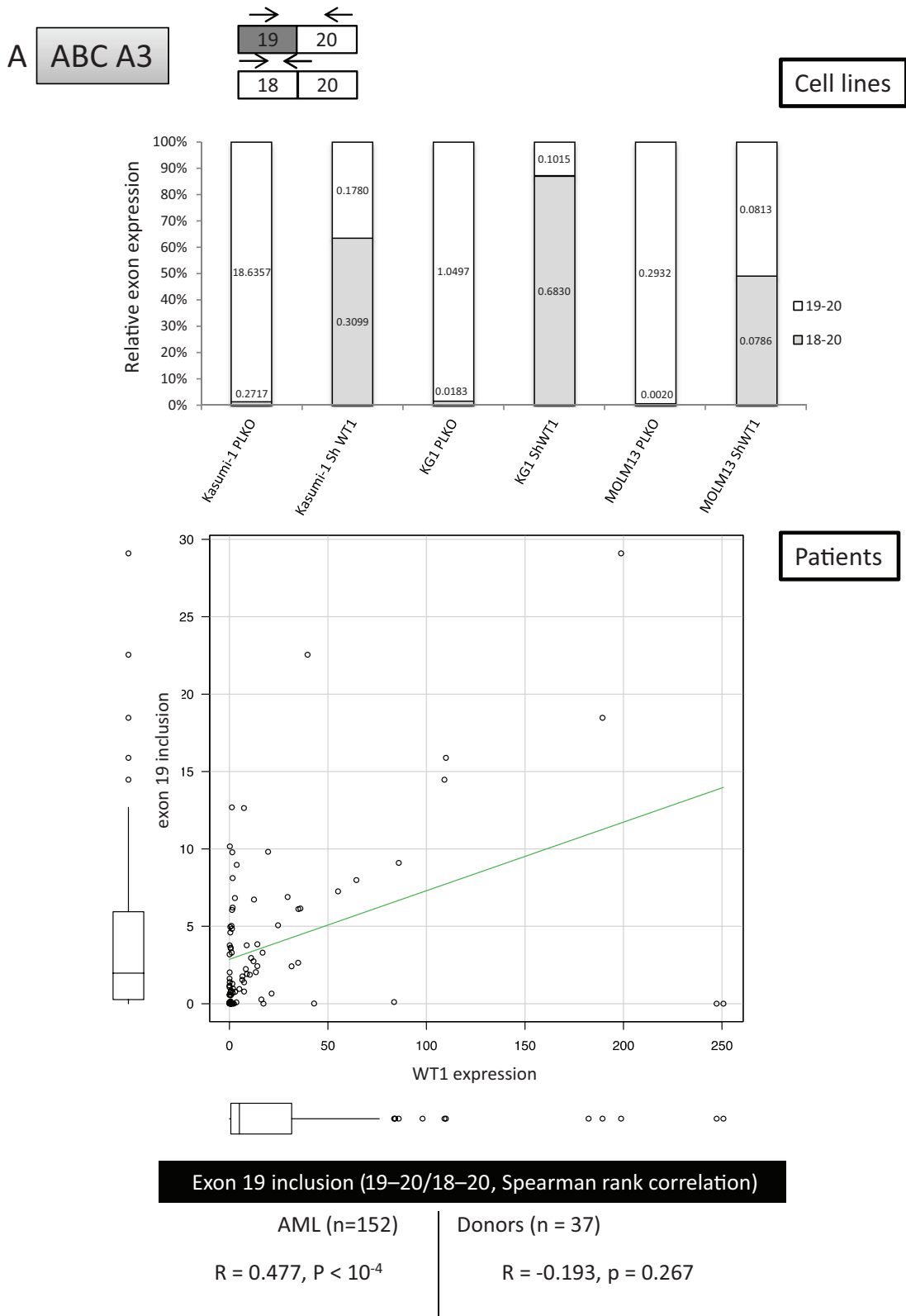


Figure 5: WT1-dependent mis-splicing of ABC transporter mRNAs in AML cells *in vivo*. The figure represents the position of oligonucleotides used for qESPCR, results of qESPCR for the three cell lines and fresh AML samples, as well as the correlation between expression levels of WT1 and ABC transporters ABC-A3 A. and ABC-A2

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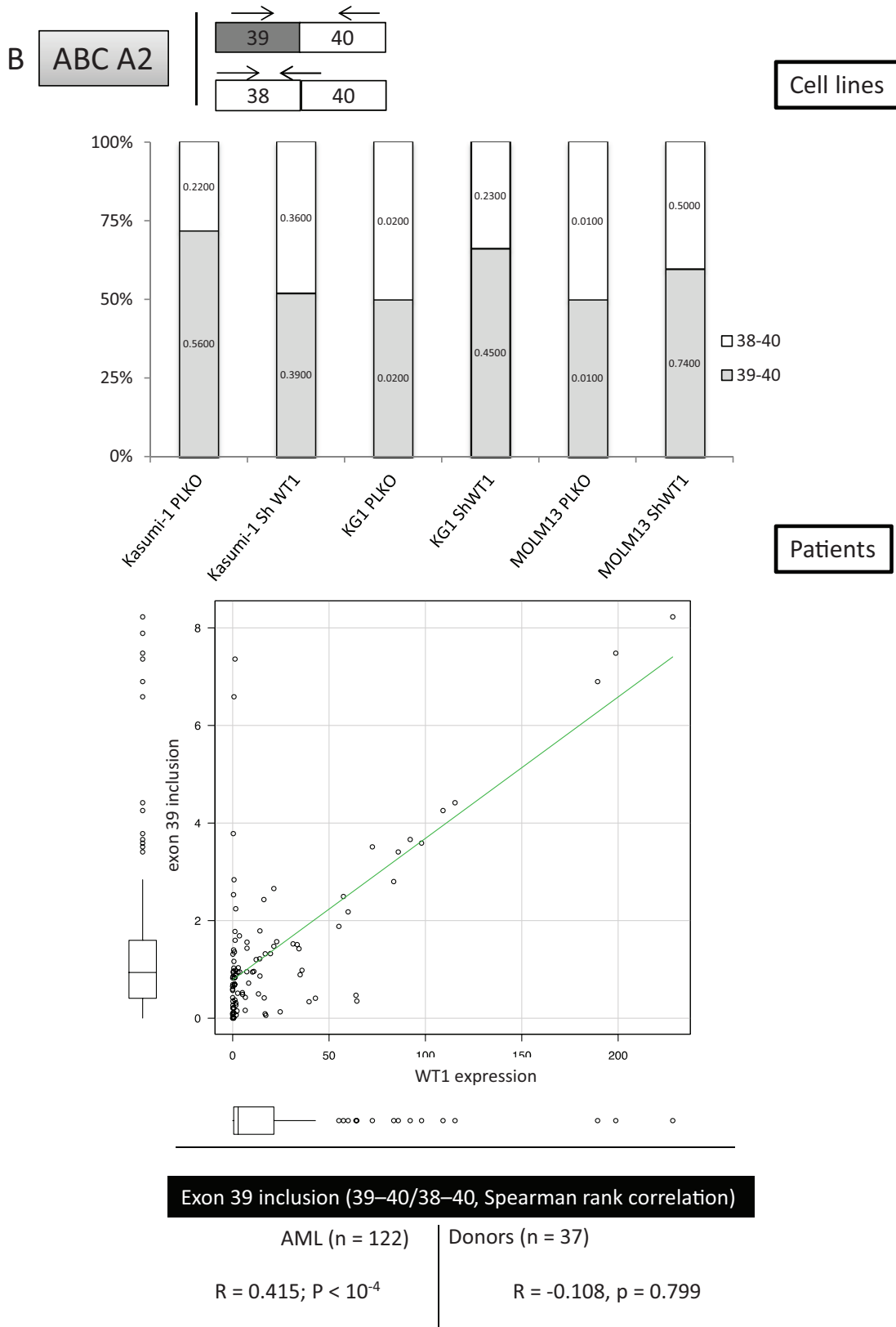
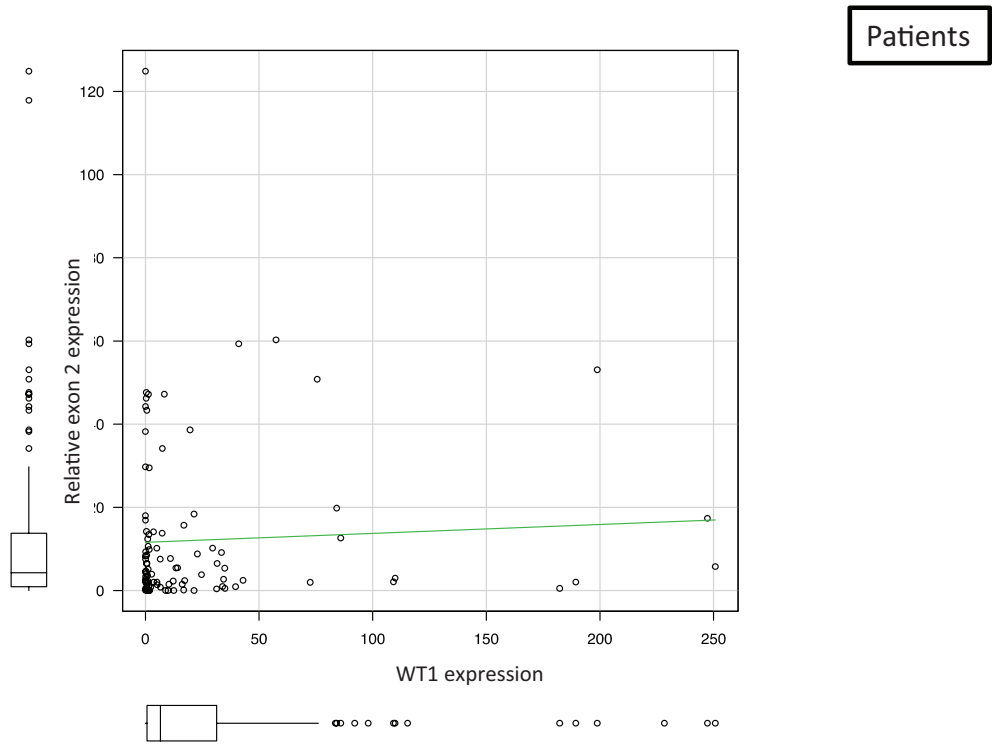
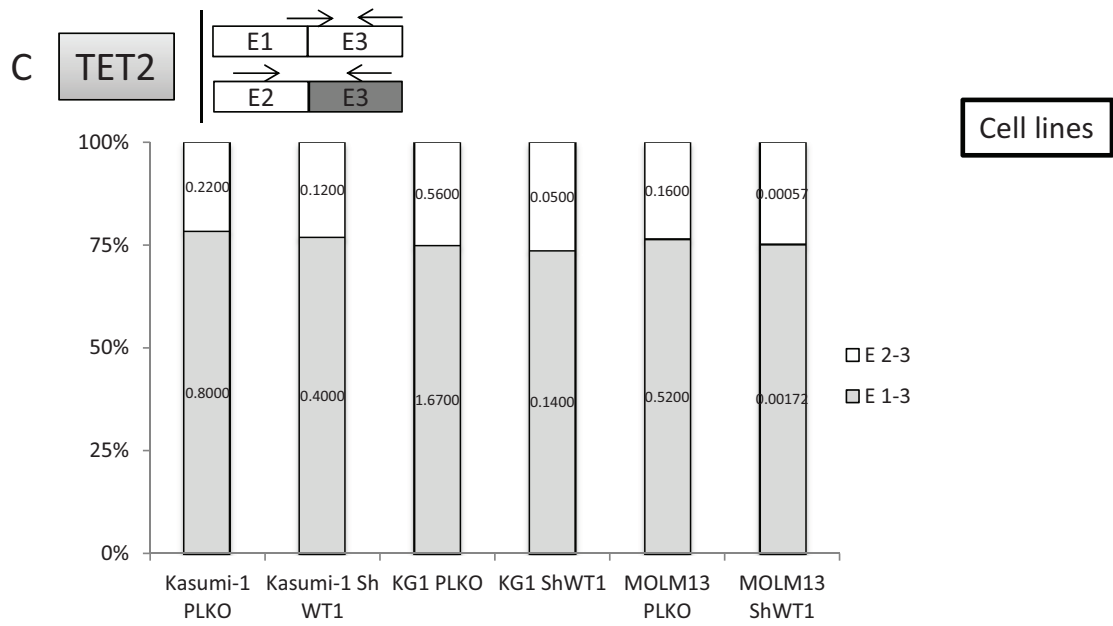


Figure 5: (Continued) B. in the 152 fresh AML samples and samples from 37 healthy donors.

(Continued)



Exon E1 inclusion (E1-E3/E2-E3, Spearman rank correlation)

AML (n = 122)	Donors (n = 37)
R = 0.041; P = 0.694	R = 0.144, p = 0.144

Figure 5: (Continued) C. Lack of correlation between WT1 and TET2 exon 2 expression *in vitro* and *in vivo*.

and AraC-resistant cells were not associated with WT1 expression in each of the three cell lines as shown by microarray and qESPCR (Figure 5C). In contrast to ABC transporter AEU, no correlation was found between WT1 expression and the distribution of the two TET2 isoforms in fresh AML samples (Figure 5C). Thus, these results identify a specific and significant correlation between WT1 expression and alternative splicing of the ABC transporters ABC-A2, -A3, -A5 and -C3 in AML samples.

DISCUSSION

AS occurs in 95% of all multi-exonic genes and can promote cell growth and survival in cancer [17, 31]. Accordingly, AS can lead to loss-of-function of tumor suppressors, activation of oncogene pathways, immune escape and resistance to treatment [32]. These events all occur in AML [4]. Several reports have found somatic mutations that occurred in splicing factors in AML, yet their frequency is significantly lower than that observed in other myeloid malignancies such as myelodysplastic syndromes [6]. In addition to spliceosome gene mutations, numerous abnormalities acquired by AML cells might disturb AS. They include epigenetic changes such as CpG methylation and histone modifications, which can interfere with RNA polymerization and AS as well as DEK and WT1 gene expression. Given this context, we wanted to assess the effect of DEK, WT1 and chemoresistance on the pattern of exon expression in AML. To this end we designed AML cell lines expressing low versus high levels of DEK and WT1 and used K562 and SKM1 cells that are resistant or sensitive to AraC, DXR or AZA. Twenty-four clinical samples derived from two groups of patients having distinct outcomes following IC were also analyzed with exon-arrays. The exon-array analysis of these 41 samples could be validated by ESPCR in 45/65 tested AEU events while 152 AML diagnosis samples and 37 control bone marrow samples were analyzed by qESPCR for TET2, ABC-A2, -A3, -A5 and -C3 isoform expression. This study identified both *in vitro* and *in vivo*-specific patterns of exon expression as they relate to oncogene expression and resistance to the main agents used to treat AML.

A recent study investigated exon expression in samples from newly-diagnosed AML patients using exon-array analysis [4]. This work gave consistent results with two cohorts of 31 and 36 patients analyzed by exon-array, and showed that about 30% of expressed genes are abnormally spliced in AML. Although not confirmed through ESPCR, the results suggested that certain AEU events involved mRNAs that were related to several oncogenes, tumor suppressor proteins, splicing factors and heterogeneous-nuclear-ribonucleoproteins, as well as proteins involved in apoptosis, cell proliferation and spliceosome assembly. Here we assessed the exon expression in several situations that provided distinct

patterns of exon-array-evidenced AEU, which were validated by ESPCR analysis of more than 60 AEU events. Regardless of the conditions, our results identified several mis-spliced mRNAs that are related to oncogenes or tumor suppressors as illustrated by GO analysis, which implicated several AEU-associated pathways consistent with AML development, persistence and resistance to treatment.

Analyzing oncogene-associated AEU events revealed that DEK and WT1 triggered specific and common AEU events. Regarding the event type, the proportion of alternative last exon, acceptor, intron-retention and promoter was significantly different between cells expressing DEK or WT1. However, whether these differences arise from different kinds of molecular interplay between the two factors and the splicing machinery remains to be investigated. In addition, 99 exon events were found to be shared between the two cell types. Interestingly, for both DEK (80%) and WT1 (59%), the majority of identified AEU events involved genes that were unmodified at the whole gene transcription level. Accordingly, exon expression analysis unmasked several important pathways, including those involved in ABC transporters, tight junctions, actin cytoskeleton regulation and the MAPK signaling pathway.

Drug resistance is a major concern in AML management. *In vitro* drug resistance appeared to generate a significantly higher number of exon events than oncogene expression. Exon array-based analysis of drug-resistant cells showed that AraC-, DXR- and AZA-resistant cells show significantly distinct patterns of exon expression. Both AEU types and the targeted genes differed markedly between cells that were resistant to AraC, DXR and AZA. The proportion of mRNAs carrying AEU events in the absence of whole gene transcriptional changes was significantly lower with drug resistance than either DEK or WT1 expression with 32%, 37% and 49% for AraC, DXR and AZA, respectively. However GO analysis showed that these latter exon events unmasked new important functional pathways (Figure 2), with more than 80% being distinct from those generated by whole gene transcription changes. Cells resistant to the pyrimidine analogs AraC and AZA shared 13 exon-associated functional pathways whereas cells that are resistant to the intercalating agent DXR and either AraC or AZA shared only six exon-associated functional pathways.

Numerous AEU events distinguished AML samples from control bone marrow samples. These AEU events involved about 3,000 genes, with 20% unmodified at the whole gene transcription level. In contrast to the different kinds of analyzed cell lines, no important fluctuations in the distribution of AEU types appeared between samples derived from the SG1 and SG3 groups, with the only significant difference being the distribution of exon/ALE events between the two sample groups. About 80% of AML-associated functional pathways were distinct from

those associated with whole gene transcription changes, yet six and five pathways were specific to cells derived from patients with chemosensitive and chemoresistant AML, respectively. However, the proportion of oncogenes and drug resistance-associated-AEUs was not significantly different between cells derived from resistant and chemosensitive AML, although the qualitative distribution of drug-associated AEUs was clearly distinct between the two cell categories. Assuming that AEU plays a role in the resistance of AML to IC, these results suggest that such effects would depend more on a propitious combination of AEU than quantitative fluctuations of individual exon events. Consistent with this line of thinking, AML cells derived from newly diagnosed patients who subsequently showed drug resistance and treatment failure were found to be significantly enriched in AEUs involving prognostically relevant genes [27, 28].

ESPCR and qESPCR are useful tools for validating exon-array data at a technical level and for analyzing exon events on a large scale. Having observed the skipping of TET2 exon 2 in AraC-resistant AML cells and in diagnosis samples from patients with chemosensitive disease, we were interested to check the status of the TET2 exon 2 in our series of 152 AML samples. qESPCR demonstrated that TET2 exon 2 is expressed at significantly lower levels in AML samples compared to normal bone marrow cells, which occurred in the absence of notable changes in the expression of the full TET2 gene. Moreover, there are approximately 50 known human ABC transporter genes encoding proteins that translocate solutes and drugs across cellular membranes. In addition to ABC-B1 (MDR1/Pgp)-mediated chemoresistance [33, 34], genetic polymorphisms [35] as well as functional [36] and transcriptional [37] analyses have shown that an increasing number of ABC transporters, including ABC-A3, ABC-C1, ABC-C3 and ABC-G2, can cause resistance to cancer chemotherapeutic agents [3, 38–40]. For the four ABC transporters examined here (ABC-A2, -A3, -A5 and -C3), we found altered splicing concurrent with WT1 expression *in vitro*. qESPCR quantification validated this correlation between WT1 expression and exon events *in vivo* for these four AEUs. As with TET2, these differences occurred in the absence of significant correlations between the expression of the full ABC-A2, -A3, -A5 and -C3 genes and WT1.

In conclusion, the present study showed for the first time that in addition to splicing factor mutations, additional AML features such as oncogene expression and drug exposure can modify the landscape of exon expression. These changes involve numerous genes that are unmodified at the whole gene transcription level, and unmasked exon-specific functional pathways. Future studies are needed to determine how oncogene expression and drug resistance can trigger these specific patterns and to validate how exon expression patterns affect protein function, as would be the case for ABC transporter drug

efflux activity. Such validation will prompt exploration of the specific triggers of certain AS events with splice-switching oligonucleotides that can inhibit AS and may represent a promising tool for treating disease-associated exon skipping [22, 23]. Previous reports have suggested that certain splicing events, such those involving WT1 [41], TP53 [42], HOXA9 [43], BAALC [44], VEGF [45] or BCL-X [46], might influence disease outcome in intensively treated AML. The present identification of numerous AEUs that are specifically associated with drug resistance *in vitro* and treatment failure *in vivo* prompts further evaluation of their prognostic implications and potential use as biomarkers.

MATERIALS AND METHODS

Patient population and samples

The medical ethics committee of the Hospices Civils de Lyon approved this study. Informed consent was obtained from patients and healthy volunteers in accordance with the Declaration of Helsinki and institutional guidelines. The experimental cohort consisted of 17 patients treated with French acute leukemia group (ALFA) protocol ALFA-0701 [26] who were selected based on treatment response and outcome. As summarized in Table x, these patients were distributed into two groups with respect to IC response and outcome. The first group included seven patients who achieved prolonged relapse-free complete remission (CR, > 4 years) without known relapse at the time of last follow-up. The second group included 10 patients with primary resistance to IC. Bone marrow cells were collected at the time of diagnosis and, in patients from the first group, at the time of complete remission. The validation cohort consisted of 226 AML patients diagnosed at Lyon's or Saint Etienne university hospital. Control cells corresponded to bone marrow mononuclear cells (BMMNCs, 37 samples) derived from bone marrow donors.

Cell lines

The human AML cell lines Kasumi-1, MOLM13 and KG1 were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), 1% L-glutamine and 1% penicillin-streptomycin (Sigma-Aldrich, Inc., St. Louis, MO, USA). The cell lines have been authenticated by ATCC. These three cell lines are representative of the main AML subtypes: Kasumi-1 harbors a t(8;21) translocation, MOLM13 was derived from an AML-M5a patient and KG1 cells originated from the bone marrow of a 59-year-old male AML patient. The SKM1 cell line was established from the peripheral blood of a 76-year-old Japanese man with acute monoblastic

leukemia (AML M5) following a myelodysplastic syndrome [47]. Azacitidine-sensitive or -resistant SKM1 cells were a kind gift from Dr. P. Auberger (Nice) and have been previously characterized [25]. AraC-sensitive, AraC-resistant (K562/AraC) and doxorubicin-sensitive, doxorubicin-resistant (K562/DXR) cells were described previously [48].

RNA interference, and lentivirus

For WT1 RNA interference, a pool of 4 validated shRNA sequences purchased from Sigma (5'-CCGGGCAGCTAACAAATGTCTGGTTACTCGAGTAACCAGACATTGTTAGCTGCTTTTTG-3', 5'-CCGGGCATCTGAGACCAGTGAGAACTCGAGTTTCTACTGGTCTCAGATGCTTTTTG-3', 5'-CCGGATGAAGTTAGGAGCCACCTTCTCGAGAAGGTGGCTCCTAAGTTCATCTTTTTG-3', 5'-CCGGTATAAGTACTAGATGCATCACCTCGAGGTGATGCATCTAGTACTTATATTTTTG-3') were used. Lentiviral particles were generated by transfection of lentiviral plasmids and packaging mix (purchased from Sigma) into HEK-293-T cells using Lipofectamine 2000 (Invitrogen). Supernatants containing viral particles were harvested between 36–72 h after transfection and purified for use in WT1 knock-down experiments. The empty PLKO.1 vector was used as a negative control. For lentiviral infection, 1.5×10^6 /mL MOLM13, KG1 or Kasumi-1 cells were incubated with recombinant lentiviruses at a multiplicity of infection of 1:5 for 72 h. The same procedure was used for DEK RNA interference with the four validated shRNA sequences. Sequences are available on request.

RNA purification and reverse transcription

RNA was isolated with TRIZOL reagent (Invitrogen) with the concentration and purity determined by UV spectrophotometry (Nanodrop). Before reverse transcription, RNA was treated with DNAase (DNAse-free™, Invitrogen) to prevent DNA contamination. cDNA was synthesized from 1 µg RNA using a random primer (High Capacity cDNA Reverse Transcription Kit, Invitrogen) and Superscript II reverse transcriptase (Invitrogen).

Affymetrix exon array hybridization and array data

One µg of total RNA was labeled and purified with TRIZOL with Affymetrix reagents according to the manufacturer's instructions. Hybridization cocktails containing 5–5.5 µg cDNA were prepared and hybridized to Affymetrix-GeneChip Human Exon HTA2 arrays (Affymetrix). Affymetrix Expression Console Software was used for quality assessments. Affymetrix exon array data treatment was performed using FASTERDB annotation (<https://fasterdb.lyon.unicancer.fr/>) and the data were

normalized using quintile normalization. The background correction and probe selection were performed as previously described [49].

Exon-specific polymerase chain reaction (ESPCR)

Splicing events were annotated on the FASTERDB database and primers were designed to encompass alternative splicing events using Primer 3. PCR reactions were performed using the Herculase II Fusion DNA Polymerase (Agilent Technologies) in 30–35 cycles and the resulting products were analyzed by agarose gel electrophoresis. Primer sequences are available on request. Samples were analyzed in duplicate.

