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Par

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ÉTUDE DES ALTÉRATIONS GÉNOMIQUES ACQUISES DANS LES LEUCÉMIES AIGUËS MYÉLOÏDES IMPLIQUANT LE CORE BINDING FACTOR

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AVANT-PROPOS

Ce travail de thèse de doctorat s'articule autour d'articles originaux publiés ou en cours de soumission en premier ou co-premier auteur dans des revues scientifiques internationales à comité de lecture :

- **Duployez N**, Marceau-Renaut A, Boissel N, *et al*. Comprehensive mutational profiling of core binding factor acute myeloid leukemia. *Blood*. 127(20), 2451–2459 (2016).
- **Duployez N**, Boudry-Labis E, Roumier C, et al. SNP-array lesions in core binding factor acute myeloid leukemia. *Submitted*.
- **Duployez N**, Micol J-B, Boissel N, *et al*. Unlike *ASXL1* and *ASXL2* mutations, *ASXL3* mutations are rare events in acute myeloid leukemia with t(8;21). *Leuk. Lymphoma*. 57(1), 199–200 (2016).
- Antony-Debré I* and **Duployez N***, Bucci M, *et al*. Somatic mutations associated with leukemic progression of familial platelet disorder with predisposition to acute myeloid leukemia. *Leukemia*. 30(4), 999–1002 (2016)(*equally contributors).

Une partie de l'introduction s'appuie également sur les publications de 2 revues générales :

- **Duployez N**, Willekens C, Marceau-Renaut A, Boudry-Labis E, Preudhomme C. Prognosis and monitoring of core-binding factor acute myeloid leukemia: current and emerging factors. *Expert Rev Hematol*. 8(1), 43–56 (2015).
- **Duployez N**, Lejeune S, Renneville A, Preudhomme C. Myelodysplastic syndromes and acute leukemia with genetic predispositions: a new challenge for hematologists. *Expert Rev Hematol*. 9(12), 1189–1202 (2016).

Certains des résultats présentés ont notamment fait l'objet de communications orales et affichées en congrès scientifiques nationaux et internationaux :

- Somatic mutations associated with leukemic progression of FPD/AML. *Congrès de l'ASH 2016*, San Diego (communication orale).
- Etude du profil mutationnel étendu des leucémies aiguës myéloïdes à core binding factor des protocoles CBF2006 et ELAM02. *Congrès de la SFH 2016,* Paris (communication orale).
- Genomic landscape of core binding factor acute myeloid leukemia. *Congrès de l'EHA 2015*, Vienne (poster avec présentation orale).
- Additional molecular aberrations leading to leukemic transformation in patients with familial platelet disorder. *Congrès de l'EHA 2015*, Vienne (poster avec présentation orale).
- Leucémies aiguës myéloïdes (LAM) avec t(8;21) et LAM avec inv(16) : 2 entités distinctes ? *Congrès de la SFH 2015*, Paris (communication orale).

Enfin, ce travail est à l'origine d'une collaboration internationale avec le Memorial Sloan Kettering Cancer Center (MSKCC, New York) et la publication d'un article original dont le résumé figure en annexe de cette thèse : Micol J-B, Pastore A, Inoue D, **Duployez N**, *et al*. ASXL2 is essential for haematopoiesis and acts as a haploinsufficient tumour suppressor in leukemia. *Nature Communications*. 8, 15429 (2017).