Quantitative Exon-specific Polymerase Chain Reaction (qPCR)

cDNA was amplified by qPCR using a Bio-Rad Chromo4: CD002161 device and 20 µl reactions with iQ SYBR Green PCR Supermix (BioRad) and 10 µM of each appropriate primer. The expression of the housekeeping gene Gus (NM_000181) was used as an internal control. A melting curve (65–92°C) was generated at the end of each run to verify primer specificity. The Pfaffl method was used for relative quantification [50]. Primer sequences are available on request.

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CONFLICTS OF INTEREST

The authors declare no competing financial interest.

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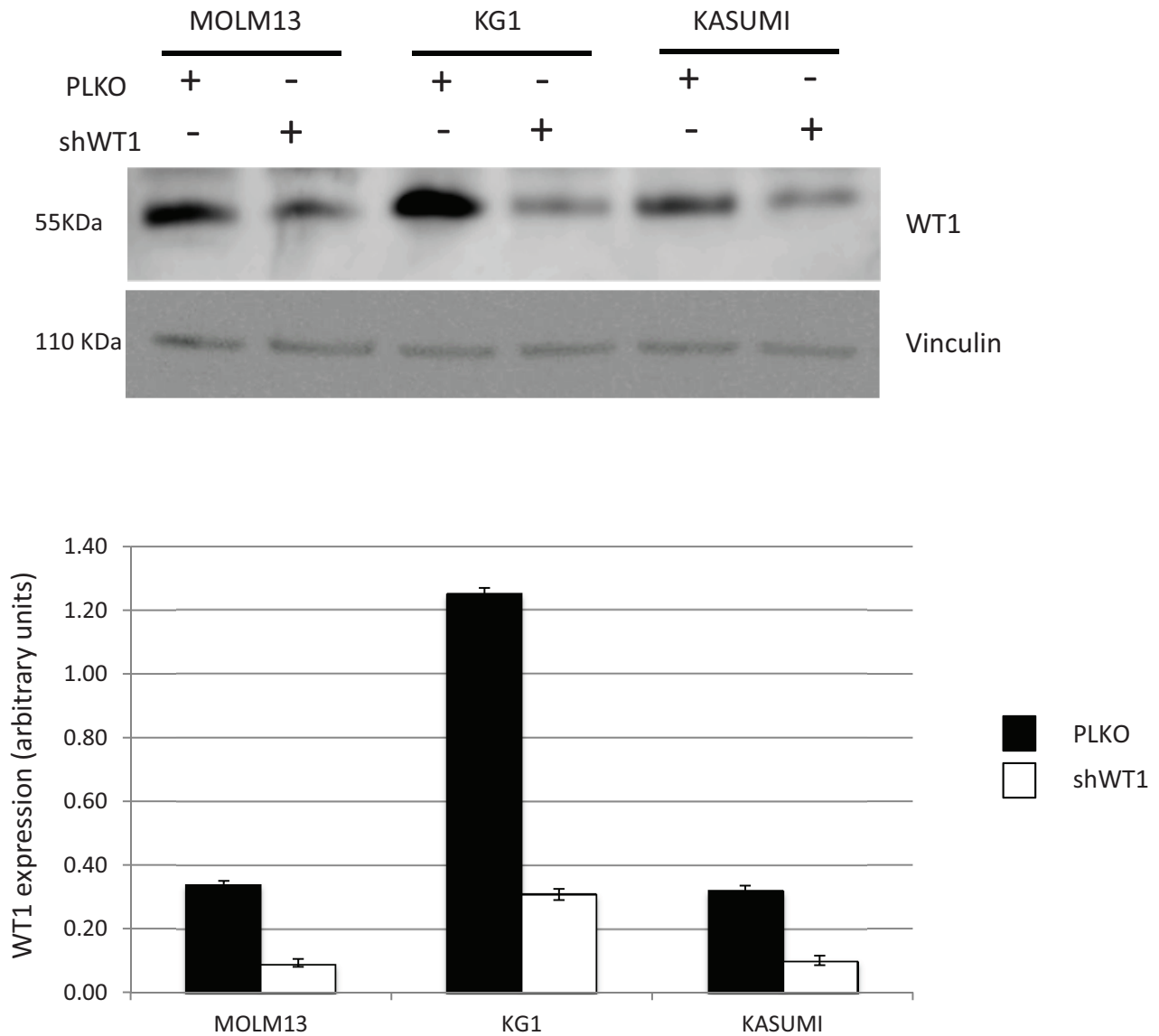
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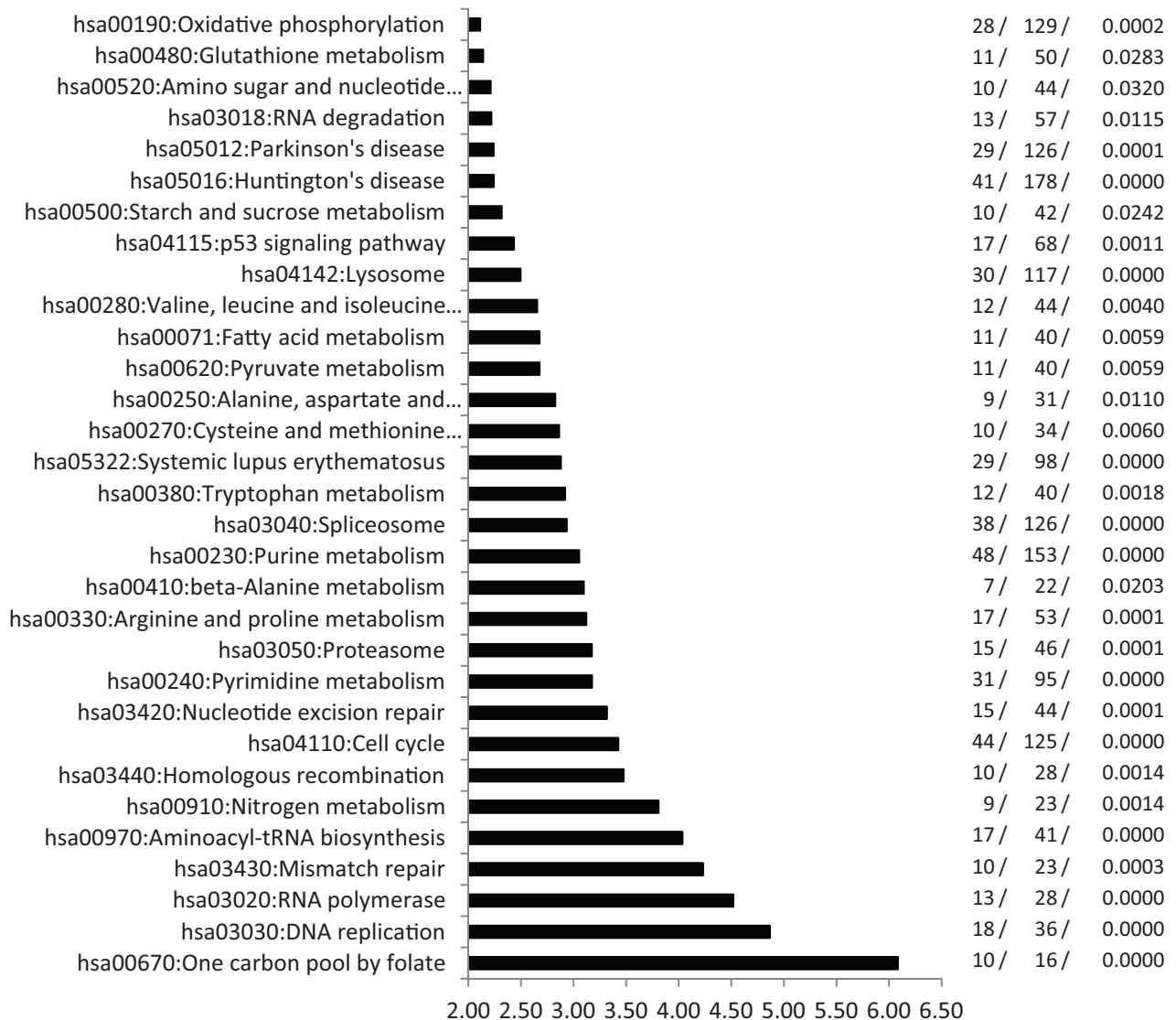
SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure S1: Knock-down of WT1 expression in AML cell lines. MOLM13, Kasumi-1, and KG1 AML cells were infected with lentiviral vectors encoding shWT1 or control vectors (PLKO). Protein lysates were prepared and western blot analysis was performed using antibodies for WT1. RNA was prepared and qRT-PCR was performed as detailed in the Methods section.

A

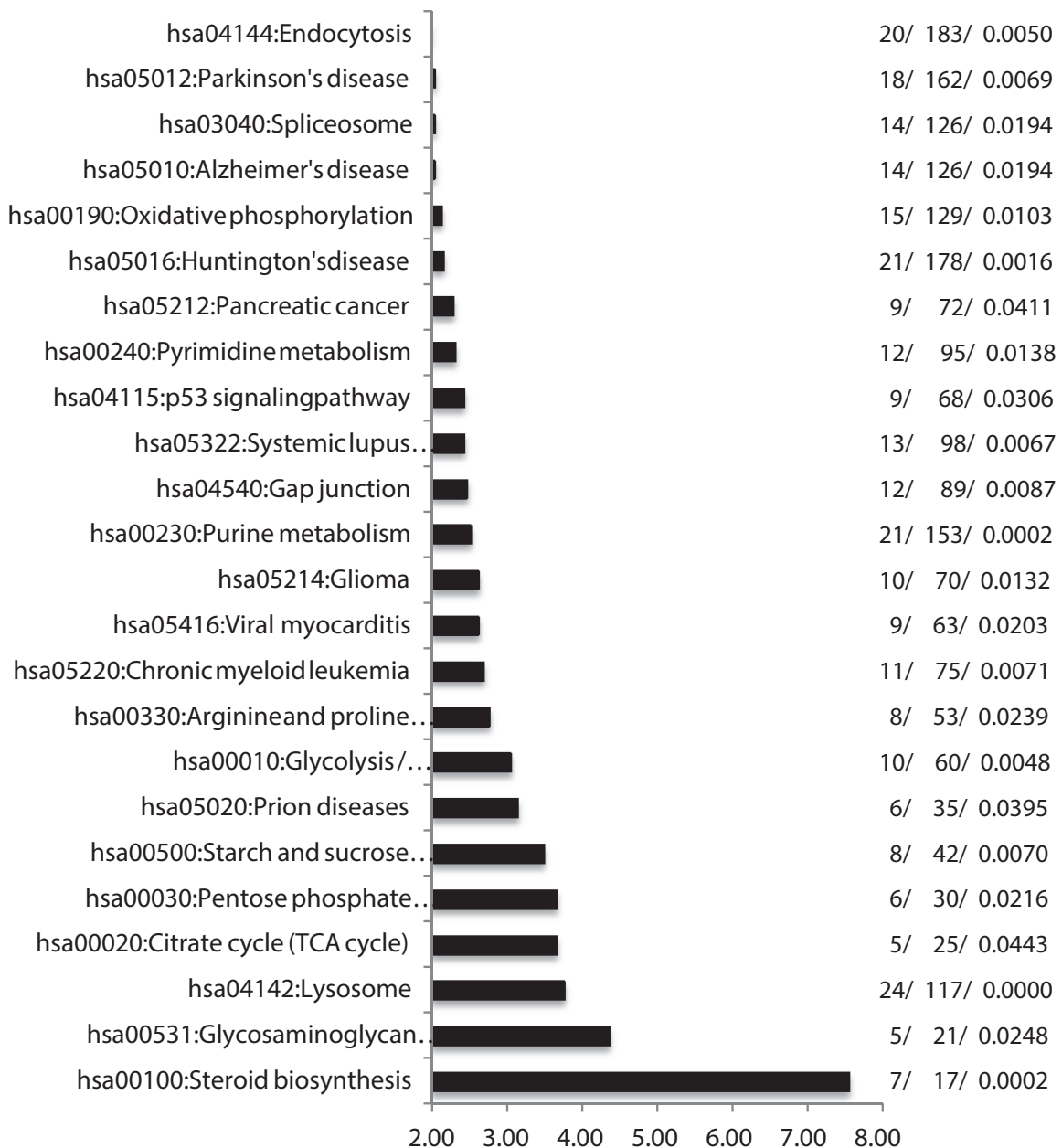
WT1, transcription



Fold enrichment

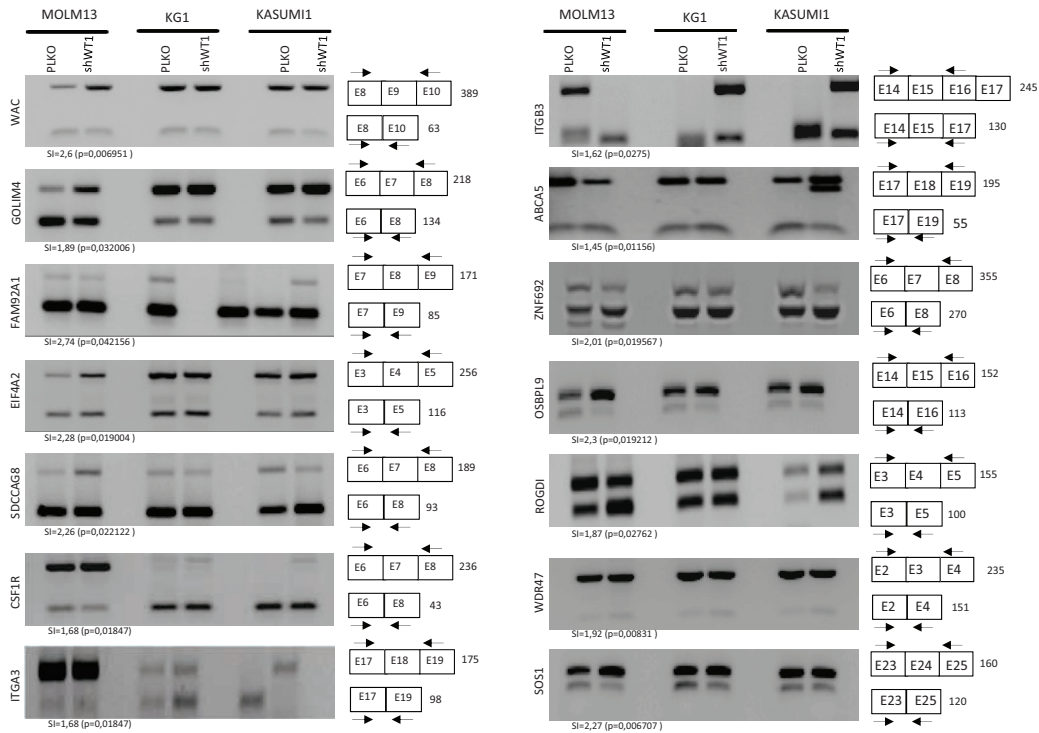
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B DEK, transcription

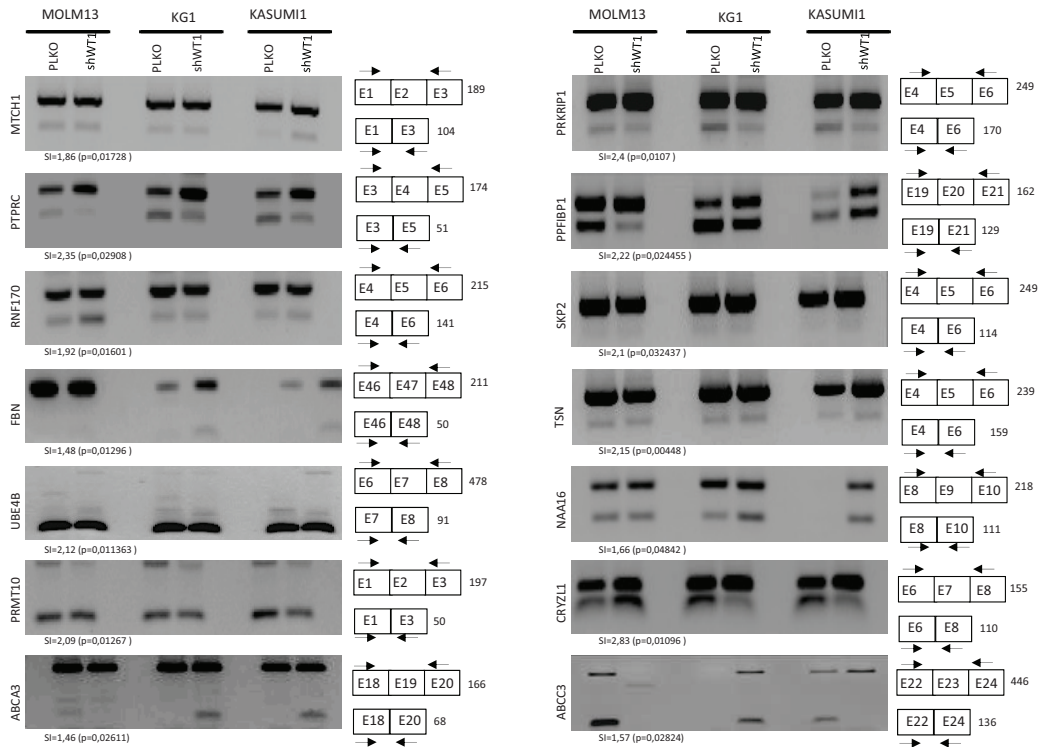


Supplementary Figure S2 (Continued): Pathway enrichment analysis in cells knocked-down for WT1 and DEK expression. For ontology analysis, gene lists were analyzed using DAVID software (KEGG pathways). The complete set of genes featured in microarrays was used as reference background. The 3 numbers on the right represent the number of deregulated mRNA, the overall number of genes within the pathway and the *p* value. Data are presented for genes quantitatively modified upon WT1 **A.** and DEK **B.** expression.

A Exon Skipping

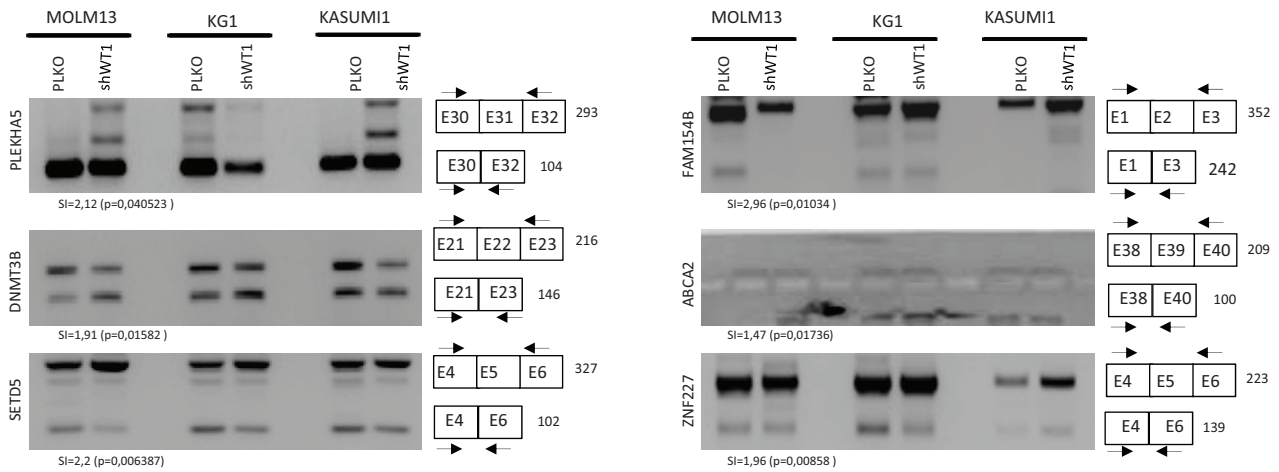


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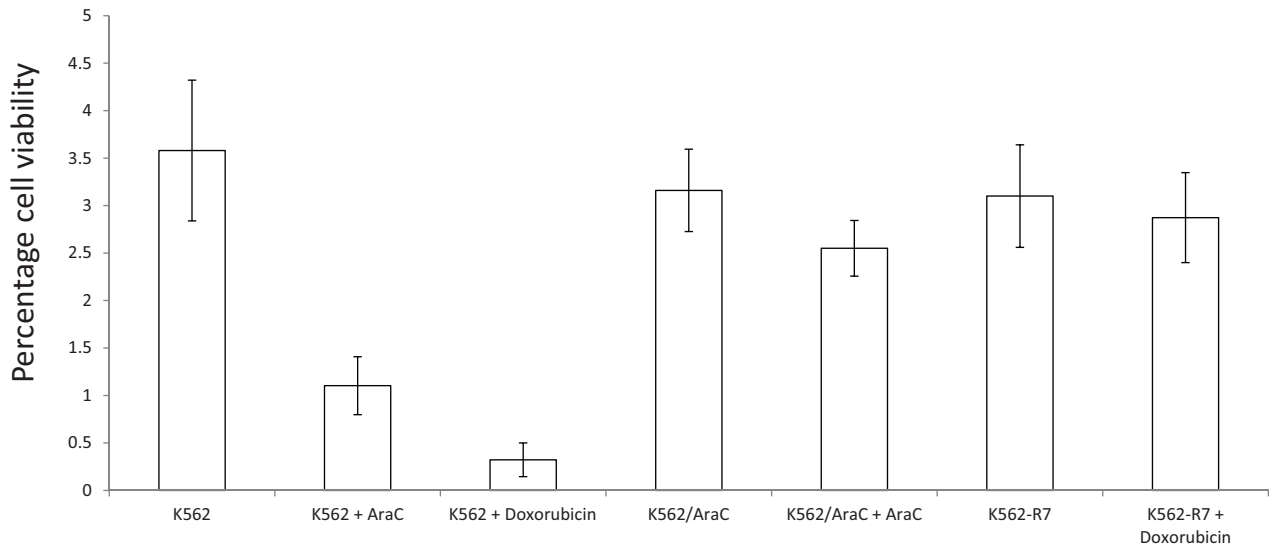


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C Exon Skipping



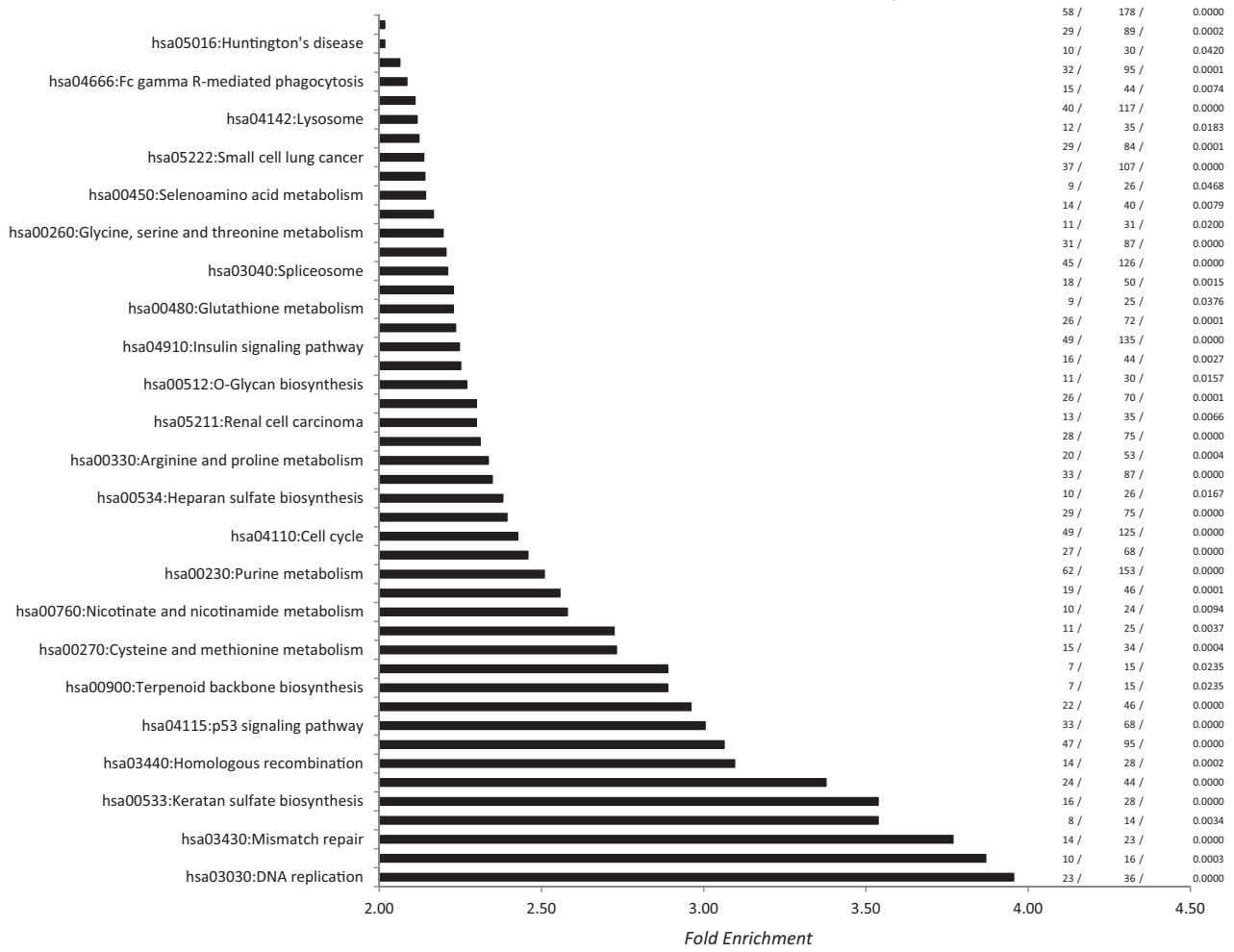
Supplementary Figure S3 (Continued): Validation of microarray-predicted exon events. Exon-specific RT-PCR assays were performed with RNA samples derived from WT1+ and WT1- MOLM13, Kaumi-1, and KG1 cells. Numbers indicate the expected band (bp) of PCR products. SI (splicing index) and p values are indicated for each exon event. $SI \geq 1.2$ was considered a significant change in exon expression and was used for comparisons.



Supplementary Figure S4: Trypan blues exclusion analysis of AraC- and DXR-resistant versus sensitive K562 cells.

A

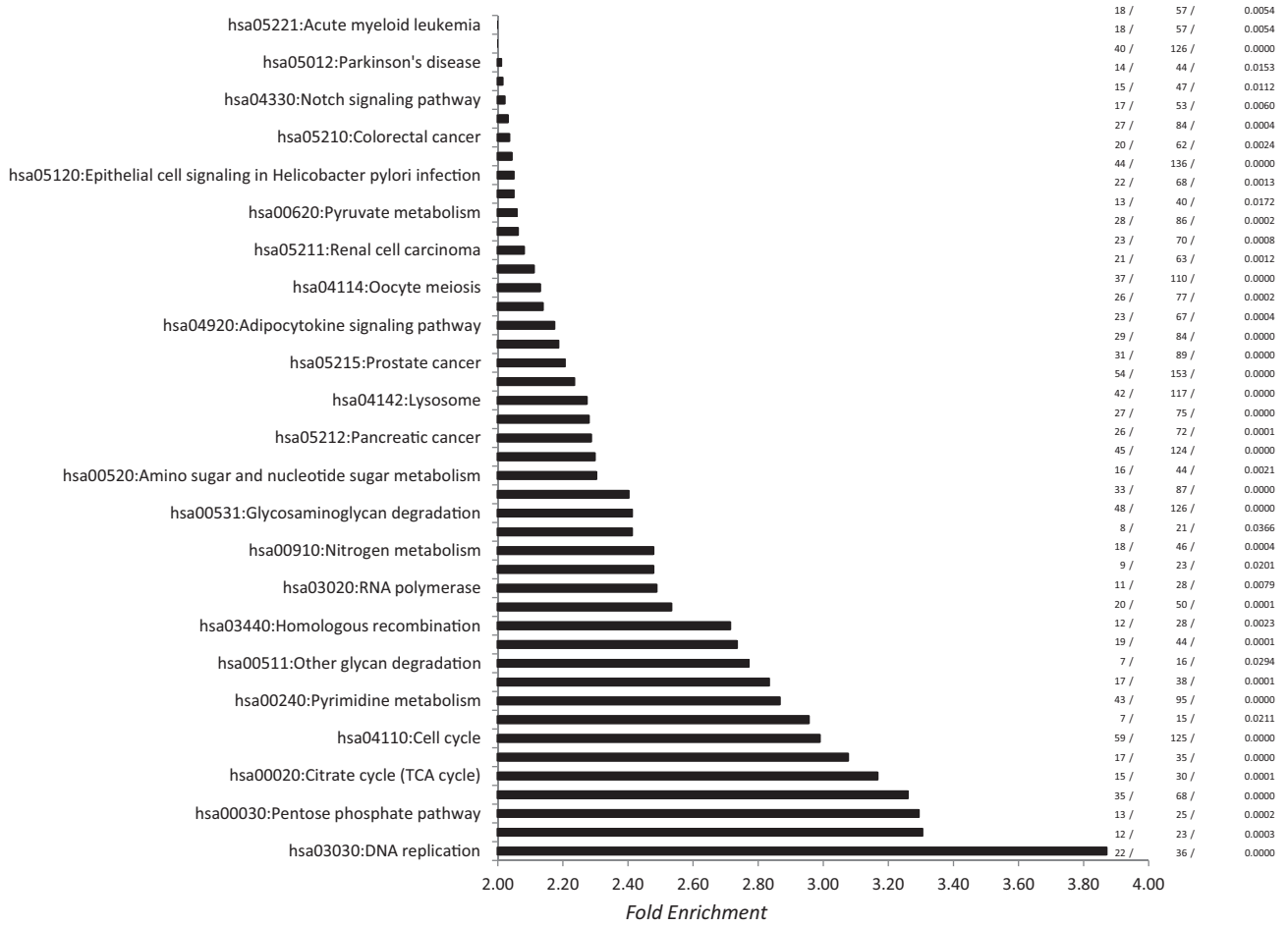
AraC, transcription



(Continued)

B

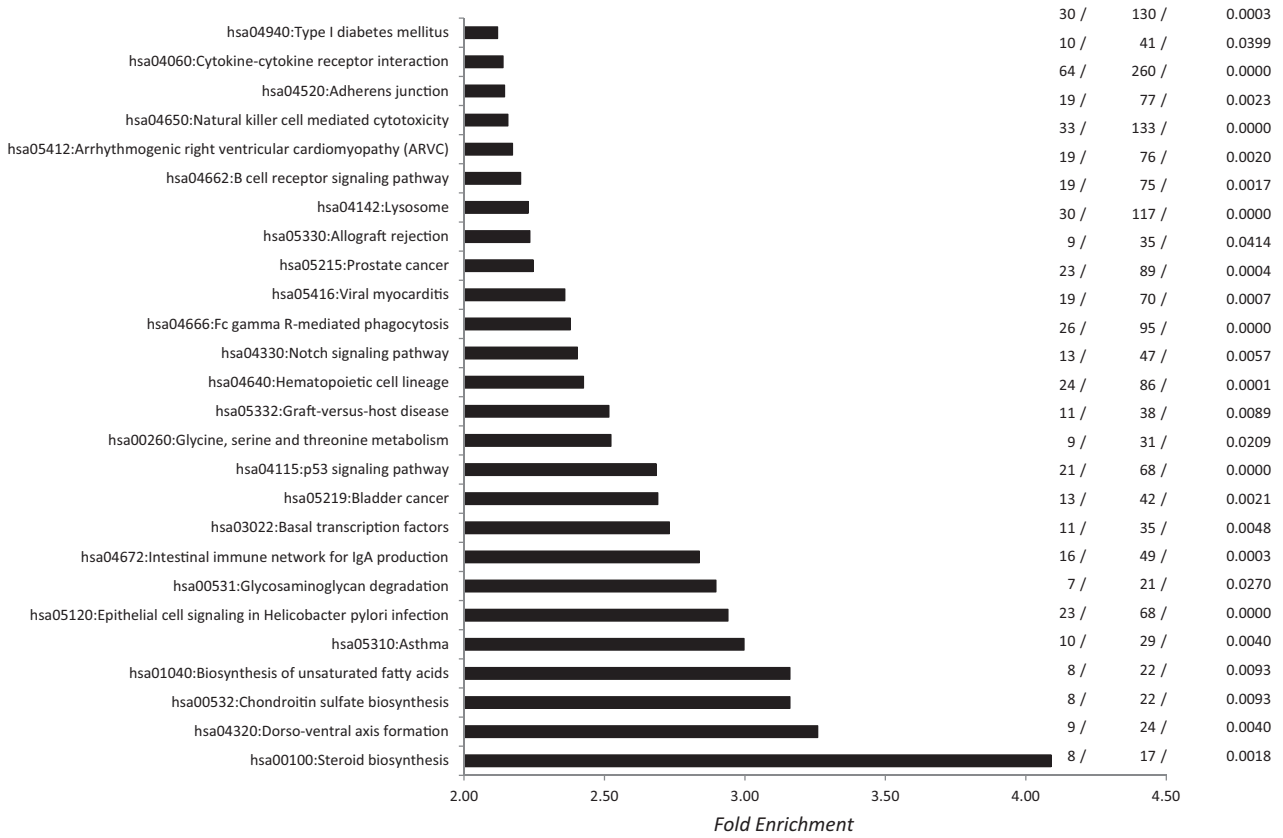
DXR, Transcription



(Continued)

C

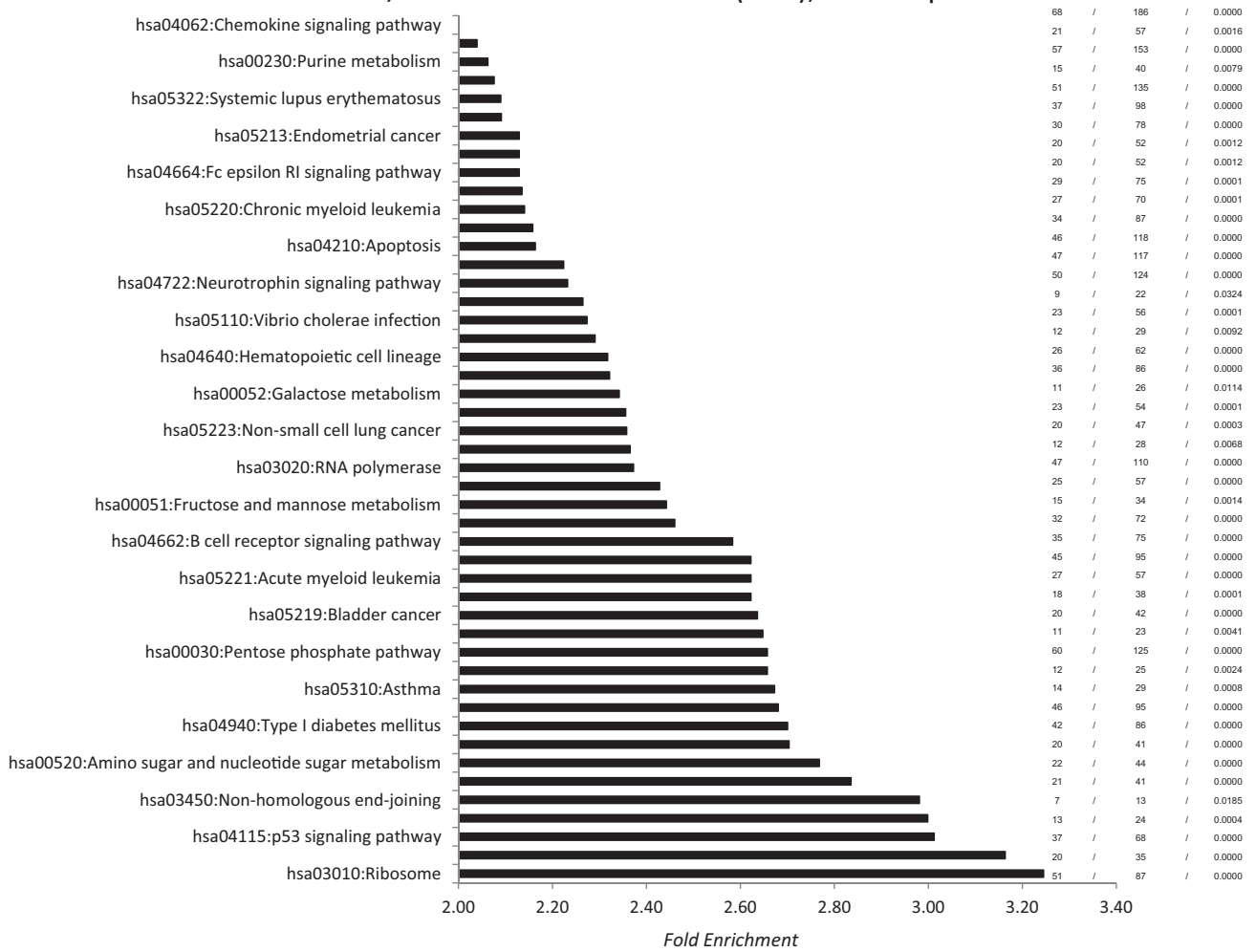
AZA, transcription



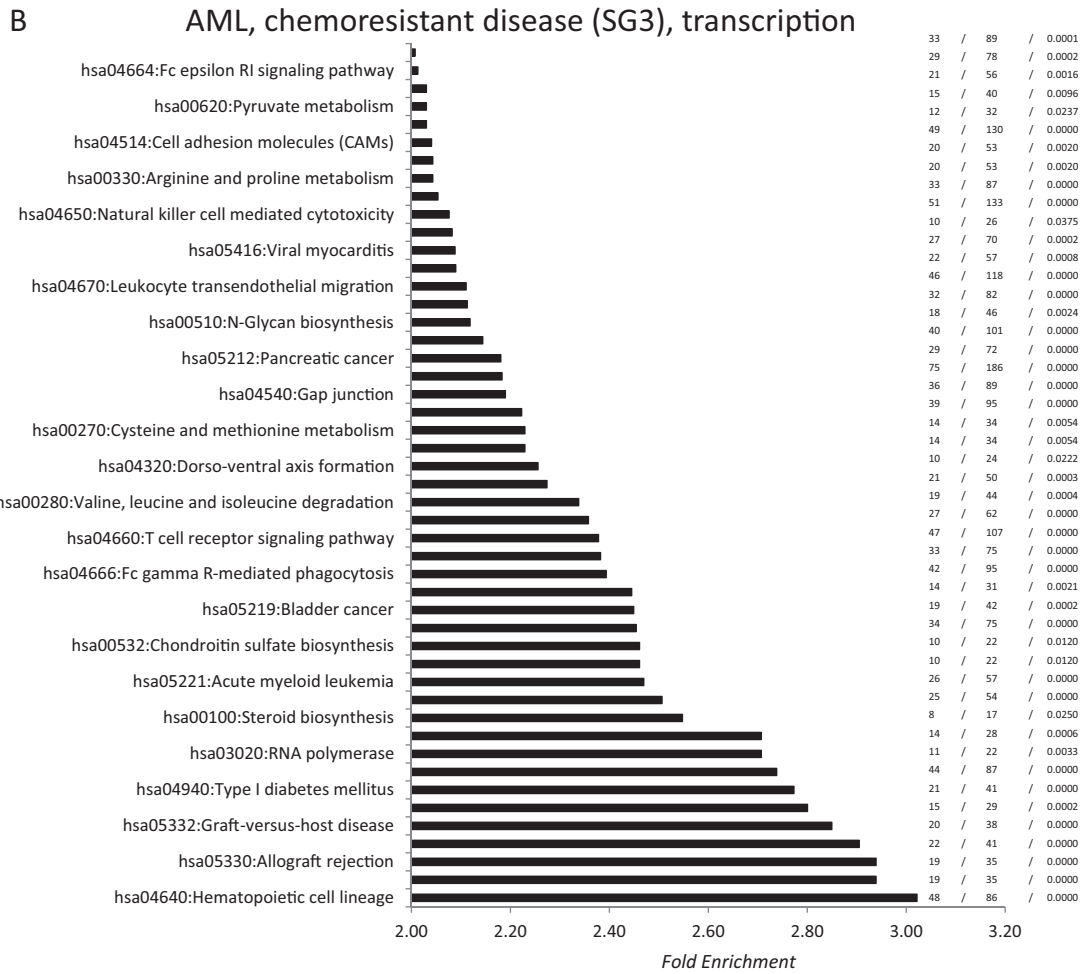
Supplementary Figure S5 (Continued): Pathway enrichment analysis in cells resistant to AraC, DXR and AZA. See the legend of supplementary Figure 2. Data are presented for genes quantitatively modified in cells resistant to AraC A., DXR B. and AZA C.

A

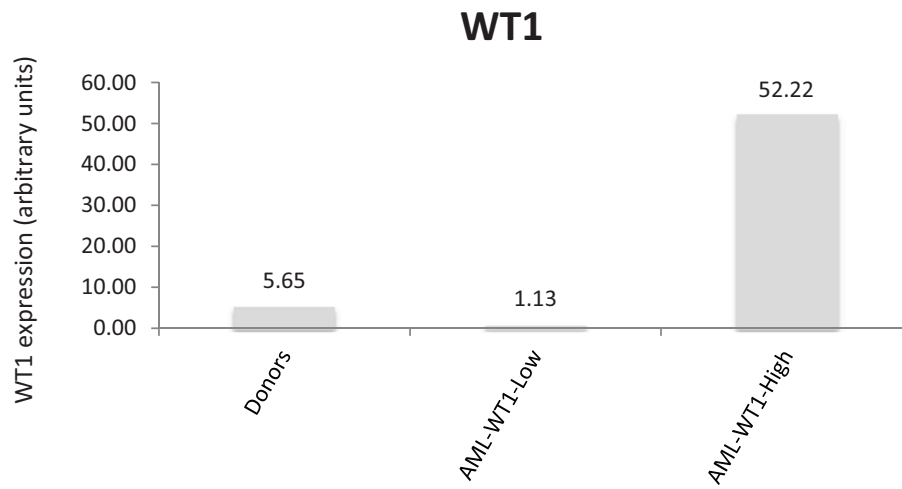
AML, chemosensitive disease (SG1), transcription



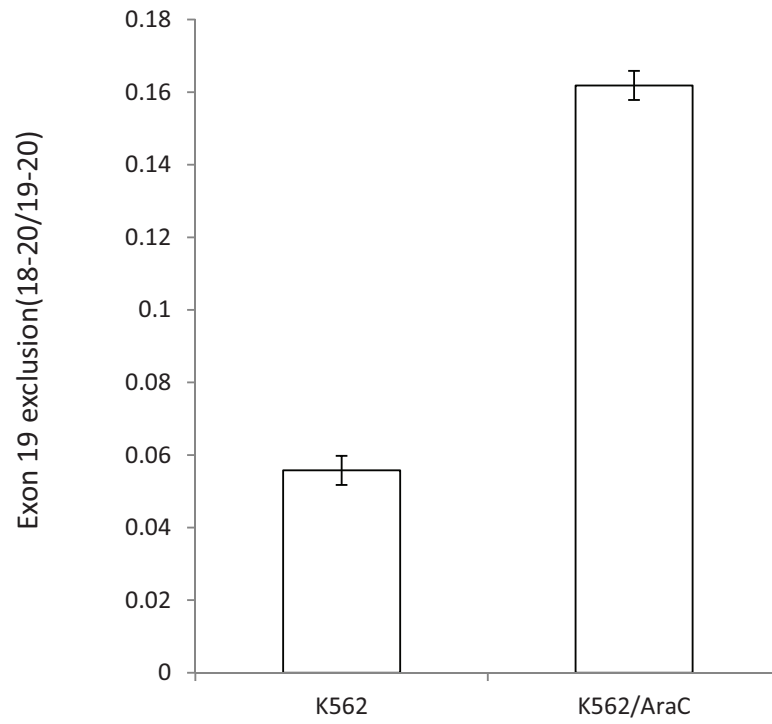
(Continued)



Supplementary Figure S6 (Continued): Pathway enrichment analysis in AML samples deriving from patients with sensitive (SG1) and resistant disease (SG3) (see Table 1). See the legend of supplementary Figure 2. Data are presented for genes quantitatively modified in samples deriving from SG1 **A.** and SG3 **B.**



Supplementary Figure S7: WT1 expression in normal bone marrow donors and AML cells. WT1 expression was measured through qRT-PCR in bone marrow samples derived from the 37 donors and the 152 AML. AML-WT1 low and AML-WT1 high correspond to the 76 and 76 AML cases with WT1 expression \leq or $>$ to the median value (9, 00 arbitrary units), respectively.



Supplementary Figure S8: ABCA-3 exon 19 exclusion in AraC-resistant cells.

Supplementary Table S1. AEU events shared by DEK and WT1-expressing cells

Supplementary Table S2. AEU events shared by AraC- and DXR-resistant cells

Supplementary Table S3. AEU events, shared by AZA- and AraC-resistant cells

Supplementary Table S4. AEU events, shared by DXR- and AZA-resistant cells

Supplementary Table S5. AEU events, shared by DEK-expressing cells and fresh AML samples

Supplementary Table S6. AEU events, shared by WT1-expressinf cells and fresh AML samples

Supplementary Table S7. AEU events, shared by AraC-resistant cells and fresh AML samples

Supplementary Table S8. AEU events, shared by DXR-resistant cells and fresh AML samples

Supplementary Table S9. AEU events,shared by AZA-resistant cells and fresh AML samples

Supplementary Table S10. Disrtibution of AEU events in gene frequently mutated in AML

II-1.3. Conclusion, perspectives

Ce travail nous a permis d'identifier des patrons d'épissages spécifiques de la résistances aux drogues de chimiothérapies et/ou de l'expression de DEK et WT1. De façon importante, 30% à 80% des évènements identifiés concernaient des gènes dont la transcription globale n'était pas modifiée. L'analyse de l'épissage permet donc d'accéder à des événements jusque là méconnu. L'annotation de ces anomalies a permis d'identifier des nouvelles voies potentiellement pertinentes au plan mécanistique, physiopathologique et thérapeutique. En développant des systèmes de PCR quantitative spécifique d'exon nous avons pu valider l'impact de WT1 sur l'épissage *in vivo*, dans des échantillons médullaire de LAM prélevé au diagnostic.

A partir de ces données, nous avons choisi d'approfondir notre étude des perturbations d'épissage en tentant d'évaluer leur utilité clinique, comme biomarqueur chez les malades traités par chimiothérapie intensive, ainsi que leur implication dans la résistance au traitement.

II-2. Anomalies d'épissage du messenger de TET2 dans les leucémies aiguës myéloïdes traitées par chimiothérapie intensive

II-2.1. Introduction

Dans ce troisième travail, nous avons cherché à valider l'utilité d'une analyse de l'épissage comme biomarqueur dans les LAM traitées par chimiothérapies intensive. Des travaux précédents ont déjà souligné l'impact pronostique de certains événements d'épissage dans les LAM, comme ceux concernant les messagers de WT1, TP53, HOXA9, BAALC, VEGF, et BCL-X².

Dans notre étude précédente, nous avons trouvé une exclusion de l'exon 2 de TET2 dans les cellules K562 résistantes à l'AraC de même que dans les échantillons blastiques médullaires de malades ayant obtenu une rémission prolongée de leur maladie après chimiothérapie intensive. L'analyse de 152 échantillons de LAM et de 37 contrôles (donneur de moelle osseuse) par RTPCR quantitative spécifique d'exon permettait de confirmer la présence d'une diminution spécifique de l'exon 2 de TET2 en l'absence de modification transcriptionnelle globale du gène. TET2 est une dioxygénase catalysant la conversion de la 5-methylcytosine en 5-hydroxymethylcytosine entraînant par ce biais une déméthylation de l'ADN. Le gene TET2 est muté dans 7–23% des AML et 25% des SMD, sans point chaud de mutation au long de la séquence codante. Ces mutations réduisent l'activité enzymatique de la protéine. Au plan clinique les mutations de TET2 confèrent un pronostic favorable au SMD de risque élevé traité par agents hypométhylants alors que leur impact pronostique dans les LAM traitées par chimiothérapie intensive fait l'objet de débat.

Nous avons quantifié spécifiquement l'expression de l'exon 2 de TET2 dans deux séries de LAM traitées par chimiothérapie intensive de même que chez des patients traités non intensivement. Notre objectif était d'évaluer l'impact pronostique de l'exclusion de l'exon 2 de TET2 et de le confronter à celui des marqueurs pronostiques utilisés en routine comme le caryotype et les réarrangements de NPM1, CEBPA et de FLT3.

II-2.2. Article soumis:

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TET2 exon 2 skipping is an independent favorable prognostic factor for cytogenetically normal acute myelogenous leukemia (AML)

TET2 exon 2 skipping in AML

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1 **Abstract**

2 In AML, approximately one-third of expressed genes are abnormally spliced, including
3 aberrant TET2 exon 2 expression. In a discovery cohort (n=99), *TET2* exon 2 skipping
4 (TET2E2S) was found positively associated with a significant reduction in the cumulative
5 incidence of relapse (CIR). Age, cytogenetics, and TET2E2S were independent prognostic
6 factors for disease-free survival (DFS), and favorable effects on outcomes predominated in
7 cytogenetic normal (CN)-AML and younger patients. Using the same cutoff in a validation
8 cohort of 86 CN-AML patients, TET2E2S^{high} patients were found to be younger than TET2^{low}
9 patients without a difference in the rate of complete remission. However, TET2E2S^{high} patients
10 exhibited a significantly lower CIR ($p < 10^{-4}$). TET2E2S and *FLT3-ITD*, but not age or *NPM1*
11 mutation status were independent prognostic factors for DFS and event-free survival (EFS),
12 while TET2E2S was the sole prognostic factor that we identified for overall survival (OS). In
13 both the intermediate-1 and favorable ELN genetic categories, TET2E2S remained significantly
14 associated with prolonged survival. There was no correlation between TET2E2S status and
15 outcomes in 34 additional AML patients who were unfit for IC. Therefore our results suggest
16 that assessments of *TET2* exon 2 splicing status might improve risk stratification in CN-AML
17 patients treated with IC.

18

1 **Introduction**

2 Intensive anthracycline and cytarabine (AraC)-based combination chemotherapy has been
3 used as the backbone of acute myeloid leukemia (AML) treatment for nearly 40 years.¹ Using
4 such a regimen, ~70–80% of patients who are 60 years old or younger with non-
5 promyelocytic AML will achieve complete remission;^{2, 3} however, most patients will ultimately
6 relapse, and the overall survival of these patients is only 40–45% at 5 years. Among patients
7 who were older than 60 year old, 40–50% of those exhibiting a good performance status can
8 achieve complete remission, but their cure rates are less than 10% and median survival is less
9 than 1 year.^{4, 5} In addition to patient-dependent factors, pretreatment cytogenetic and
10 molecular abnormalities, such as *NPM1* (nucleophosmin gene 1) and *CEBPA* (CCAAT/enhancer
11 binding protein alpha gene) mutations or *FLT3*-ITD (internal tandem duplications in the fms
12 related tyrosine kinase 3 gene), represent the strongest prognostic factors.⁶ Nevertheless,
13 despite several attempts to precisely stratify patients based on these cytogenetic and
14 molecular subcategories, the prognosis of many patients with AML, especially CN-AML,
15 remains uncertain and the optimal post-remission therapy for such patients is unclear.
16 Consequently, some patients remain undertreated and rapidly relapse, whereas others are
17 overtreated and unnecessarily exposed to treatment-related toxicity.

18 Recently, AML cells have been found to harbor a huge number of alternatively expressed
19 exons.⁷ Expression of these exons can be caused by numerous factors, such as spliceosome
20 mutations,⁸ histone acetylation/methylation, DNA CpG methylation, and WT1 expression.⁹
21 Alternative exon usage (AEU) can also play a role in the plasticity of tumor cells and may
22 thereby influence patient responses to treatment. Previous reports have suggested that
23 certain splicing events, such those involving WT1,¹⁰ TP53,¹¹ HOXA9,¹² BAALC,¹³ VEGF,¹⁴ or
24 BCL-X,¹⁵ might influence disease outcomes.

25 TET2 is a dioxygenase that catalyzes the conversion of 5-methylcytosine to 5-
26 hydroxymethylcytosine and thereby promotes DNA demethylation.¹⁶ *TET2* somatic mutations
27 occur in 7–23% of AML and 25% of myelodysplastic syndrome (MDS) patients, and they are
28 distributed across the entire coding sequence without obvious hot spots.¹⁷ These mutations
29 decrease TET2 enzymatic activity by either truncating the protein or affecting its catalytic
30 activity.¹⁶ *TET2* mutations confer favorable outcomes in high-risk MDS treated with
31 hypomethylating agents.^{18, 19} In intensively treated AML patients, several studies have
32 investigated the impact of TET2 mutations at the time of diagnosis on outcomes, and have
33 yielded conflicting results as mutations have both been reported to exert unfavorable²⁰ or
34 neutral effects on patient outcomes.²¹⁻²³ Recently, fluctuations in TET2 exon 2 expression have

1 been detected in leukemic cells.²⁴ *TET2* exon 2 skipping (TET2E2S) has been identified in AML
2 cell lines that are resistant to AraC and in fresh diagnosis bone marrow samples deriving from
3 patients subsequently cured with IC.²⁴

4 Herein, we hypothesize that TET2E2S may serve as biomarker for outcomes in AML patients
5 who undergo intensive treatment. In 219 AML patients (discovery cohort, 99; validation
6 cohort, 86; AML unsuitable for IC, 34), the TET2E2S status at diagnosis was found to be
7 associated with a reduced relapse rate and prolonged survival in intensively treated patients,
8 independently of routinely used clinical, molecular, and cytogenetic prognostic markers. This
9 positive effect was most robust in CN-AML and younger patients, suggesting that assessments
10 of *TET2* exon 2 splicing status might improve AML risk stratification.

11

1 **Methods**

2 **Patients and Sample Collection**

3 The medical ethics committee of the Hospices Civils de Lyon approved this study. Informed
4 consent was obtained from each patient and healthy volunteer in accordance with the
5 Declaration of Helsinki and institutional guidelines. The discovery cohort included 99
6 consecutive non-APL AML patients who were homogeneously treated at Lyon's University
7 Hospital following the French acute leukemia group (ALFA) protocols 9801, 9802, and 9803
8 between 2001 and 2007 (Table 1). The validation cohort included 86 CN-AML patients who
9 were more recently treated at Lyon and Saint-Etienne's University Hospitals according to
10 either the ALFA 0701, ALFA 0702, or Groupe Ouest Est d'Etude des Leucémies aiguës et
11 Autres Maladies du Sang (GOELAMS) protocols LAM 2006 IR/LAM SA 2007 between 2008 and
12 2013 (Table 2). As controls, bone marrow mononuclear cells (BMMNCs, 38 samples) were
13 obtained from bone marrow donors. In CN-AML from the validation cohort, FLT3-ITD²⁵ and
14 mutations in NPM1 (exon 12)²⁶ were assessed as previously described. CEBPA mutations were
15 assessed in CN-AML in the absence of a FLT3-ITD or NPM1 mutation. Patients with double
16 mutant CEBPA were classified in the favorable ELN genetic group.

17 **RNA Purification and Reverse Transcription**

18 RNA was isolated with TRIzol reagent (Invitrogen). RNA concentrations and purity were
19 determined using UV spectrophotometry (Nanodrop). Prior to carrying out reverse
20 transcription reactions, RNA was treated with DNAase (DNAse-freeTM, Invitrogen) to prevent
21 DNA contamination. Then, cDNA was synthesized from 1 µg RNA using a random primer (High
22 Capacity cDNA Reverse Transcription Kit, Invitrogen) and Superscript II reverse transcriptase
23 (Invitrogen).

24 **Quantitative Exon-Specific Polymerase Chain Reaction (qPCR)**

25 For qPCR, cDNA was amplified in 20 µl reactions using iQ SYBR Green PCR Supermix (BioRad)
26 with 10 µM of each appropriate primer. Thermocycling was carried out using a Bio-Rad
27 Chromo4: CD002161. Expression of a housekeeping gene, *Gus* (NM_000181), was used as an
28 internal control. A melting curve (65–92°C) was generated at the end of each run to verify
29 specificity of the amplicon. The Pfaffl method was used for relative quantification of transcript
30 levels.²⁷ Primer sequences are available upon request.

31 **Statistics**

32 Associations between categorical variables were analyzed using Fisher's exact test. Central
33 tendency differences between groups were compared using Mann–Whitney or Kruskal–Wallis
34 tests. Associations between continuous variables were assessed using Spearman's correlation.

1 OS was defined as the time from initiating IC treatment to death from any cause. EFS was
2 defined as the time from diagnosis to either treatment failure, relapse, or death. DFS was
3 defined as the time from achievement of CR to the date of relapse or death. Patients who
4 remained alive were censored at the last follow-up visit. Allograft recipient patients were not
5 censored at the time of transplantation. TET2 exon 2 skipping was quantified by calculating
6 the E1E3/E2E3 ratio.²⁴ For categorical and survival analyses, the E1E3/E2E3 ratio was
7 dichotomized using a cutoff point that was based using the Maximally Selected Rank
8 Statistics²⁸ (maxstat R-statistics package, from the R-Project for Statistical Computing: R 3.2.0
9 software package). For each patient or control population, samples with a level of TET2 exon
10 2 skipping that was higher than that corresponding to the cutoff value were termed
11 TET2E2S^{high}, whereas those with a value lower than the cutoff were termed TET2E2S^{Low}.
12 Survival rates and DFS were calculated using the Kaplan–Meier method.²⁹ Log rank tests were
13 used to compare survival rates between groups. To identify prognostic factors for OS, DFS,
14 and EFS, multivariate analyses were performed using a Cox proportional hazards model.³⁰ A
15 stepwise backwards procedure was used with a threshold significance level of $P < 0.10$ to select
16 factors in the model. All tests were two-sided. The final type I error rate was fixed at 0.05 to
17 identify factors associated with “time to event” outcomes. The cumulative incidence of relapse
18 (CIR) analysis only included patients who achieved a CR, for which the time was calculated
19 from the date of CR until relapse. Patients who remained alive without relapse were censored,
20 whereas those who died without relapse [non-relapse mortality (NRM)] were counted as a
21 competing cause of failure. The mean CIR with standard error (SE) were estimated using the
22 method of Gray,³¹ and differences between groups were analyzed using a test developed by
23 Gray³² with the RcmdrPlugin.EZR script from R-software.³³

1 **Results**

2 **TET2 exon 2 skipping is an independent favorable prognostic factor** 3 **among intensely treated AML patients from the discovery cohort**

4 Patient characteristics are summarized in Table 1. We found TET2E2S to be associated with
5 DFS, so we divided the 99 intensely treated patients from the discovery cohort into two
6 categories based on the TET2E2S value. Patients were dichotomized according to the cutoff
7 determined using maximally selected log-rank statistics (Figure 1A, Table 2)²⁸. The cutoff was
8 0.99 (Figure 1A) and TET2E2S^{high} patients were defined as patients with TET2E2S > 0.99,
9 whereas TET2E2S^{low} patients had a TET2E2S value ≤ 0.99. In 4 patients, the level of E2E3
10 expression was below the sensitivity threshold for the PCR assay; these cases were considered
11 to be TET2E2S^{high}. The two groups were well matched for age, gender, and cytogenetics,
12 while the proportion of allografted patients was not significantly different between TET2E2^{high}
13 and TET2E2^{low} patients (Table 2). TET2E2S^{high} patients had a significantly lower CR rate
14 compared with TET2E2^{low} patients (Table 2). However, the CIR was significantly lower in the
15 TET2E2S^{high} than in the TET2E2S^{low} group (Table 2, Figure 1), while there was no significant
16 difference in the NRM rate between these groups (Figure 1). The median follow-up duration
17 was 106 months. TET2E2S^{high} patients had a significantly longer DFS compared with TET2E2S^{low}
18 patients (Table 2, Figure 1C), while there was a trend for a longer EFS and OS in TET2E2S^{high}
19 patients (Table 2). Multivariate analysis showed that age, cytogenetics, and TET2E2S were
20 independent prognostic factors for DFS (Table 3). The overall level of *TET2* expression, as
21 measured based on qRT-PCR amplification of a 10 exon sequence, was not significantly
22 associated with the response rate, relapse rate, CIR, DFS, EFS, or OS (not shown).

23 In the 49 CN-AML cases from the discovery cohort, the overall response rate was 84% and
24 there was no significant difference between the TET2E2S^{High} (15/21, 71%) and TET2E2S^{Low}
25 AML (26/28, 93%) patients. The 5-year CIR remained significantly lower in the TET2E2S^{high}
26 group compared with the TET2E2S^{low} group (24% [95% CI 8–44] vs. 79% [95% CI 56–90],
27 p=0.0003 by the Gray test; Supplementary Figure 1A). Univariate analysis showed that
28 TET2E2^{high} CN-AML patients had a significantly longer DFS compared with TET2E2^{low} patients
29 (log rank test p=0.019), while there was a trend for longer EFS and OS in TET2E2S^{high} cases
30 (log-rank test p=0.22 for EFS and 0.36 for OS, respectively; Supplementary Figure 1B). In the
31 50 AML cases with an abnormal karyotype, TET2E2S^{high} patients had a significantly lower CR
32 rate compared with TET2E2^{low} patients (10/21 [52%] vs. 25/29 [86%], p=0.004, Fisher's
33 exact test). However, the 5-year CIR was significantly lower in the TET2E2S^{high} group
34 compared with the TET2E2S^{low} group (19% [95% CI 5.5–38.8] vs. 75.9% [95% CI 53.7–
35 88.4], p=0.0003 by the Gray test; Supplementary Figure 2A). Univariate analysis showed that

1 TET2E2^{high} patients with an abnormal karyotype had a significantly longer DFS compared with
2 TET2E2^{low} patients (log rank test $p=0.023$; Supplementary Figure 2B).

3 In the 55 patients who were younger than 60 years old, the overall response rate was 82%
4 and there was no significant difference between TET2E2S^{high} (18/24, 75%) and TET2E2S^{low}
5 AML patients (27/31, 87%, $p=0.078$), while the 5-year CIR was significantly lower in the
6 TET2E2S^{high} group than in the TET2E2S^{low} group (16.7% [95% CI 5–34.3] vs. 71% [95% CI
7 51–84], $p=0.00011$ by the Gray test; Supplementary Figure 3A). Univariate analysis showed
8 that for patients younger than 60 years old, TET2E2^{high} patients had significantly longer rates
9 of DFS (log rank test $p=0.00234$; Supplementary Figure 3B), EFS (log rank test $p=0.034$;
10 Supplementary Figure 3C), and OS compared with TET2E2^{low} patients (log rank test $p=0.039$;
11 Supplementary Figure 3D). In the 44 patients who were greater than 60 years old, the CR rate
12 was 71% and TET2E2S^{high} patients had a significantly lower CR rate than TET2E2^{low} patients
13 (7/18 [39%] vs. 24/26 [92%], $p<10^{-4}$), whereas the 5-year CIR was significantly lower in the
14 TET2E2S^{high} group than in the TET2E2S^{low} group (27.8 [95% CI 8.4–51.5] vs. 84.6 months
15 [95% CI 58.5–94.9], $p=0.006$ by the Gray test; Supplementary Figure 4). In patients greater
16 than 60 years old, TET2E2S showed no significant impact on OS, DFS, or EFS (not shown).

17 **TET2 exon 2 skipping is an independent favorable prognostic factor** 18 **for CN-AML patients**

19 The aforementioned results suggested that although TET2E2S is associated with a decreased
20 CR rate, the CR duration is significantly longer in TET2E2S^{high} AML patients, suggesting that
21 TET2E2S has a more favorable effect on the outcome of CN-AML that develops in younger
22 individuals. Accordingly, we next investigated the effects of TET2 exon 2 skipping in a
23 validation cohort of 86 additional consecutive CN-AML patients treated with IC. All patients
24 were diagnosed between 2008 and 2013 and were tested for *FLT3*-ITD and *NPM1* exon 12
25 mutations; in addition, we searched for *CEBPA* mutations in patients without *FLT3*-ITD or
26 *NPM1* mutations. Patients from the validation cohort were divided into 2 categories according
27 to the TET2E2S cutoff (0.99) that was determined by maximally selected log-rank statistics for
28 the discovery cohort (Table 4). TET2E2S^{high} patients were found to be significantly younger
29 than TET2E2^{low} patients (Table 4); these two groups of patients were well balanced for sex,
30 ELN genetic group distribution, and *FLT3*-ITD, *NPM1*, and *CEBPA* mutation status (Table 4).
31 The proportion of allograft recipient patients was not significantly different between TET2E2^{high}
32 and TET2E2^{low} patients (Table 4). Similarly to CN-AML patients from the discovery cohort,
33 there was no significant difference in the CR rate between TET2E2^{high} and TET2E2^{low} patients
34 from the validation cohort (Table 4). The CIR was significantly lower in the TET2E2S^{high}

1 compared with the TET2E2S^{low} patients (Table 4, Figure 2). The median duration of follow-up
2 was 24 months. Univariate analysis showed that TET2E2S^{high} patients had a significantly
3 longer DFS, EFS, and OS compared with TET2E2S^{low} patients (Table 4, Figure 2,
4 Supplementary Figure 5). These differences persisted for DFS, EFS, and OS in the 43 patients
5 who were younger than 60 years old and for DFS and EFS in the 43 patients who were greater
6 than 60 years old (not shown). In both the ELN genetic favorable and intermediate-1 groups,
7 TET2E2S remained a favorable prognostic factor for OS, DFS, and EFS (Supplementary Figure
8 6). Differences between the TET2^{high} and TET2^{low} groups were significant for DFS, EFS, and OS
9 in the favorable ELN genetic group. In the intermediate-1 ELN group, differences were
10 statistically significant for DFS and EFS, while there was a trend for improved OS in the
11 TET2^{high} patients (Supplementary Figure 6). Multivariate analysis showed that TET2E2S and
12 *FLT3*-ITD, but not age or *NPM1* mutation (with or without *FLT3*-ITD), were independent
13 prognostic factors for DFS and EFS, while TET2E2S was the sole prognostic factor for OS
14 (Table 5).

15 **Prognostic impact of TET2 exon 2 skipping in AML patients who were** 16 **unsuitable for intensive chemotherapy**

17 The prognostic effect of TET2E2S was evaluated in an additional series of 34 AML patients
18 who were unsuitable for IC (Table 1) and were instead treated with hydroxyurea (9 patients),
19 azacitidine (5 patients), decitabine (2 patients), low dose AraC (10 patients), or supportive
20 care alone (8 patients). TET2E2S showed no significant impact on the response rate, relapse
21 rate, OS, DFS, or EFS in this cohort (not shown).

22

1 **Discussion**

2 In AML, abnormal alternative splicing involves genes that encode various oncogenes, tumor
3 suppressor proteins, splicing factors, and heterogeneous-nuclear-ribonucleoproteins, which
4 encode proteins involved in apoptosis, cell proliferation, and spliceosome assembly.⁷ This
5 suggests the possibility that such deregulated patterns of alternative splicing likely have
6 additional pathogenic, diagnostic, prognostic, and therapeutic implications. This present study
7 was carried out to gain insights into the potential clinical consequences of TET2 exon
8 deregulation in AML. Recently, an analysis of exon arrays permitted TET2E2S to be identified
9 in AraC-resistant cells *in vitro* and allowed for the diagnosis of samples from AML cases who
10 did not relapse with a >4-year follow-up period.²⁴ Among the 99 intensively treated patients
11 from the discovery cohort, TET2E2S status at the time of diagnosis was associated with a
12 significantly reduced CR rate. This effect appeared to be restricted to older patients and AML
13 patients with an abnormal karyotype, while it was not observed in younger patients from the
14 discovery cohort, including CN-AML patients from both the discovery and validation cohorts.
15 Furthermore, in the discovery cohort, TET2E2S status was associated with a significantly
16 reduced CIR, especially in CN-AML and younger patients. This finding was confirmed in 86 CN-
17 AML patients from the validation cohort in whom the cutoff value of TET2E2S was defined by
18 maximally selected log-rank statistics in the discovery cohort, which allowed the validation
19 cohort to be dichotomized into 2 groups that exhibited clearly distinct outcomes. Accordingly,
20 high levels of TET2E2S were found associated with reduced CIR and prolonged OS, EFS, and
21 DFS, independently of routinely used clinical and molecular prognostic markers. In contrast to
22 intensively treated AML, TET2E2S exhibited no prognostic impact for cases of AML who were
23 unsuitable for IC.

24 *TET2* exon 2 is spliced in a mutually exclusive manner compared with exon 1, and it uses an
25 alternative promoter (<https://fasterdb.lyon.unicancer.fr/>). However, *TET2* exon 2 is not
26 translated into a protein and its role in the regulation of *TET2* expression remains unknown.
27 Using qES-PCR, we have recently shown that levels of TET2E2S are significantly higher in AML
28 samples compared with normal bone marrow mononuclear cells.²⁴ In accord with *in vitro*
29 findings obtained using AraC-resistant cells, TET2E2S was found to be associated with a
30 significantly lower response rate in intensively treated patients from the discovery cohort. This
31 effect was strongest in older patients and AML patients with an abnormal karyotype, while it
32 was absent in CN-AML and younger patients. TET2E2S did not influence the response rate in
33 the 86 CN-AML patients from the validation cohort irrespective of age. In accord with *in vivo*
34 findings obtained by microarray,²⁴ TET2E2S was found to be associated with a significantly
35 reduced CIR in intensively treated patients from the discovery cohort. This favorable effect

1 was strongest in younger patients and CN-AML patients, and could be confirmed using the
2 same cutoff value in the validation cohort irrespective of the age of the patient. Overall,
3 TET2E2S was found to be independently associated with a favorable outcome in both cohorts.
4 In the discovery cohort, this favorable effect was independent of age and cytogenetics, while
5 it remained strongest in younger and CN-AML patients. In the validation cohort, the prognostic
6 impact of TET2E2S was found to be independent of age, *FLT3*-ITD, and *NPM1* mutation
7 status, and TETE2S remained a favorable prognostic factor for DFS, OS, and EFS in both the
8 ELN favorable and intermediate-1 groups (Supplementary Figure 1). Together, these results
9 suggest that the favorable prognostic impact of TET2E2S on predicting against relapse.
10 Accordingly, at 5 years 78% \pm 6.5% of TET2E2S^{high}/CN-AML patients from the validation
11 cohort were predicted to be alive and in CR compared with only 7.5% \pm 6.6% of
12 TET2E2S^{low}/CN-AML patients ($p < 10^{-4}$, Figure 1). Because this present report is based on a
13 retrospective analysis, further studies will be necessary to confirm these findings.

14 To date, the prognostic impact of TET2 in hematological malignancies has been assessed at
15 the genomic level. In MDS, *TET2* mutations have been associated with a higher rate of
16 response to hypomethylating agents.^{18, 19} In AML patients who receive intensive therapy,
17 Metzeler *et al* identified *TET2* mutations as a negative prognostic factor restricted to patients
18 with CN-AML and favorable genetic aberrations (*NPM1* mutated/*FLT3* no ITD, or *CEBPA*
19 mutated).²⁰ This finding was not confirmed in other studies.^{17, 22, 23} By contrast, Chou *et al*
20 found that *TET2* mutations were a negative prognostic factor for patients with an intermediate
21 cytogenetic risk.³⁴ However, the presence of *TET2* mutations lost its independent significance
22 if additional genetic aberrations were also considered.³⁵ To the best of our knowledge, these
23 present findings are the first to link aberrant *TET2* mRNA splicing with clinical outcomes in
24 intensively treated CN-AML patients. As detailed in the Methods section, we measured *TET2*
25 exon 2 skipping using qES-PCR by calculating the ratio E1E3/E2E3, while overall *TET2*
26 expression was measured based on the qRT-PCR amplification of a sequence from exon 10,
27 which is not known to be alternatively spliced (<https://fasterdb.lyon.unicancer.fr/>). Using
28 these two methods to analyze patient samples, our results indicate that in contrast to
29 TET2E2S, fluctuations in the overall mRNA transcript expression levels of TET2 did not
30 correlate with patient responses to IC, relapse risk, CIR, NRM, DFS, EFS, or OS. This finding
31 demonstrates that aberrant *TET2* splicing, but not transcription influences the outcome of
32 intensively treated AML patients. Accordingly, these present findings suggest that, at least in
33 the case of *TET2*, measurements of AEU might be more appropriate than measurements of
34 overall gene expression for assessments of disease severity.

1 In conclusion TET2E2S, but not overall *TET2* gene expression levels, was found to be
2 independently associated with a favorable DFS, EFS, and OS in intensively treated CN-AML
3 patients, but not in AML patients who were unsuitable for IC. This finding highlights the need
4 for assessing AEU rather than overall gene expression levels in prognostic assessments. As
5 they indicate that the level of TET2E2S is significantly associated with survival for currently
6 accepted cytogenetic and molecular prognostic categories, our findings suggest that assessing
7 levels of TET2E2S will permit to better assessments of disease risk, thereby improving patient
8 management and outcomes. Accordingly, our study suggests that TET2 exon 2 skipping
9 should be considered in the diagnostic workup of AML patients.

10

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10

1 **Conflict of Interest**

2

3 The authors have no conflicts of interest to declare.

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7 **Supplementary information is available at Leukemia's website**

8

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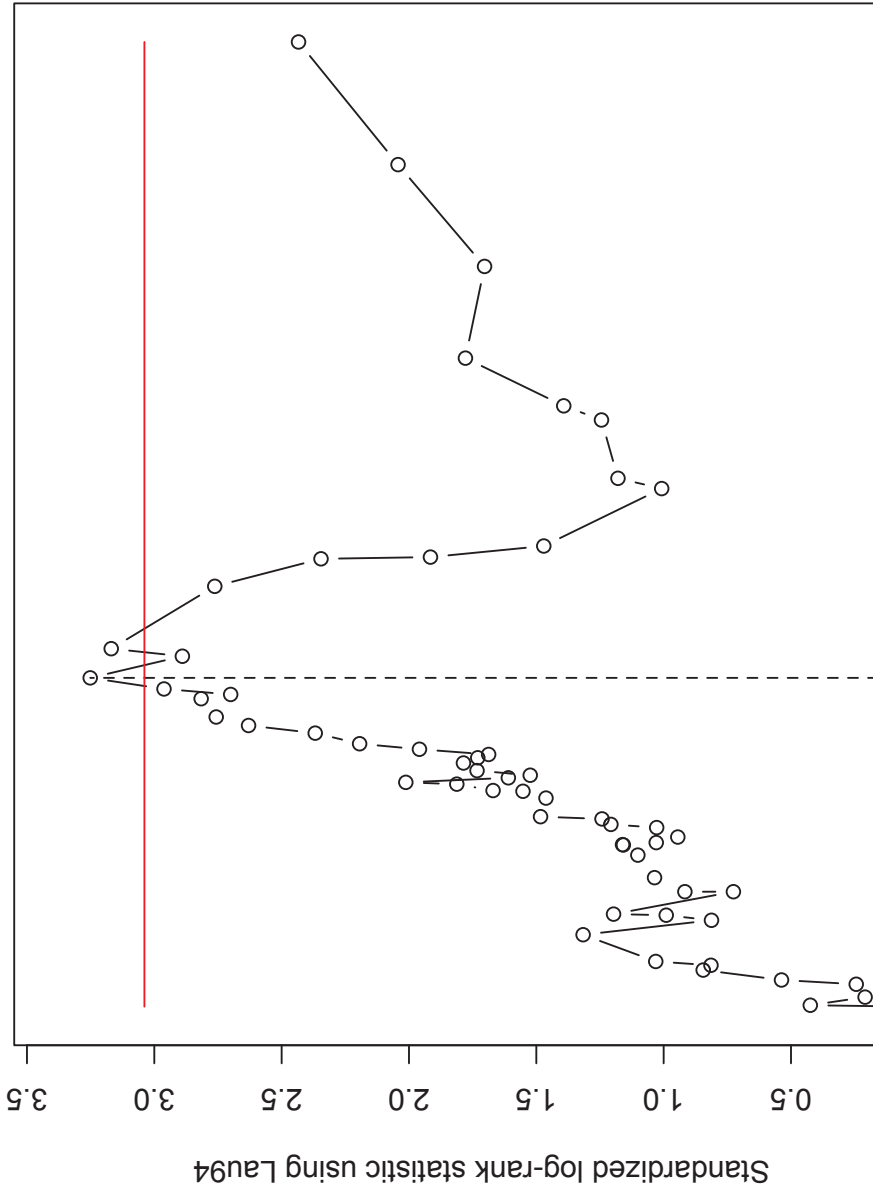
1 **Figure Legends**

2

3 **Figure 1.** Effects of TET2 exon 2 skipping on disease-free survival in intensively-treated
4 patients in the discovery cohort. A. Absolute standardized log-rank statistics and significance
5 based on improved Bonferroni inequality. B. Cumulative incidence of relapse and non-relapse
6 mortality. C. Disease-free survival.

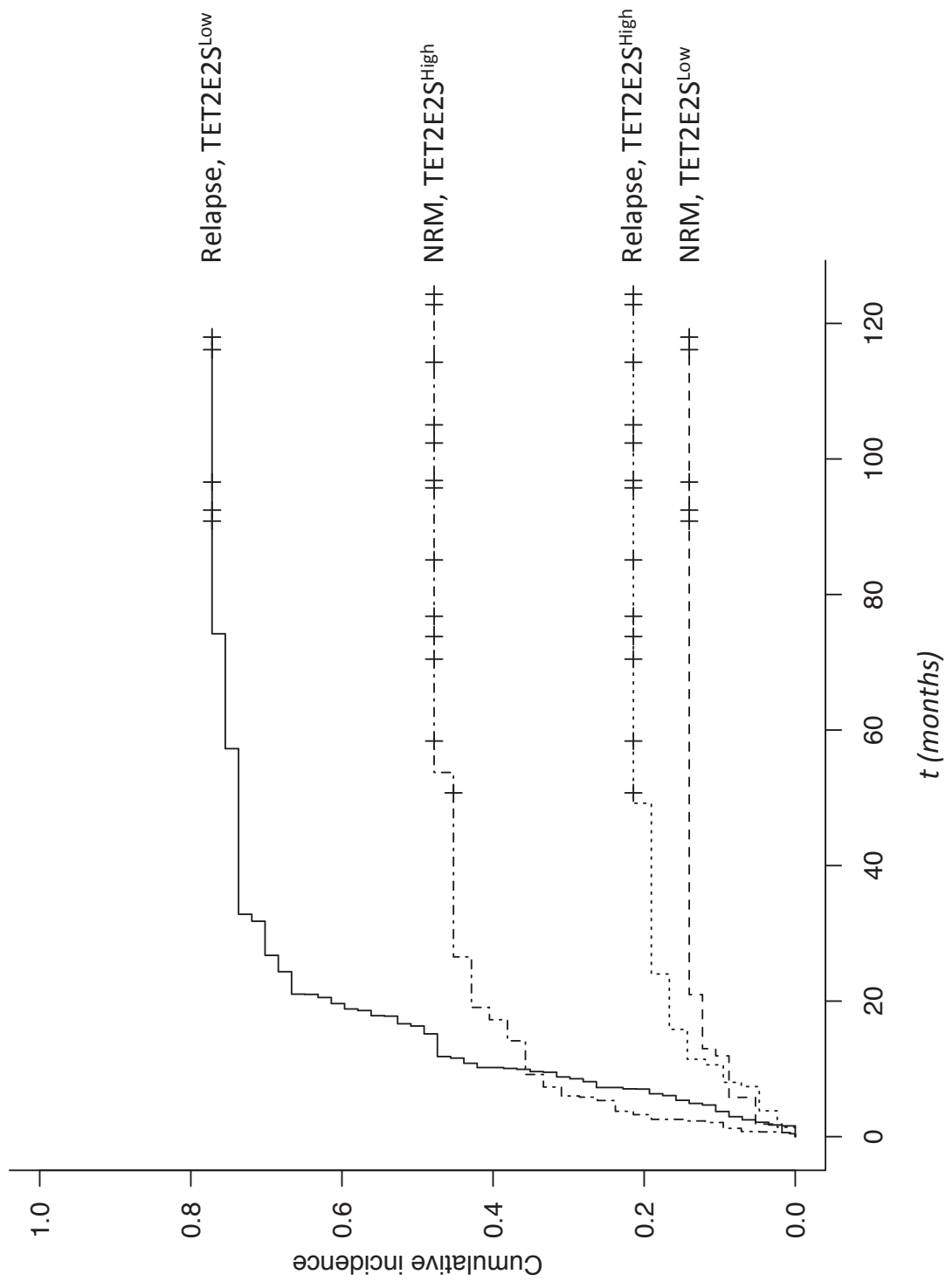
7 **Figure 2.** Effects of TET2 exon 2 skipping on the cumulative incidence of relapse, non-relapse
8 mortality (A) and overall survival (B) in 86 CN-AML patients from the validation cohort.

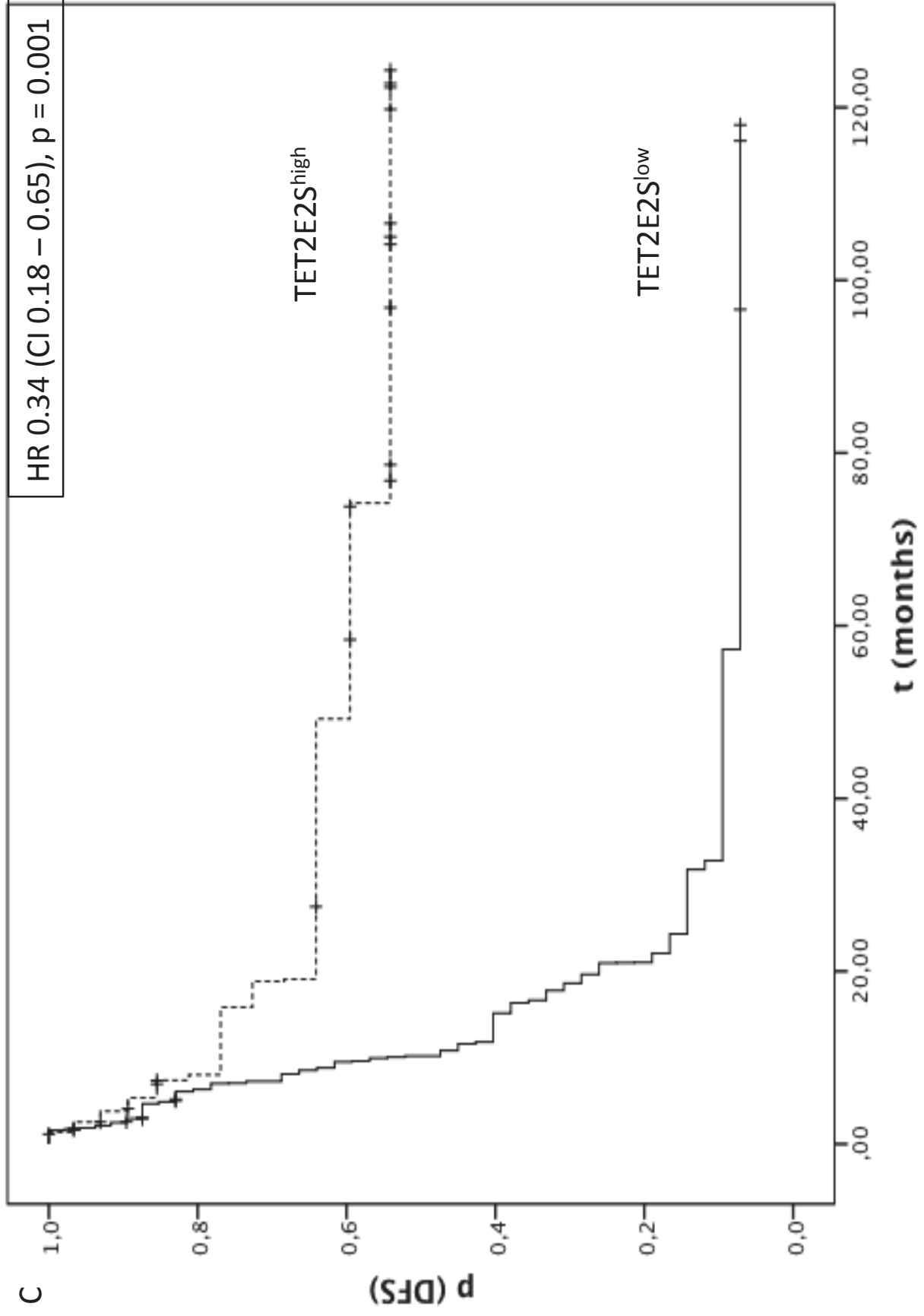
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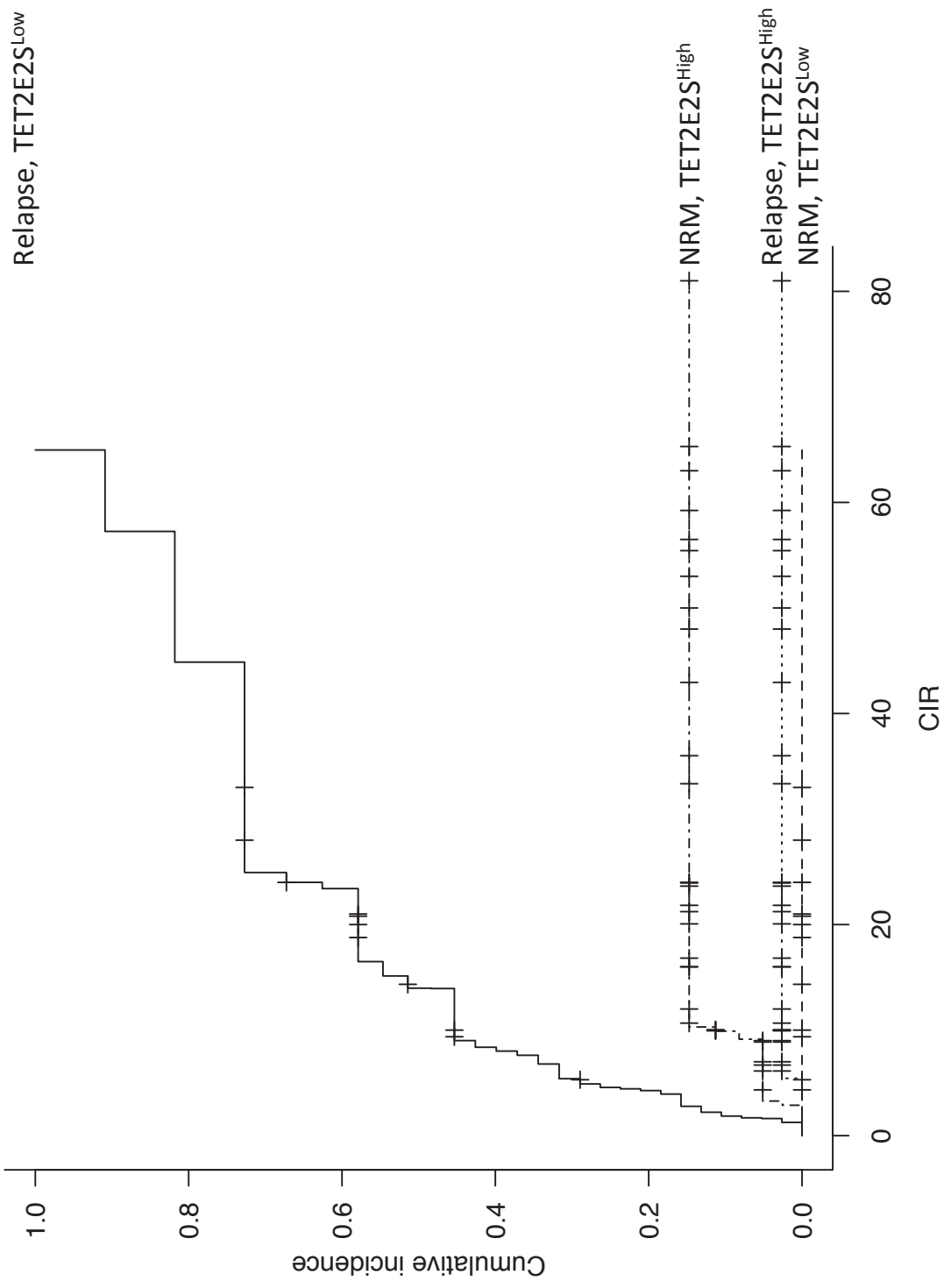
A

B

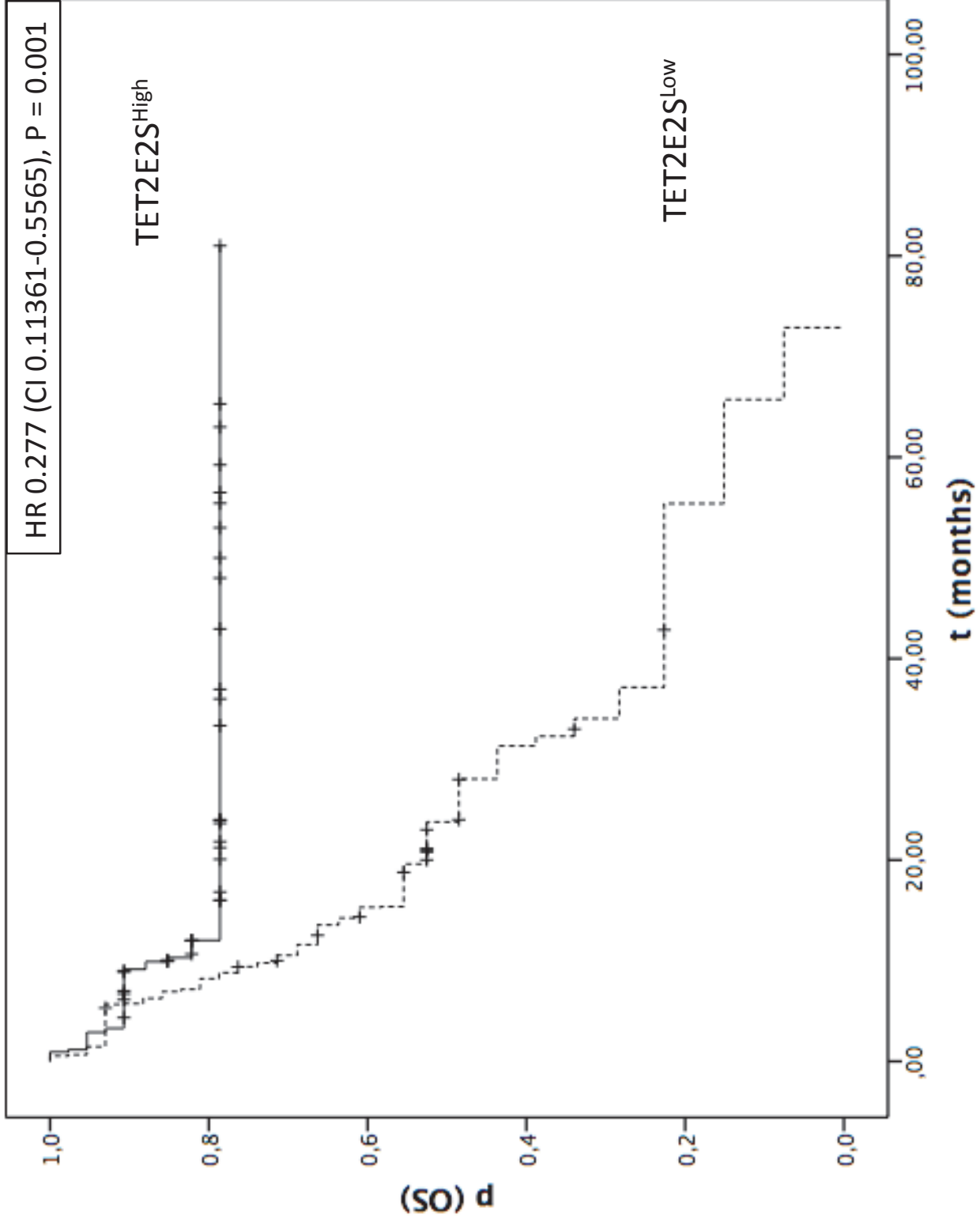




A



B



TET2 exon 2 skipping is an independent favorable prognostic factor for cytotogenetically normal acute myelogenous leukemia (AML)

TET2 exon 2 skipping in AML

Supplementary Figures

Supplementary Figure legends

Supplementary Figure 1. Effect of TET2 exon 2 skipping on CIR, NRM (A), and DFS (B) in the 49 CN-AML patients from the discovery cohort.

Supplementary Figure 2. Effect of TET2 exon 2 skipping on CIR, NRM (A), and DFS (B) in the 50 patients with abnormal karyotype from the discovery cohort.

Supplementary Figure 3. Effect of TET2 exon 2 skipping on CIR, NRM (A), DFS (B), EFS, and OS in younger patients from the discovery cohort.

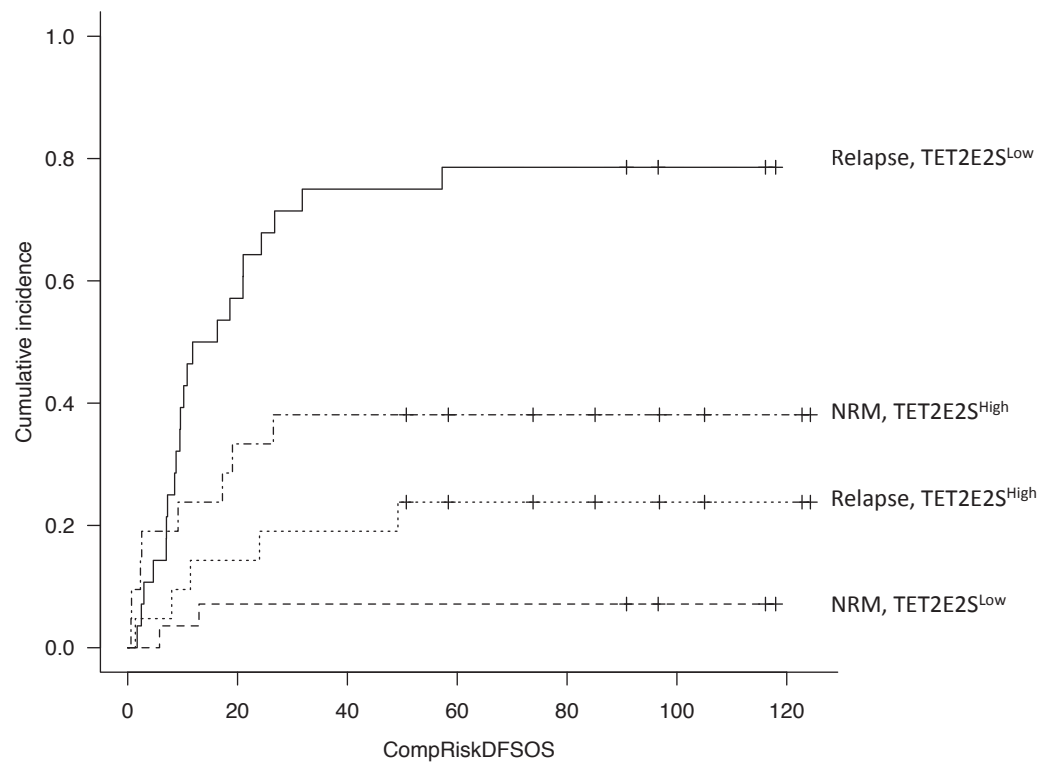
Supplementary Figure 4. Effect of TET2 exon 2 skipping on CIR and NRM in older patients from the discovery cohort.

Supplementary Figure 5. Effect of TET2 exon 2 skipping on DFS (A) and EFS (B) in the 86 patients from the validation cohort.

Supplementary Figure 6. Effect of TET2 exon 2 skipping on DFS, EFS, and OS in patients from the validation cohort belonging in Favorable (A, B, C) and intermediate-1 (D, E, F) ELN genetic categories.

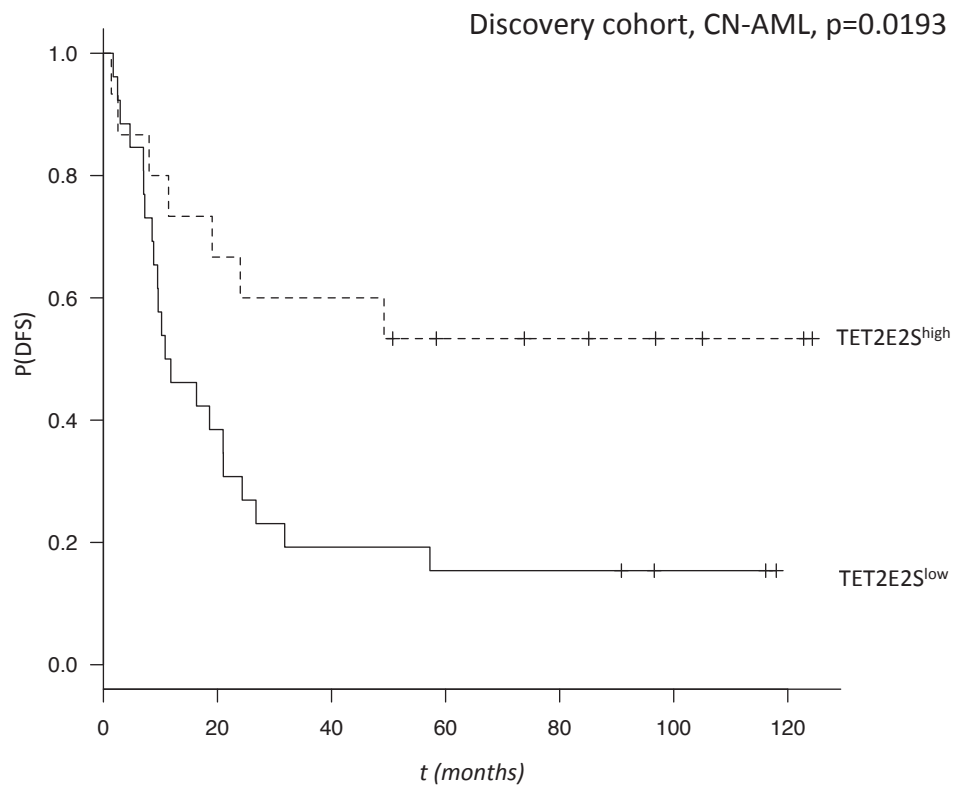
A

Discovery cohort, CN-AML: CIR, $p=0.0003$; NRM, $p=0.009$



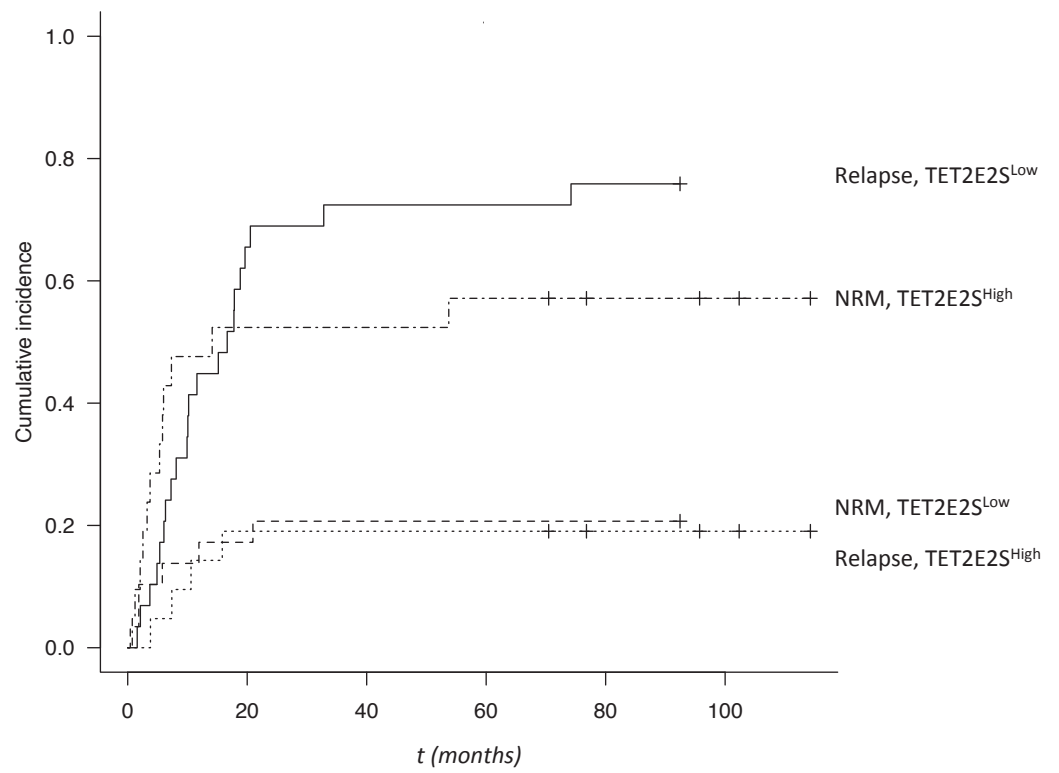
Mint Mohamed *et al.*, Supplementary Figure 1

B



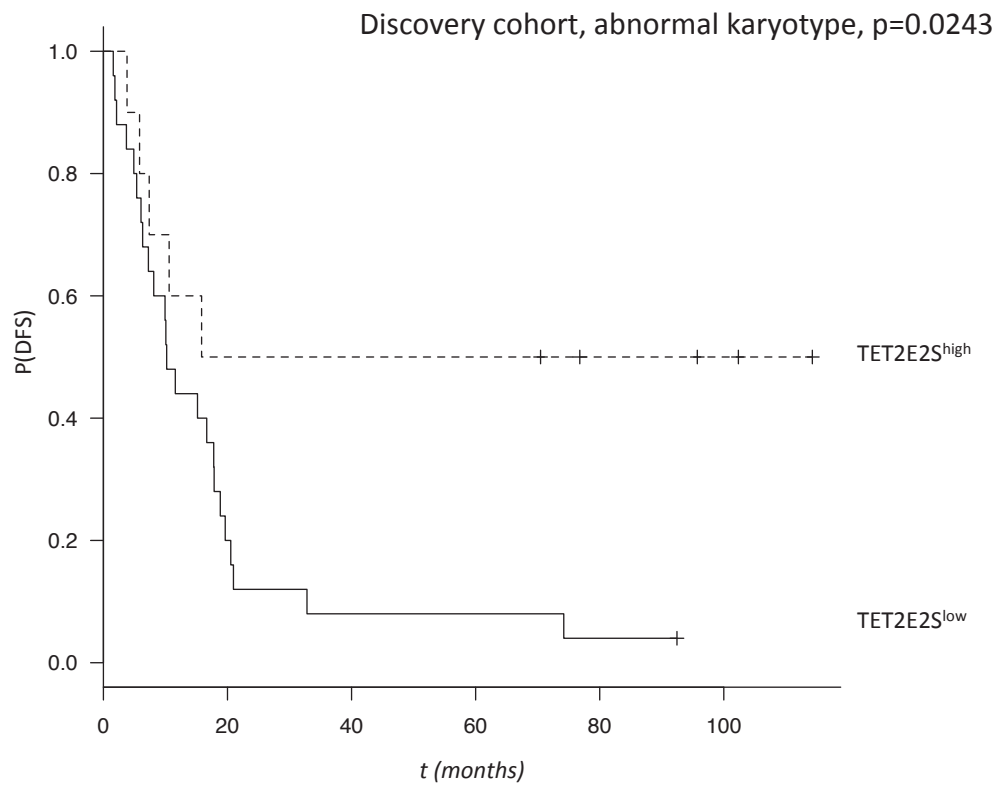
Mint Mohamed *et al.*, Supplementary Figure 1

A Discovery cohort, abnormal karyotype; CIR, $p=0.0005$, NRM, $P=0.011$



Mint Mohamed *et al.*, Supplementary Figure 2

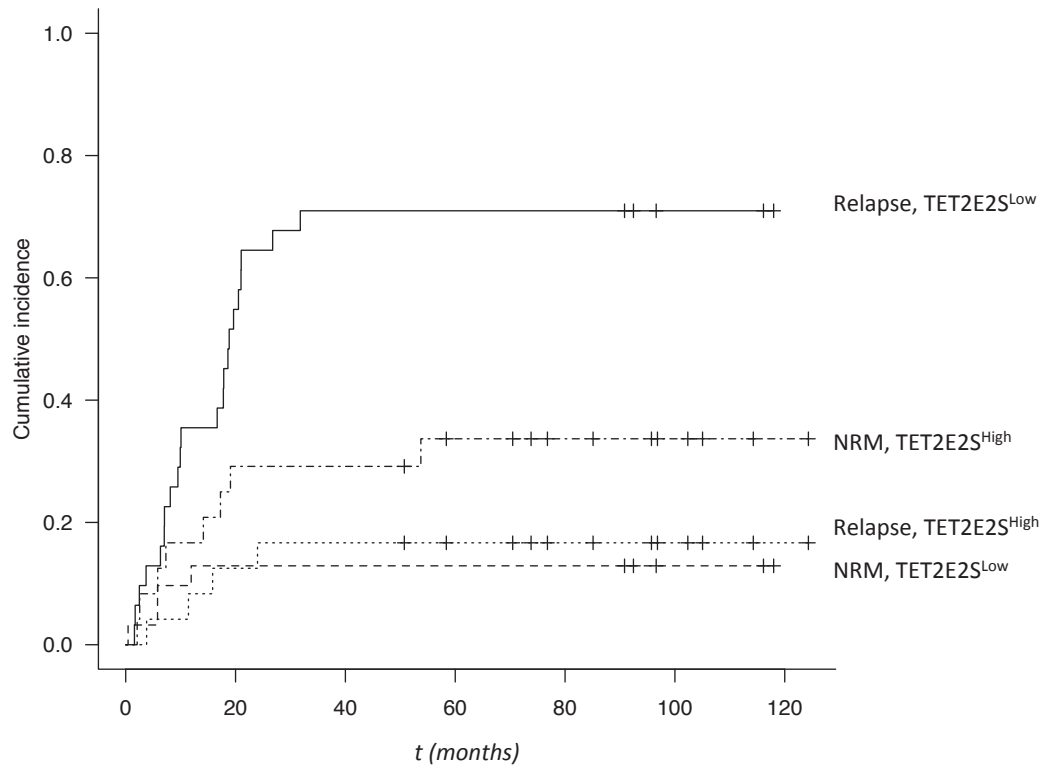
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Mint Mohamed *et al.*, Supplementary Figure 2

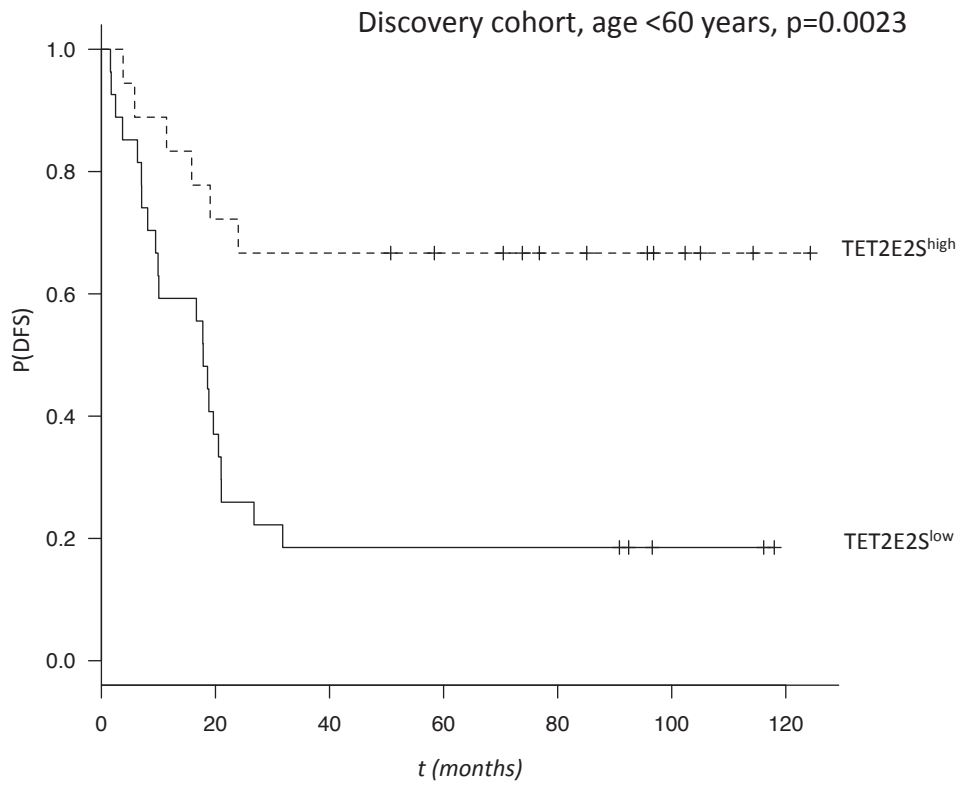
A

Discovery cohort, age <60 years; CIR, $p=0.0001$, NRM, $p=0.087$



Mint Mohamed *et al.*, Supplementary Figure 3

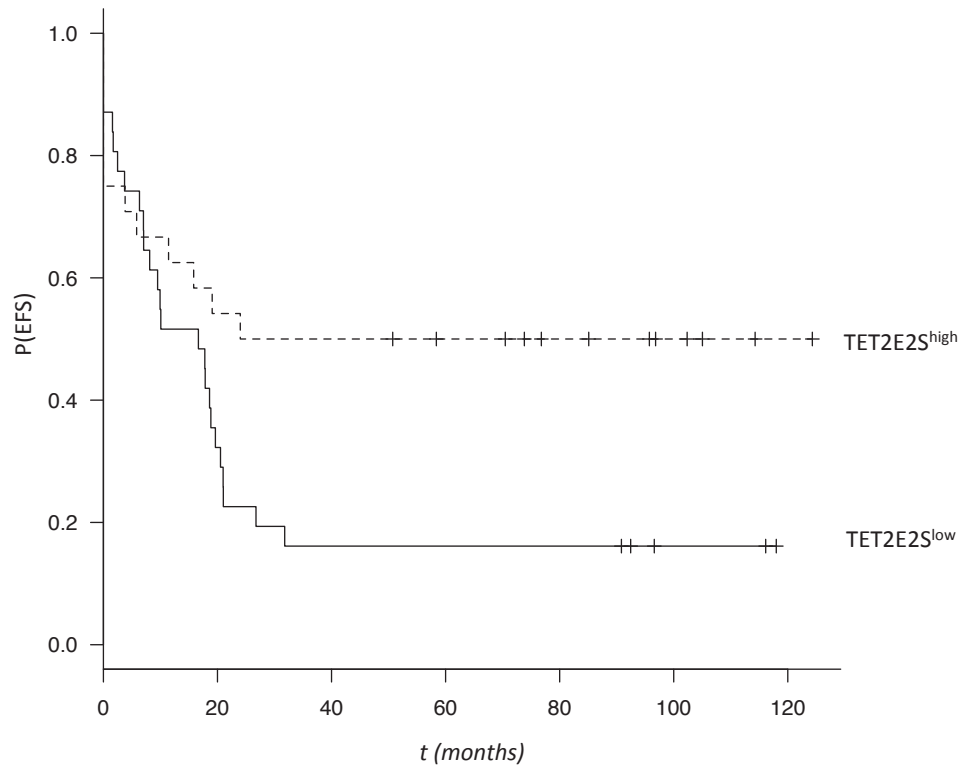
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Mint Mohamed *et al.*, Supplementary Figure 3

C

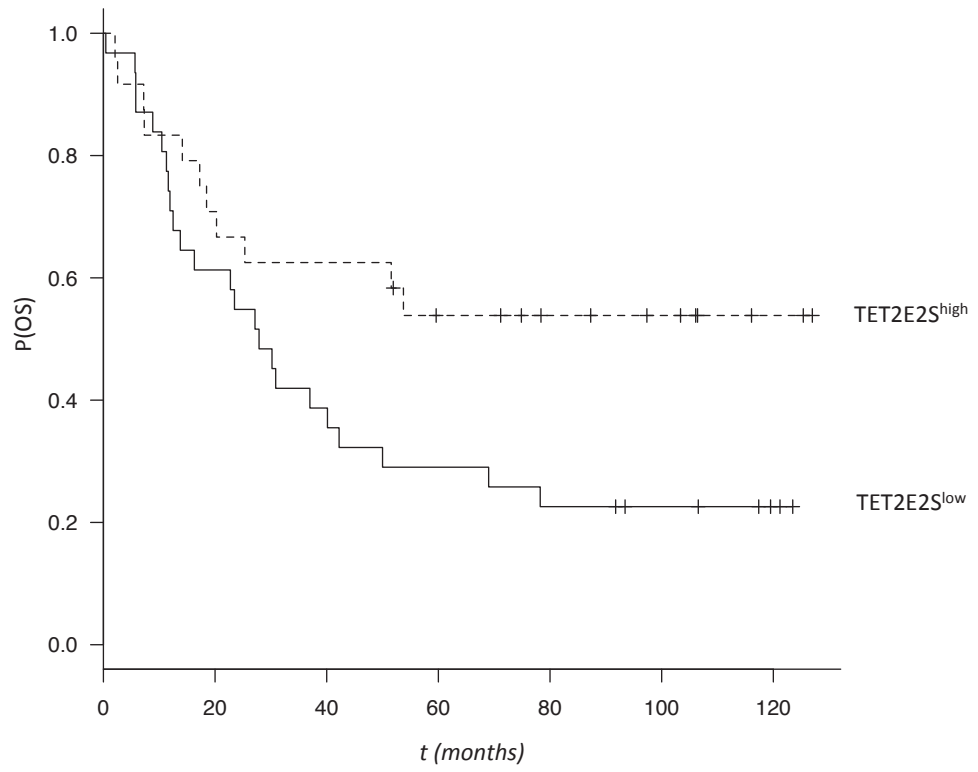
Discovery cohort, age <60 years, $p=0.0034$



Mint Mohamed *et al.*, Supplementary Figure 3

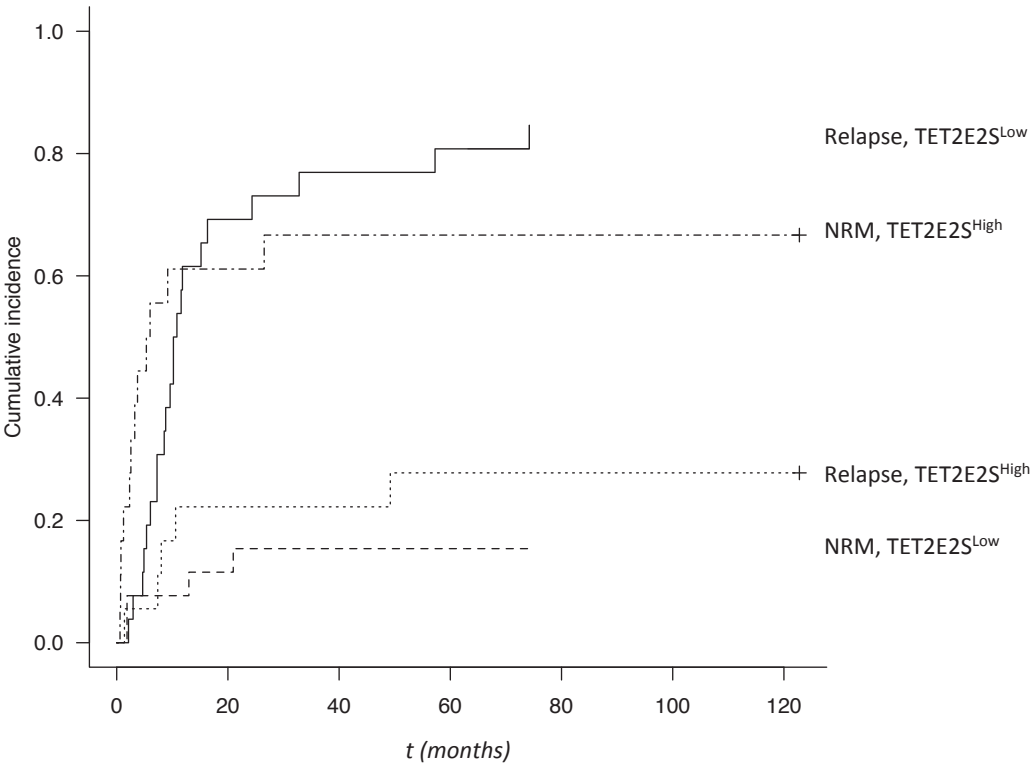
D

Discovery cohort, age <60 years, $p=0.0039$



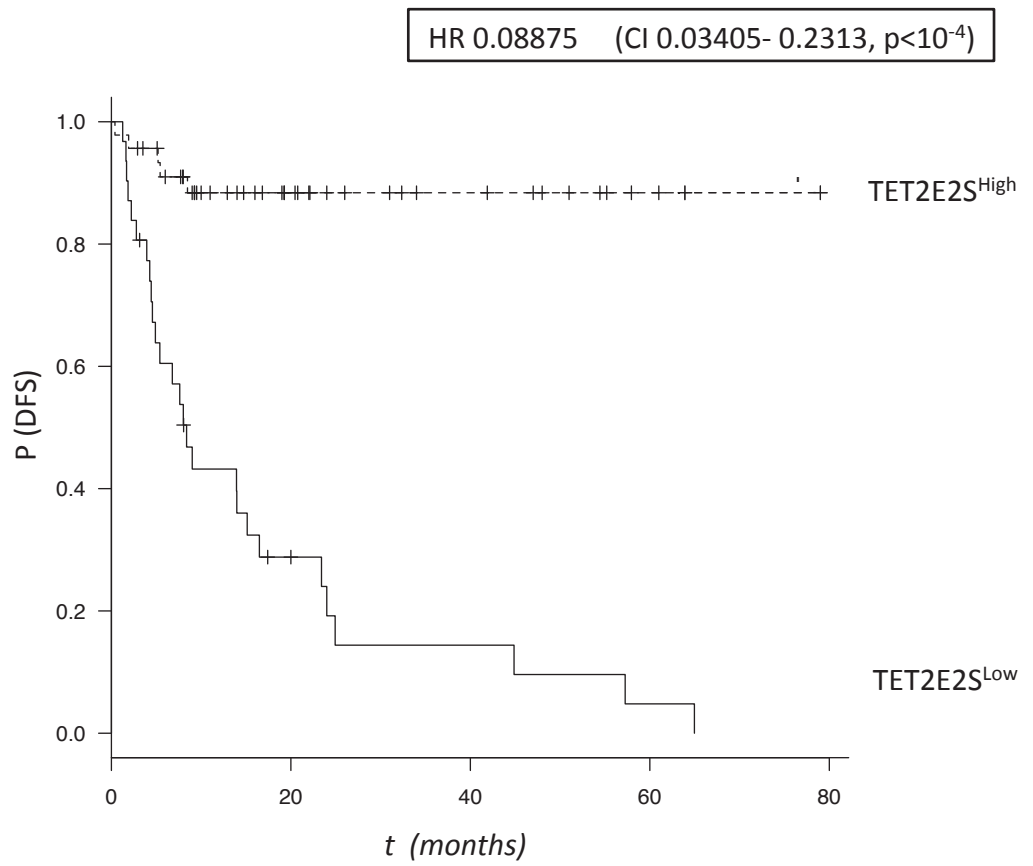
Mint Mohamed *et al.*, Supplementary Figure 3

Discovery cohort, age >60 years; CIR, p= 0.0061, NRM, p=0.0004



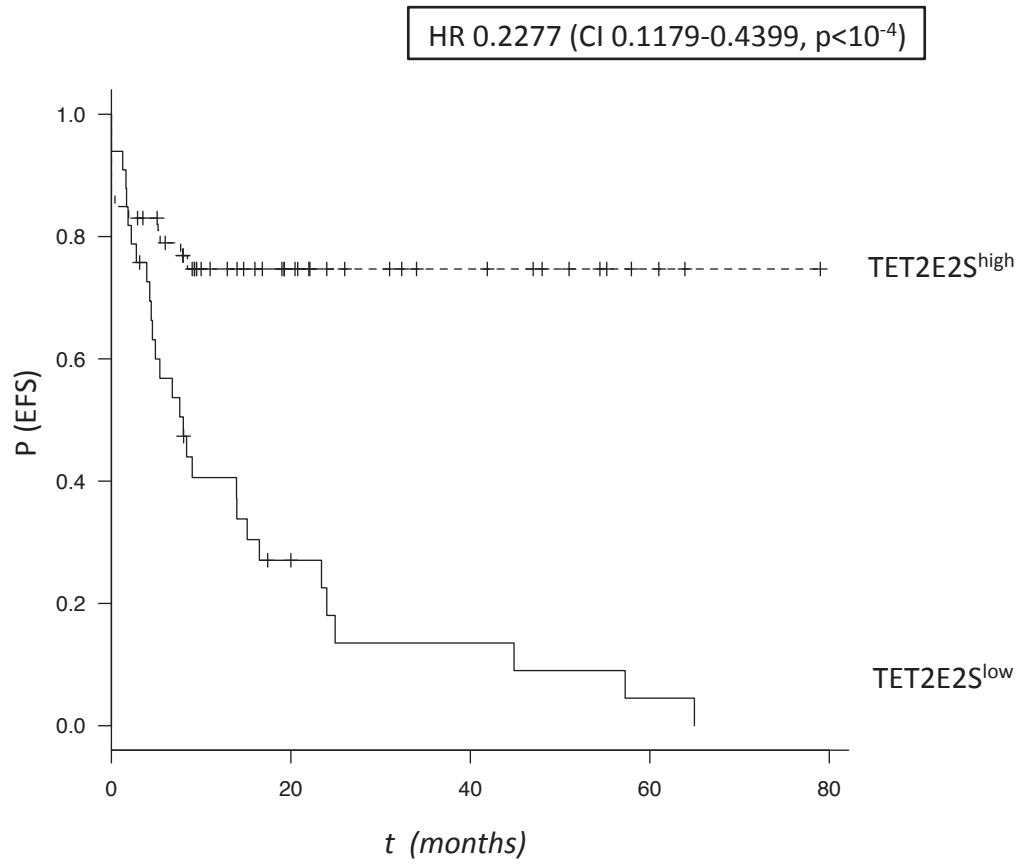
Mint Mohamed *et al.*, Supplementary Figure 4

A



Mint Mohamed *et al.*, Supplementary Figure 5

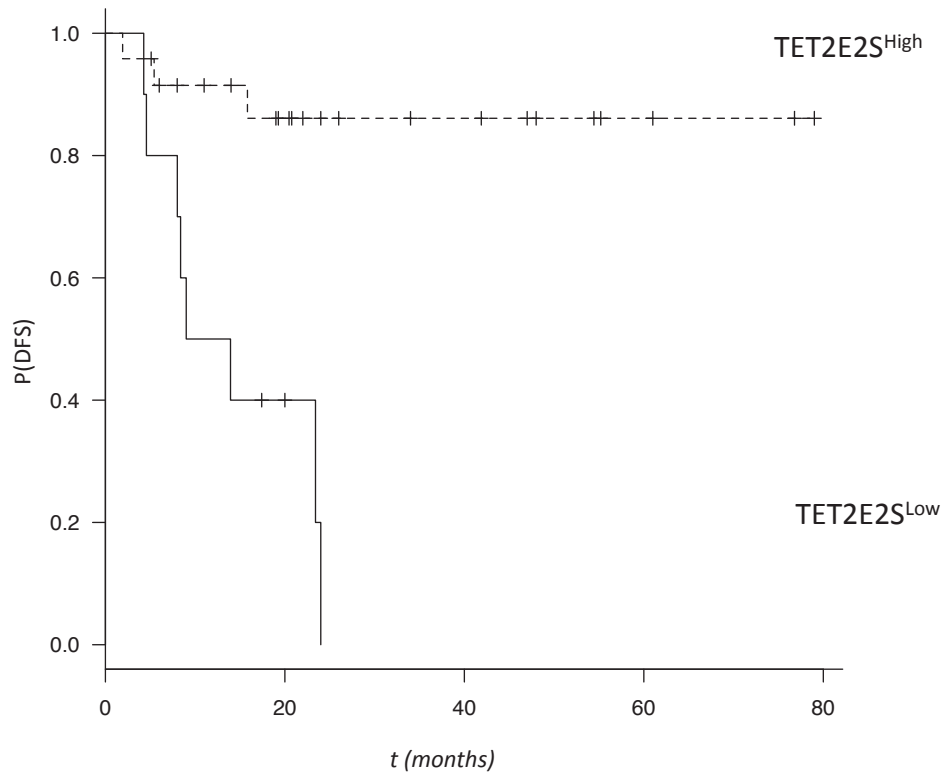
B



Mint Mohamed *et al.*, Supplementary Figure 5

A

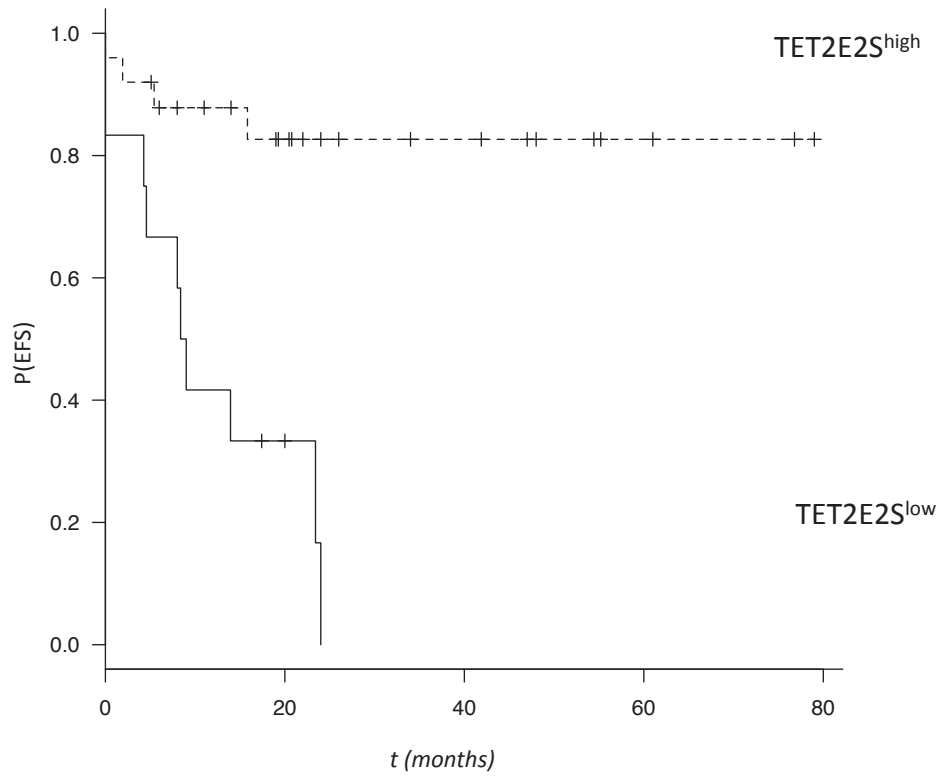
ELN Favorable; HR 0.1056 (CI 0.02711-0.4117), p= 0.0012



Mint Mohamed *et al.*, Supplementary Figure 6

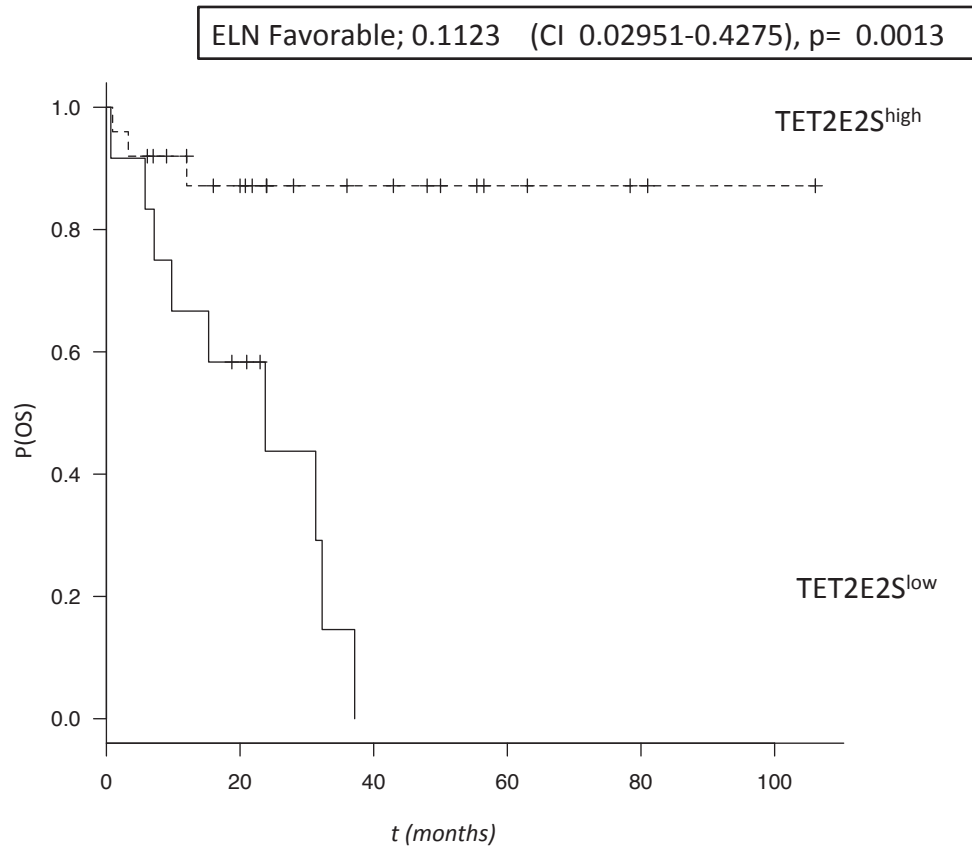
B

ELN Favorable; HR 0.1259 (CI 0.03834-0.4132), p= 0.00063



Mint Mohamed *et al.*, Supplementary Figure 6

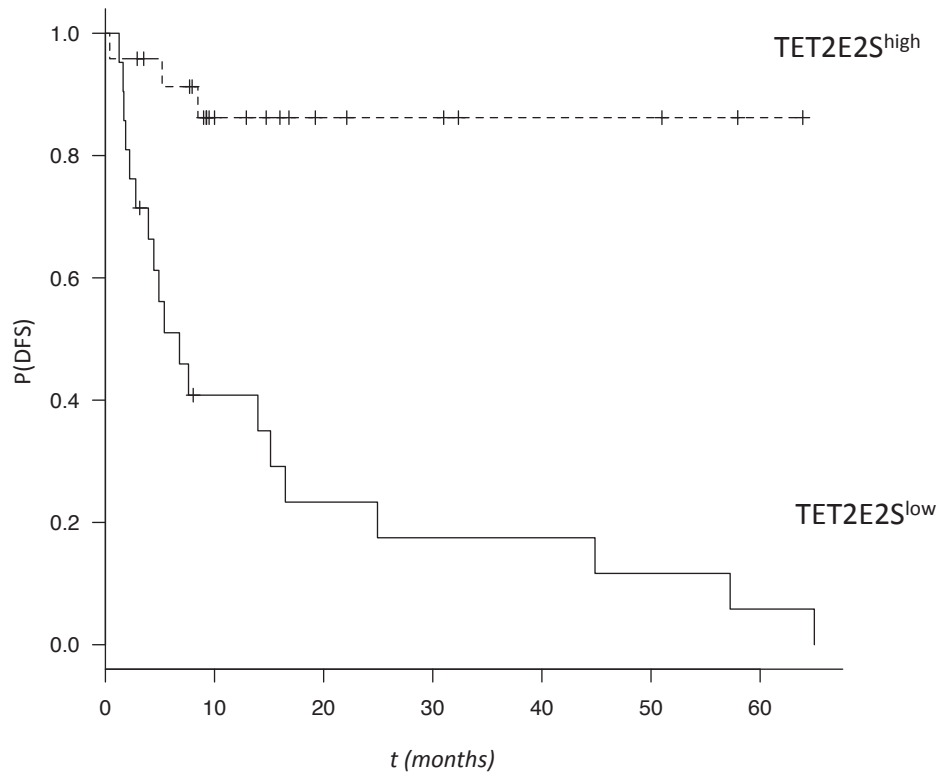
C



Mint Mohamed *et al.*, Supplementary Figure 6

D

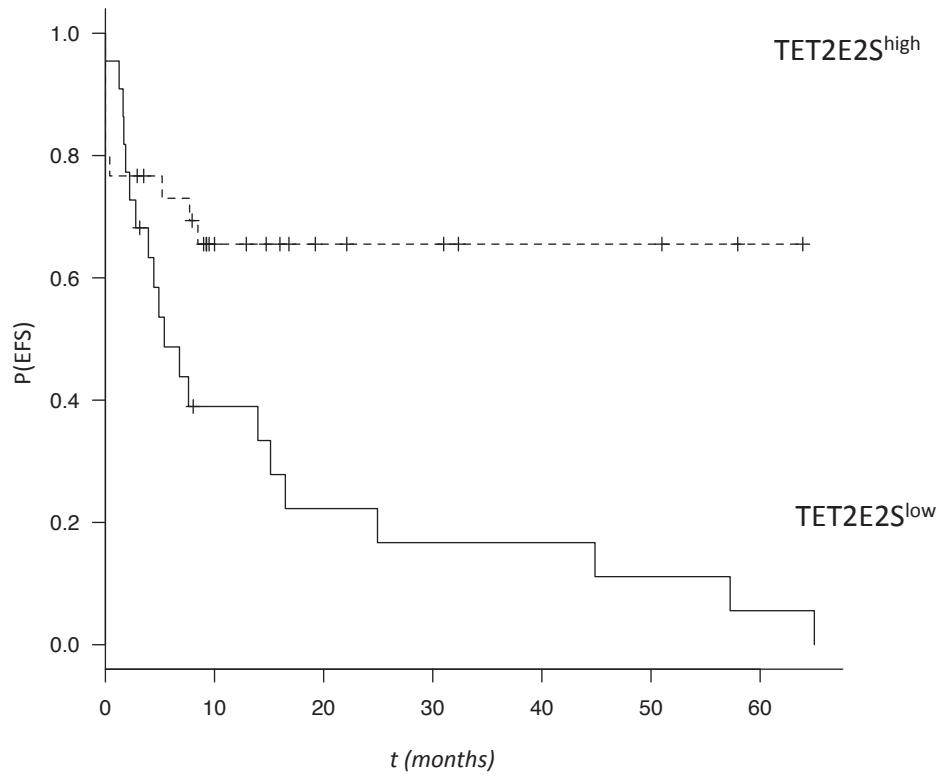
ELN Intermediate-1; HR 0.111 (CI 0.03255- 0.3789), p= 0.0004



Mint Mohamed *et al.*, Supplementary Figure 6

E

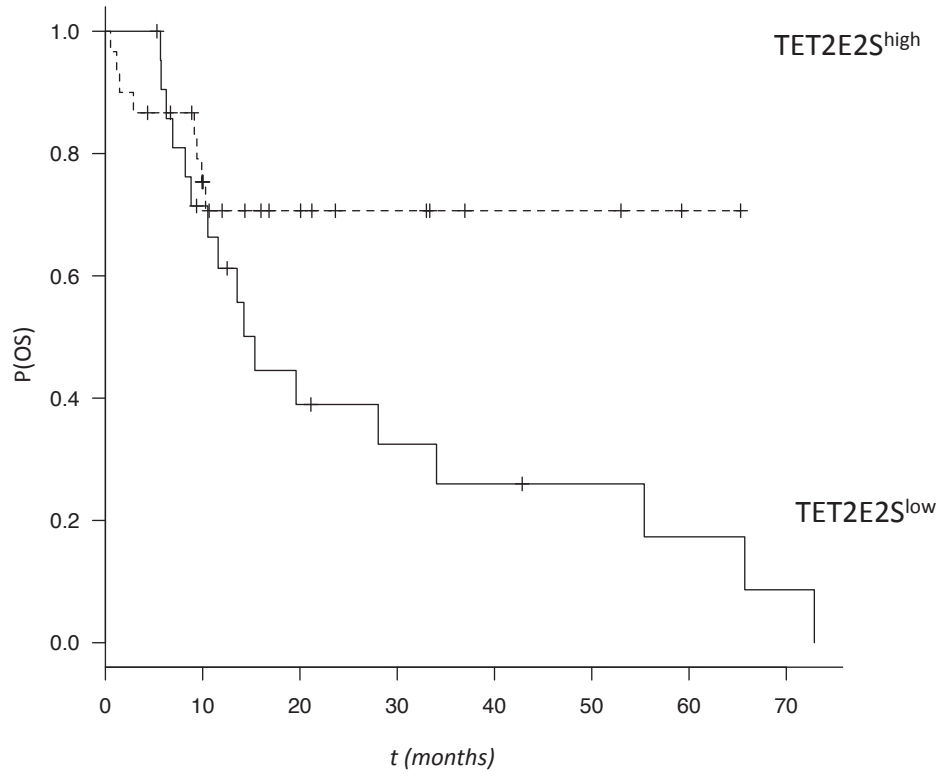
ELN Intermediate-1; HR 0.3556 (CI 0.1645- 0.7686), p= 0.0086



Mint Mohamed *et al.*, Supplementary Figure 6

F

ELN Intermediate-1; HR 0.4403 (CI 0.186 - 1.042), p= 0.0620



Mint Mohamed *et al.*, Supplementary Figure 6

II-2.3. Conclusion, perspectives

Ce travail, basé sur l'analyse bioclinique de trois séries informatives de patients traités pour LAM, montre que l'exclusion de l'exon 2 de TET2, mais pas l'expression globale du gène, est indépendamment associée à une évolution favorable de la maladie chez les patients traités par CTI. Comme le suggéraient les résultats de notre précédente étude, ces données confirment l'intérêt d'étudier les modifications d'épissage. Le fait que cette analyse permette ici d'améliorer la précision pronostique de la classification ELN, ce travail suggère que l'analyse de l'expression de l'exon 2 de TET2 pourrait offrir un nouveau biomarqueur à même de préciser le pronostic et ainsi de guider le traitement des LAM chez l'adulte jeune. Nous projetons d'appliquer cette analyse aux patients adultes inclus dans le protocole ALFA-0701 de même que chez les enfants inclus dans le protocole ELAM 02. Au plan mécanistique, il sera intéressant d'évaluer le rôle de l'exon 2 de TET2 dans les propriétés catalytiques de la protéine. Cet exon n'est pas exprimé au niveau protéique et pourrait avoir un rôle régulateur sur l'expression du gène.

II-3. Anomalies d'épissage du messenger du transporteur ABCA3 dans les leucémies aiguës myéloïdes traitées par chimiothérapie intensive

II-3.1. Introduction

Comme nous l'avons détaillé dans l'introduction (page 18), les transporteurs ABC jouent un rôle important dans la résistance des LAM aux chimiothérapies. Dans une précédente étude², nous avons identifié des anomalies d'épissages concernant 13 transporteurs ABC. Ces anomalies concernaient des cellules résistantes à l'AraC, la daunorubicine ou l'azacitidine de même que des cellules exprimant WT1 ou DEK ou provenant de patients ayant eu des réponses variable à la chimiothérapie intensive². Parmi ces modifications d'épissage, une exclusion de l'exon 19 d'ABCA3 était retrouvée dans des cellules myéloïdes infectées par un lentivirus inhibant WT1 de même que dans des cellules K562 résistantes à l'AraC. *In vivo* par qESPCR, l'expression de l'exon 19 d'ABCA3 était retrouvée corrélée à l'expression de WT1 chez 152 malades mais pas chez 38 donneurs de moelle. Nous-nous sommes donc intéressé à l'impact de l'exclusion de l'exon 19 d'ABCA3 sur la résistance des cellules de LAM aux chimiothérapies *in vitro* et *in vivo*.

II-3.2. Résultats

II-3.2.1. In silico, la délétion de l'exon 19 d'ABCA3 favoriserait l'hydrolyse de l'ATP

La Figure 8 montre que par modélisation, la délétion de l'exon 19 d'ABCA3 concerne une région riche en acides aminés chargés positivement et située entre les deux NBD. Cette modification est compatible avec une activité catalytique augmentée de la protéine.

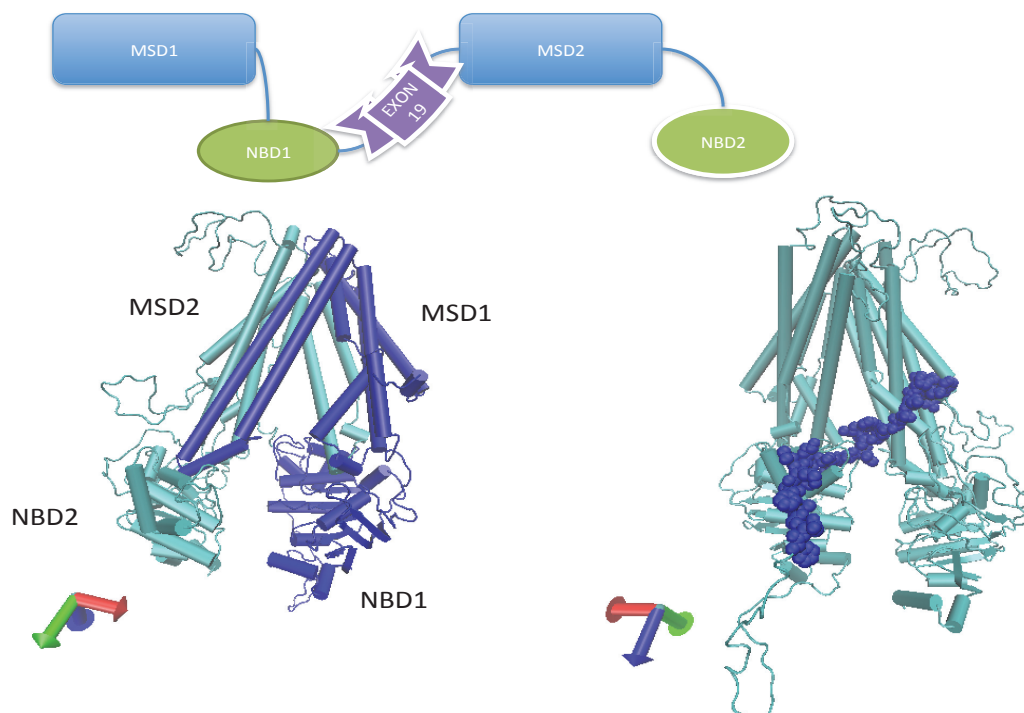


Figure 8. Modélisation d'ABCA3. La modélisation a été effectuée après alignement de l'ABCA3 humain avec l'ABCB1 murin. L'exon 19 code une région riche en acides aminés positivement chargés située entre NBD1 et MSD2. Cette modification prédit une activité catalytique accrue de la protéine. NBD, *Nucleotide Binding Domain*, MSD *membrane spanning domains*.

II-3.2.2. Les cellules leucémiques sous-exprimant l'exon 19 d'ABCA3 résistent à la daunorubicine et au VP16.

Nous avons comparé l'effet de l'expression de l'exon 19 d'ABCA3 sur la sensibilité des cellules des lignées KASUMI, MOLM13 et KG1. Pour ce faire nous avons infecté les cellules des trois lignées avec un shRNA anti-WT1 ou un lentivirus contrôle (PLKO). Par qESPCR nous avons précédemment montré que le shRNA anti-WT1 favorisait l'exclusion de l'exon 19 d'ABCA3². La figure 9 montre que pour les trois lignées, la répression de l'exon 19 d'ABCA3, associée à l'expression du shRNA anti-WT1, est associée à une résistance des cellules à la daunorubicine et au VP16.

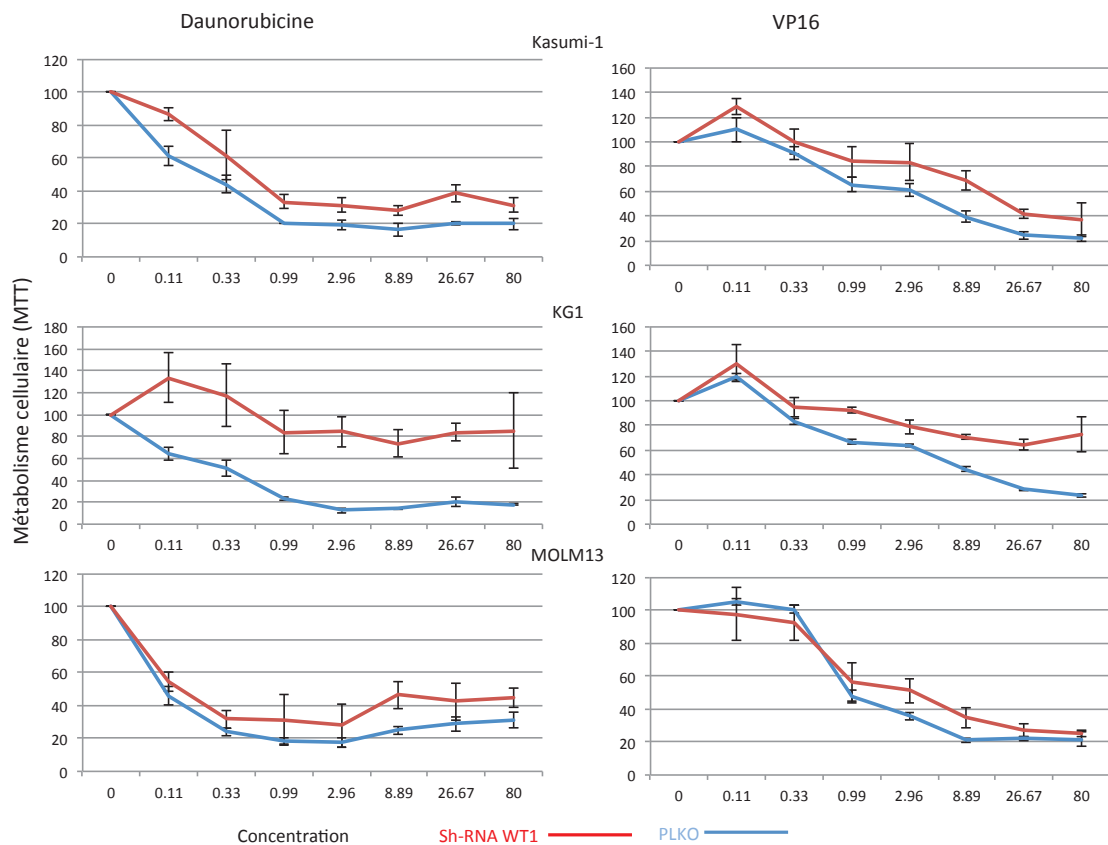


Figure 9. Sensibilité des cellules leucémiques à la daunorubicine et au VP16 en fonction de l'expression de wt&. Les cellules infectées par le shRNA-WT1 sont caractérisées par une exclusion de l'exon 19 d'ABCA3². Les résultats correspondent à trois expériences.

Afin de mieux spécifier le lien entre l'exclusion de l'exon 19 d'ABCA3 et la résistance aux drogues, l'expression globale des principaux transporteurs ABC impliqués dans le phénomène de résistance multidrogue [ABCA3, ABCC1 (MRP1), ABCG2 (BCRP) et ABCB1 (P-gp)] a été évaluée par blot de western dans les deux catégories de cellules et la Figure 10 montre que l'expression de WT1 ne modifie pas l'expression globale de ces quatre transporteurs. Ces premiers résultats suggéraient que l'exclusion de l'exon d'ABCA3, liée à l'expression du shRNA-WT1, pourrait favoriser la résistance à la daunorubicine et au VP16.

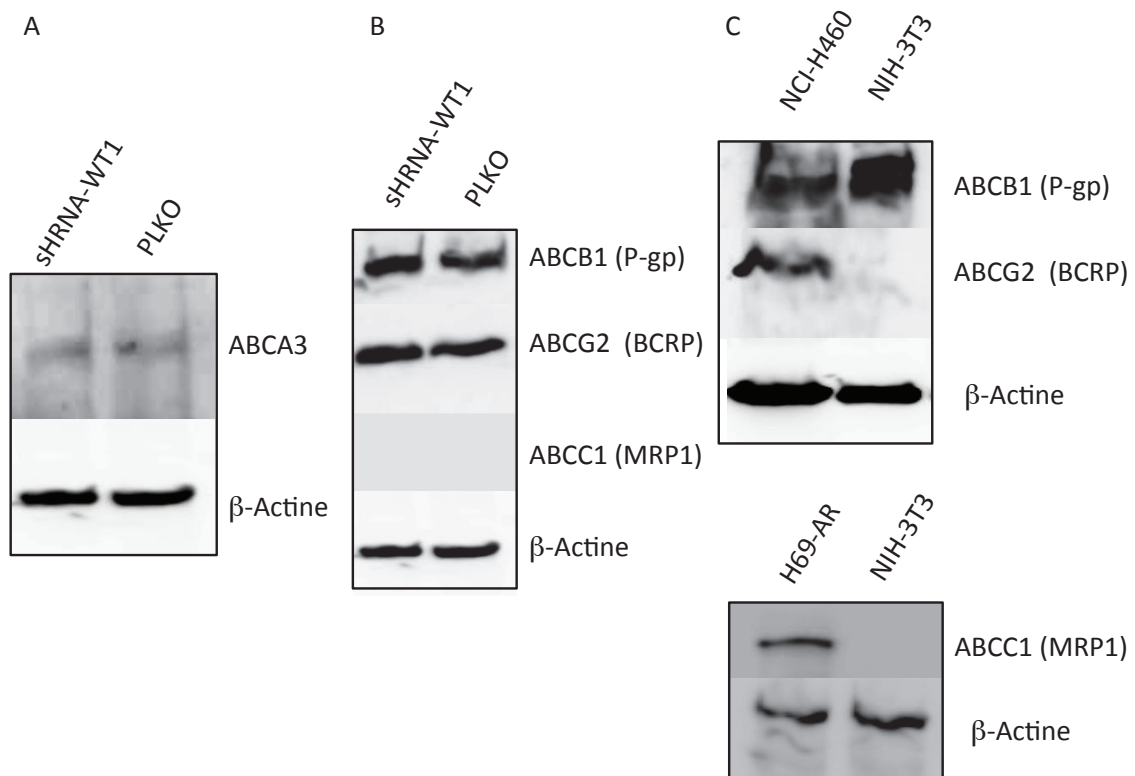


Figure 10. Expression des principaux transporteurs ABC dans les cellules leucémiques KG1 infectée par un lentivirus exprimant un shRNA-WT1. PLKO correspond au lentivirus contrôle. A expression d'ABCA3. B expression d'ABCB1 (P-gp), d'ABCG2 (BCRP) et d'ABCC1 (MRP1). En C sont représentés les contrôles cellulaires positifs et négatifs pour l'expression d'ABCB1, ABCG2 et ABCC1.

II-3.2.2. L'expression ectopique d'un vecteur ABCA3 dépourvu de l'exon 19 renforce la résistance cellulaire à la daunorubicine et à l'AraC.

Afin de relier spécifiquement l'expression de l'exon 19 d'ABCA3 à la résistance aux chimiothérapies, nous avons construit deux vecteurs d'expression plasmidiques d'ABCA3 correspondant à la forme sauvage (Figure 11) ainsi qu'à un mutant dépourvu de l'exon 19.

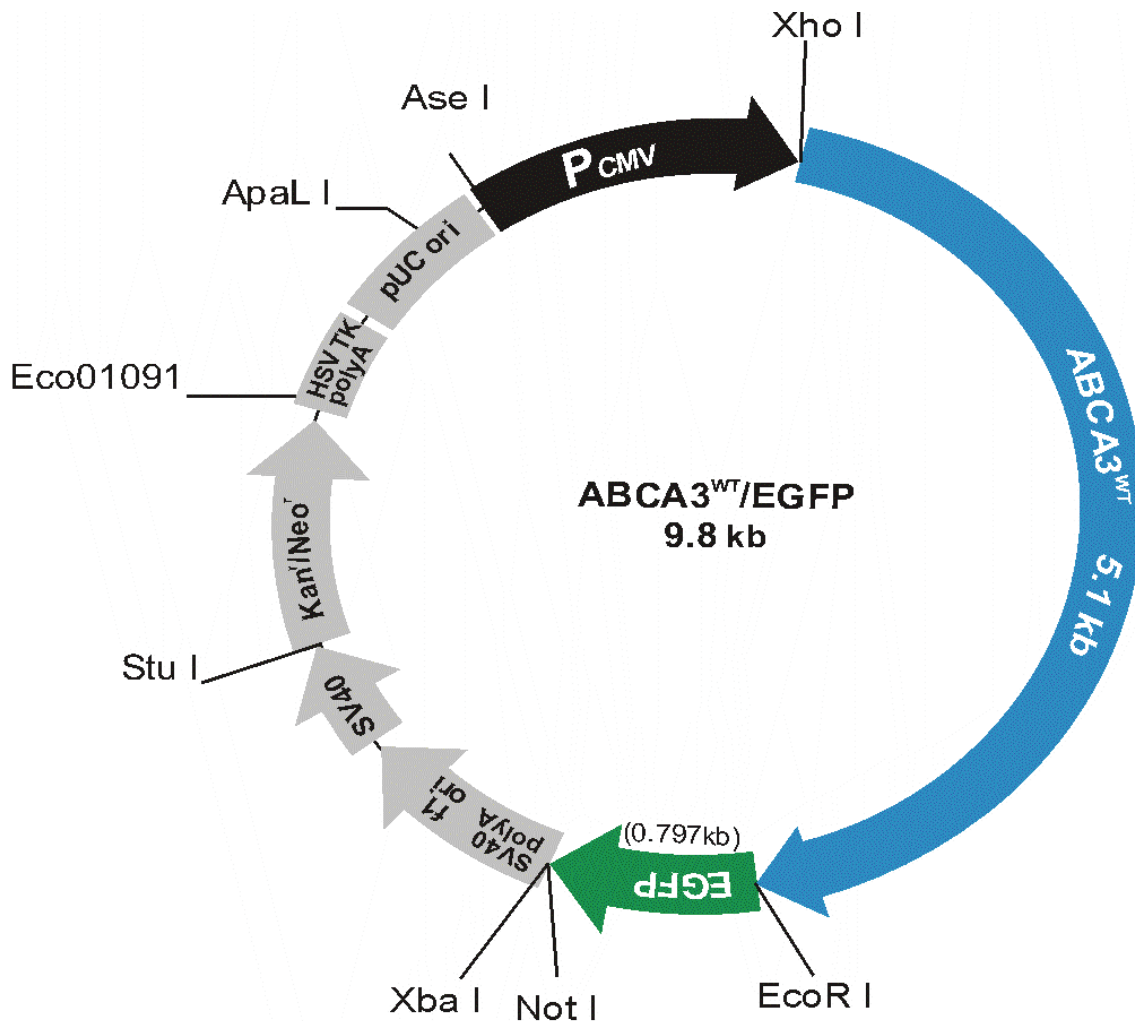


Figure 11. Vecteur d'expression ABCA3 (Accession NCB1 #NM_001089). Le vecteur initial est un don de Michael F Beers (Surfactant Biology Laboratories, Translational Research Center – Philadelphia)³⁵. La mutagenèse a été effectuée par la firme Genwizz avec contrôle par séquençage.

Après transfection de cellules HEK-293T à la lipofectamine, l'efficacité de transfection était estimée à 40-80% par le comptage des cellules exprimant la GFP. Après incubation pendant heure avec différentes concentrations de daunorubicine, la viabilité cellulaire était mesurée au MTT. La Figure 12 montre que la délétion de l'exon 19 d'ABCA3 augmente la résistance cellulaire à la daunorubicine, par rapport au résultat obtenu avec le vecteur ABCA3 sauvage. Les expériences avec l'AraC sont en cours.

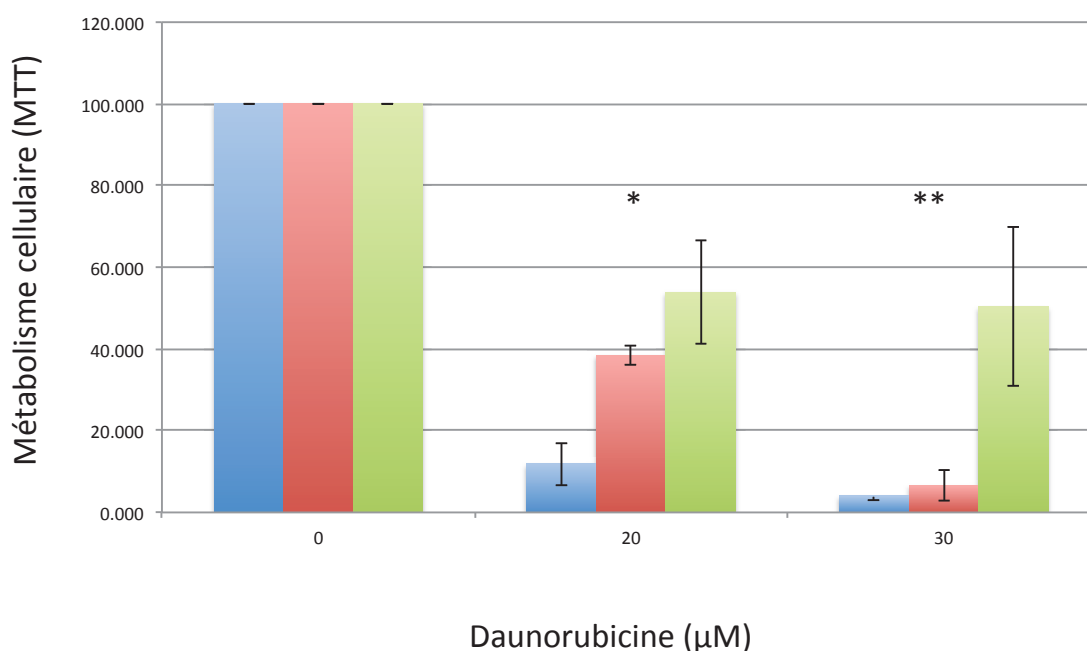


Figure 12. Sensibilité des cellules HEK-293T à la daunorubicine après transfection par les vecteurs pCMV vide (bleu), ABCA3 sauvage (Rouge) et ABCA3 sans exon 19 (vert). Les résultats correspondent à trois expériences de transfection. Les barres verticales représentent les écart-types. * $p < 0.05$, ** $p < 0.005$

II-3.2.3. Effet des isoformes d'ABCA3 sur l'efflux de daunorubicine

Les données de la littérature détaillées dans l'introduction (page 18) indiquent qu'ABCA3 induit un phénotype MDR concernant un large éventail de drogues. Concernant son rôle dans l'efflux, plutôt que d'exclure les drogues hors de la cellule, ce transporteur redirige ses substrats dans des compartiments intracellulaires comme les lysosomes. Son expression a ainsi été reliée à une accumulation cellulaire de daunorubicine. Afin d'évaluer l'effet de l'exclusion de l'exon 19 d'ABCA3 sur l'efflux de daunorubicine, les mêmes cellules que celles utilisées pour l'expérience de sensibilité (Figure 12) ont été analysées par FACS avec une excitation à 488 nm avec analyse à 560 nm. La figure 13 montre que l'expression ectopique de la forme sauvage d'ABCA3 augmente significativement la rétention cellulaire de daunorubicine. Ce résultat confirme celui obtenu par Chapuy *et al.*, (2008)³⁹. Alors que les expériences de sensibilité montraient que l'exclusion de l'exon 19 d'ABCA3 augmentait la résistance des cellules HEK-293T à la daunorubicine (Figure 12), l'analyse par FACS indique que par rapport au vecteur exprimant le gène sauvage, l'expression de la forme dépourvue de l'exon 19 possède un effet d'efflux significativement inférieur (Figure 13).

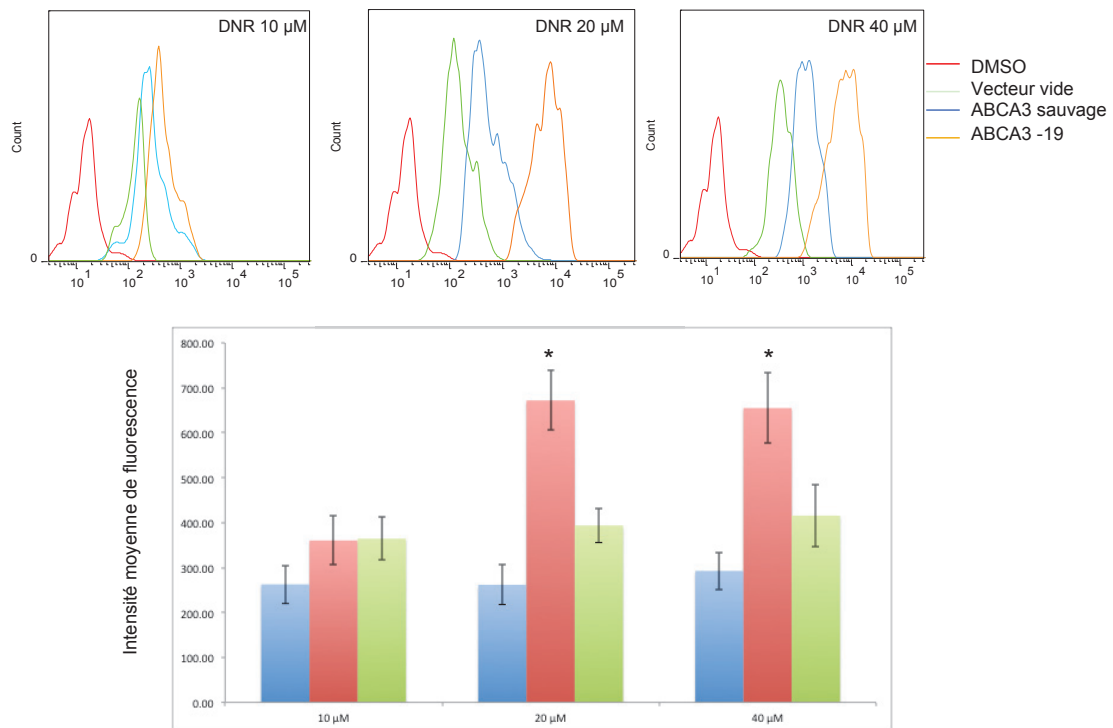


Figure 13. Effet des isoformes d'ABCA3 sur l'efflux de daunorubicine dans les cellules HEK-293T. Après transfection et incubation avec trois concentration de daunorubicine (DNR), les cellules ont été analysées par cytométrie en flux. Les données correspondent à trois expériences de transfection. La partie supérieure de la figure est un élément représentatif de ces trois expériences. Les barres d'erreur représentent les écart-types (* $p < 0.05$).

II-3.2.4. Implication pronostique de l'exclusion de l'exon 19 d'ABCA3 dans les LAM traitées par chimiothérapie intensive

L'exclusion de l'exon 19 d'ABCA3 a tout d'abord été quantifiée dans la moelle osseuse de 106 patients traités par CTI pour LAM, incluant 99 LAM non promyélocytaire. Les données cliniques correspondant à cette cohorte expérimentale sont détaillées dans le tableau 3. La technique de PCR quantitative spécifique d'exon (qESPCR) est détaillée dans l'article n°2². Concernant la réponse à la CTI, les patients avec un niveau d'exclusion de l'exon 19 d'ABCA3 supérieur à la valeur médiane de la population (A3S19^{high}) avaient un taux de réponse significativement réduit : 37/53 vs 46/53 (69.8% versus 86.8%, $p = 0.029$). Ces valeurs étaient de 34/50 versus 42/49 pour les 99 cas de LAM non promyélocytaires (68.0% versus 85.7%, $p = 0.031$). Chez les patients ayant obtenu une rémission complète (RC), l'exclusion de l'exon 19 d'ABCA3

était associée à un taux de rechute élevé : 27/37 versus 27/46 (73.0% versus 58.7%), la différence n'étant toutefois pas statistiquement significative ($p=0.13$).

Tableau 3. Données cliniques des 192 patients traités par chimiothérapie intensive (D'après Mint-Mohamed *et al.*, article en préparation).

	Discovery cohort n=106	Validation cohort n=86
Age at diagnosis (years)	56	57
Gender n (%)		
Male	60 (57%)	45 (52%)
Female	46 (43%)	41 (48%)
Cytogenetics n (%)		
Favorable	10 (9%)	-
Intermediate	72 (68%)	86 (100%)
Adverse	24 (23%)	-
Molecular features n (%)		
<i>NPM1</i> mutation	na	58 (67%)
<i>FLT3</i> -ITD	na	32 (37%)
Outcome		
Complete response to induction regimen	83 (78%)	77 (89%)
Overall survival (5 years)	30% ($\pm 5\%$)	42% ($\pm 8\%$)
Risk of relapse (5 years)	70% ($\pm 5\%$)	57% ($\pm 10\%$)

Le suivi médian était de 24,8 mois. La Figure 14 montre que l'exclusion de l'exon 19 d'ABCA3 était associée à une survie brève, avec une relation inverse entre le niveau d'exclusion et la survie globale, sans maladie ou sans événement. Le même effet était retrouvé chez les 49 malades avec un caryotype normal. Chez les 99 malades sans LAP, l'analyse multivariée retrouvait l'âge, le caryotype (selon l'ELN) et l'exclusion de l'exon 19 d'ABCA3 comme facteurs pronostiques indépendants pour la survie globale, sans maladie et sans événement (Tableau 4). L'âge et l'exclusion de l'exon 19 d'ABCA3 représentaient les deux facteurs pronostiques indépendants pour ces trois paramètres chez les 49 patients avec caryotype normal.

Pour valider ces résultats nous avons évalué l'impact pronostique de l'exclusion de l'exon 19 d'ABCA3 dans une cohorte de confirmation de 110 patients consécutifs dont 86 étaient traités par CTI (Tableau 3). Nous avons sélectionné des LAM à caryotype normal qui représentent une catégorie pronostique hétérogène nécessitant l'apport de

nouveaux outils prédictifs en termes de réponse au traitement et d'évolution. Les malades étaient testés pour les mutations de CEBPA, de NPM1 ainsi que pour FLT3-ITD. Avec un suivi médian de 15,3 mois, l'analyse univariée retrouvait une relation négative entre le niveau d'exclusion de l'exon 19 d'ABCA3 et la survie globale, sans maladie ou sans événement (figure 15). L'analyse multivariée retrouvait l'âge et l'exclusion de l'exon 19 d'ABCA3 comme seuls facteurs pronostiques indépendant pour la survie globale alors ces deux facteurs étaient associés à FLT3-ITD pour la survie sans maladie ou sans événement. Chez les 55 malades classés intermédiaire-1 selon l'ELN, l'exclusion de l'exon 19 d'ABCA3 conservait son impact pronostique négatif.

Chez les 34 patients des deux cohortes traités sans chimiothérapie intensive, l'expression de l'exon 19 d'ABCA3 n'était pas corrélées à l'évolution.

II-3.3. Article en préparation :

Aminetou Mint Mohamed, Morgan Thenoz, Catherine Koering, Delphine Boulch, Marie Balsat, Léa Payen, Mylène Honorat, Mohamed El-Hamri, Tigaud, Sandrine Hayette, Charles Dumontet, Emeline Cros, Christian Olivier Nibourel, Claude Preudhomme, Xavier Thomas, Franck-Emmanuel Françoise Solly, Pascale Flandrin-Gresta, Denis Guyotat, Lydia Campos, Mortreux, and Eric Wattel. **Exon skipping of the ABC-A3 transporter causes multidrug resistance and independently predicts survival myeloid leukemia (AML) patients treated with intensive chemotherapy.**

II-3.3. Discussion, conclusion, perspectives

La mise en évidence d'une exclusion de l'exon 19 d'ABCA3 dans des cellules résistantes à l'AraC ou sous-exprimant WT1 nous a conduit à évaluer l'effet de cet événement d'épissage sur la résistance à la chimiothérapie *in vitro* et *in vivo*.

In vitro nos résultats suggèrent que l'exclusion de l'exon 19 d'ABCA3 confère une résistance à la daunorubicine ainsi qu'au VP16. Ces données ont été obtenues avec des lignées leucémiques ainsi que dans des cellules épithéliales dans lesquelles les deux isoformes d'ABCA3 étaient transfectées. La résistance à l'AraC est en cours d'analyse dans ces deux systèmes cellulaires. Concernant le mécanisme impliqué dans cette résistance à la daunorubicine, nous avons analysé par FACS l'efflux de cette drogue. Nos résultats confirment ceux obtenus par d'autres études en montrant qu'ABCA3 augmente la rétention cellulaire de la daunorubicine (Figure 13). Paradoxalement, alors que l'isoforme ABCA3 dépourvu de l'exon 19 entraîne une résistance accrue à la daunorubicine *in vitro* (Figures 9 et 12), son effet sur la rétention de la drogue apparaît significativement plus faible que celui observé avec le transporteur sauvage (Figure 13). Ce résultat obtenu après trois expériences de transfection mérite confirmation par une nouvelle série d'expérience. Sa confirmation nécessiterait d'évaluer si une distribution qualitative de la drogue sous l'effet de l'exclusion de l'exon 19 d'ABCA3 pourrait expliquer les différences en termes de sensibilité. En pratique il serait intéressant de tracer l'efflux de la daunorubicine dans différents compartiments cellulaires incluant notamment le noyau, le réticulum endoplasmique et les exosomes. Le premier peut être analysé en FACS après séparation des noyaux. Le second est analysable à l'aide de marqueurs comme LAMP1, LAMP2 et le colorant LysoTracker rouge. Le troisième est analysable à l'aide des anticorps ciblant flotillin-2, alix, CD9, CD63, CD55 et CD59⁴¹.

L'expression d'ABCA3 est connue pour entraîner une résistance à l'AraC par un mécanisme non encore élucidé³⁹. La résistance aux nucléoside repose avant tout sur des anomalies de leur transporteurs comme les mutations d'ENT1⁴⁷ et surtout sur un défaut de phosphorylation pouvant résulter de mutations de gènes de kinases⁴⁸. Récemment des études ont montré qu'une résistance aux agents nucléosidiques incluant l'AraC pouvait être causé par un efflux dépendant de transporteurs ABC. Ainsi Hopper-Borge *et al.*, ont montré que MRP7 (ABCC10) déclenchait un efflux de l'AraC des cellules HEK293⁴⁹. Actuellement les deux transporteur ABC connus comme

impliqués dans la résistance aux agents nucléosidiques sont ABCC10 et ABCG2⁴⁸. Alors qu'ABCA3 entraîne une résistance à l'AraC³⁹, son effet sur l'efflux ou la rétention cellulaire de cette drogue n'a pas été étudié à notre connaissance. Nous projetons d'évaluer l'impact de l'exclusion de l'exon 19 d'ABCA3 sur la sensibilité à cette drogue.

Au plan clinique nos résultats suggèrent que l'exclusion de l'exon 19 d'ABCA3 représente un facteur pronostique indépendant dans les LAM traitées par chimiothérapie intensive. A notre connaissance ce travail est le premier démontrant l'effet pronostique de l'épissage anormal d'un transporteur ABC dans les LAM. En fait, plusieurs études ayant relié l'impact pronostique de l'expression de transporteurs ABC avec l'évolution des LAM sous traitement ont basé leurs conclusions sur des résultats de RTPCR quantitatives effectuées à l'aide d'amorces non spécifique d'exon. Ainsi ces résultats publiés ne permettent pas de trancher entre l'effet de l'expression globale du gène de celui lié à l'expression du seul exon analysé par RTPCR. Dans notre étude, l'utilisation d'oligonucléotides spécifiques d'exon a permis de relier clairement l'impact de l'exclusion de l'exon 19 d'abCA3 sur la survie.

Alors que le phénomène de résistance multidrogue représente un facteur pronostique négatif important dans la prise en charge des LAM, son ciblage par des drogues interférant avec l'efflux comme la quinine, la ciclosporine, le vérapamil ou le PSC833 n'a jusqu'ici pas permis d'améliorer significativement l'évolution de ces affections. En dehors de la quinine, ces drogues interfèrent avec le métabolisme des anthracycline, nécessitant d'en réduire les doses. Les agents de dernière génération comme le zosuquidar n'ont pas cet inconvénient mais une étude récente n'a pas retrouvé d'avantage clinique à l'utilisation de cet agent dans les LAM traitées intensivement⁵⁰. Il est possible d'inhiber ou de favoriser un évènement d'épissage grâce à l'utilisation d'oligonucléotides bifonctionnel recrutant des facteurs d'épissages inhibiteurs ou activateur au niveau d'un site précis. Une telle approche thérapeutique est en développement dans des affections génétiques associées à des anomalies d'épissage⁵¹,⁵². Nos résultats suggèrent qu'inhiber l'exclusion de l'exon 19 d'ABCA3 pourrait représenter une approche ciblée destinée à restituer une sensibilité à la daunorubicine. Nous sommes en train d'évaluer l'effet de tels oligonucléotide sur l'expression de l'exon 19 d'ABCA3.

Tout comme pour l'étude de l'épissage de TET2, les résultats obtenus avec ABCA3 nous conduisent maintenant à évaluer l'effet de cet événement d'épissage sur le pronostic des LAM pédiatriques et des LAM de l'adulte correspondant aux patients inclus dans le protocole ALFA-0701. Ces deux cohortes d'environ 400 et 200 malades permettront de confronter l'exclusion de l'exon 19 d'ABCA3 à la plupart des facteurs pronostiques connus des LAM incluant les données du séquençage haut débit. La validation de nos résultats sur ces deux cohortes permettra de proposer l'utilisation des présents marqueurs comme outil pronostique et de développer le ciblage de l'épissage par oligonucléotides bifonctionnels.

III - Conclusions

Au plan bibliographique ce travail de thèse a permis d'identifier les possibles mécanismes pouvant modifier l'épissage dans les cellules de LAM. Au plan expérimental, nous avons exploré les conséquences de l'expression de DEK et WT1 sur l'épissage. Les résultats identifient des signatures spécifiques de ces deux oncogènes ainsi que des patrons d'épissages associés à la résistance aux principales drogues utilisées dans la prise en charge des LAM. De façon intéressante ces événements surviennent pour la plupart indépendamment des modifications d'expression globale des gènes concernés. Soixante-dix pour cent des résultats obtenus en microarray ont été confirmés par PCR spécifique d'exon. Le lien observé entre l'expression de WT1 et l'épissage observé *in vitro* a été confirmé *in vivo* dans des échantillons tumoraux de LAM.

L'annotation des événements d'épissage permet d'identifier des voies (*pathways*) dérégulées. Dans nos travaux, ces voies ont été analysées à l'aide d'algorithmes ne prenant en compte que les gènes concernés par les événements d'épissage identifiés (ensemble du gène pour un événement d'épissage). De nouveaux outils bioinformatiques sont maintenant disponibles qui permettent de définir des *pathways* exon-spécifiques. Nous projetons d'analyser nos résultats à l'aide de ces outils dans le but d'affiner notre connaissance des perturbations cellulaires issues des anomalies d'épissage. Nous avons par ailleurs analysé en microarray 54 échantillons de malades inclus dans le protocole ALFA-0701. Ces échantillons correspondent à des malades ayant eu des réponses et des évolutions différentes sous traitement. L'analyse des « *pathways*-exons » nous permettra d'être plus précis dans l'identification de biomarqueurs potentiels. Ces biomarqueurs seront ensuite confrontés aux marqueurs pronostiques habituels de même qu'aux données de NGS obtenues chez les 280 malades de l'étude⁵³

Au plan clinique nous avons montré que certains événements d'épissages représentaient des facteurs pronostiques indépendants chez les malades traités par CTI. L'analyse des échantillons correspondants aux patients adultes du protocole ALFA-0701 ainsi qu'aux 400 enfants du protocole ELAM 02 chez lesquels les données NGS sont aussi disponibles. Outre la validation de nos résultats et l'identification de nouveaux marqueurs pronostiques, ces travaux permettront d'identifier de nouvelles voies impliquées dans la persistance tumorale. Nous avons fait synthétiser des oligonucléotides bifonctionnels (OBF) dans le but d'inhiber ou d'activer l'épissage de

l'exon 19 d'ABCA3. Dans une première étape nous allons déterminer l'efficacité de ces séquences par qESPCR. Nous testerons ensuite l'intérêt de ces OBF sur la résistance aux drogues *in vitro* puis *in vivo*, chez l'animal de laboratoire.

Au plan fonctionnel il devient intéressant d'évaluer les conséquences des anomalies d'épissage de TET2 et d'ABCA3 sur les propriétés des protéines correspondantes. Ces points ont été détaillés dans les chapitres correspondants.

IV – Références

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