TABLE DES MATIÈRES

ABRÉVIATIONS

ADN : acide désoxyribonucléique

ALFA : *Acute Leukemia French Association*

ARN : acide ribonucléique

CBF : *core binding factor*

CGH : *comparative genomic hybridization*

CSH : cellule souche hématopoïétique

ELN : *European LeukemiaNet*

FAB : *French-American-British*

FILO : *French Innovative Leukemia Organization*

FISH : *florescence in situ hybridization*

FPD/AML : *familial platelet disorder with propensity to myeloid malignancies*

GO : gemtuzumab ozogamicin

GOELAMS : Groupe Ouest-Est d'Etude des Leucémies et Autres Maladies du Sang

HDAC : cytarabine à hautes doses

HLA : *human leukocyte antigen*

iAMP21 : amplification intrachromosomique du chromosome 21

IL2 : interleukine-2

InVs : Institut de veille sanitaire

ITD : *internal tandem duplication*

ITK : inhibiteur de tyrosine kinase

KI : *knock-in*

KO : *knock-out*

LAL : leucémie aiguë lymphoblastique

LAM : leucémie aiguë myéloïde

LAP : leucémie aiguë promyélocytaire

LMMC : leucémie myélomonocytaire chronique

MRD : *minimal residual disease*

OMIM : *online mendelian inheritance in man*

OMS : Organisation Mondiale de la Santé

PCR : *polymerase chain reaction*

RC : rémission complète

RQ-PCR : *real-time quantitative-PCR*

RTK : récepteur à activité tyrosine kinase

RT-PCR : *reverse transcriptase-PCR*

SMD : syndrome myélodysplasique

SMP : syndrome myéloprolifératif

SNP : *single nucleotide polymorphism*

TKD : *tyrosine kinase domain*

LEXIQUE DES GÈNES

Gènes	Nom complet (nomenclature HGNC*)	Localisation
ASXL1	additional sex combs like 1, transcriptional regulator	20q11.21
ASXL2	additional sex combs like 2, transcriptional regulator	2p23.3
ASXL3	additional sex combs like 3, transcriptional regulator	18q12.1
BAP1	BRCA1 associated protein 1	3p21.1
BCOR	BCL6 corepressor	Xp11.4
BCORL1	BCL6 corepressor-like 1	Xq26.1
CALR	calreticulin	19p13.13
CBFB	core-binding factor beta subunit	16q22.1
CBL	Cbl proto-oncogene	11q23.3
CBX5	chromobox 5	12q13.13
CCDC26	CCDC26 long non-coding RNA	8q24.21
CCND1	cyclin D1	11q13.3
CCND2	cyclin D2	12p13.32
CDCA5	cell division cycle associated 5	11q13.1
CEBPA	CCAAT/enhancer binding protein alpha	19q13.11
CNOT4	CCR4-NOT transcription complex subunit 4	7q33
CREBBP	CREB binding protein	16p13.3
CSF3R	colony stimulating factor 3 receptor	1p34.3
DHX15	DEAH-box helicase 15	4p15.2
DNMT3A	DNA methyltransferase 3 alpha	2p23.3
EED	embryonic ectoderm development	11q14.2
EP300	E1A binding protein p300	22q13.2
ETV6	ETS variant 6	12p13.2
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit	7q36.1
FLT3	fms related tyrosine kinase 3	13q12.2
FOXP1	forkhead box P1	3p13
GATA1	GATA binding protein 1	Xp11.23
GATA2	GATA binding protein 2	3q21.3
HCFC1	host cell factor C1	Xq28
IDH1	isocitrate dehydrogenase (NADP(+)) 1, cytosolic	2q34
IDH2	isocitrate dehydrogenase (NADP(+)) 2, mitochondrial	15q26.1
IKZF1	IKAROS family zinc finger 1	7p12.2
JAK2	Janus kinase 2	9p24.1
KDM1A	lysine demethylase 1A	1p36.12
KDM6A	lysine demethylase 6A	Xp11.3
KIT	KIT proto-oncogene receptor tyrosine kinase	4q12
KMT2A	lysine methyltransferase 2A	11q23.3
KMT2C	lysine methyltransferase 2C	7q36.1
KRAS	KRAS proto-oncogene, GTPase	12p12.1
MAU2	MAU2 sister chromatid cohesion factor	19p13.11

MGA	MGA, MAX dimerization protein	15q15
MPL	MPL proto-oncogene, thrombopoietin receptor	1p34.2
MYB	MYB proto-oncogene, transcription factor	6q23.3
MYC	v-myc avian myelocytomatosis viral oncogene homolog	8q24.21
MYH11	myosin heavy chain 11	16p13.11
NCOA1	nuclear receptor coactivator 1	2p23.3
NCOR1	nuclear receptor corepressor 1	17p12-p11.2
NCOR2	nuclear receptor corepressor 2	12q24.31
NF1	neurofibromin 1	17q11.2
NIPBL	NIPBL, cohesin loading factor	5p13.2
NPM1	nucleophosmin	5q35.1
NRAS	neuroblastoma RAS viral oncogene homolog	1p13.2
PDS5A	PDS5 cohesin associated factor A	4p14
PDS5B	PDS5 cohesin associated factor B	13q13.1
PHF6	PHD finger protein 6	Xq26.2
PML	promyelocytic leukemia	15q24.1
PTEN	phosphatase and tensin homolog	10q23.31
PTPN11	protein tyrosine phosphatase, non-receptor type 11	12q24.13
RAD21	RAD21 cohesin complex component	8q24.11
RARA	retinoic acid receptor alpha	17q21.2
RUNX1	runt related transcription factor 1	21q22.12
RUNX1T1	RUNX1 translocation partner 1	8q21.3
SETBP1	SET binding protein 1	18q12.3
SF3B1	splicing factor 3b subunit 1	2q33.1
SIN3A	SIN3 transcription regulator family member A	15q24.2
SMC1A	structural maintenance of chromosomes 1A	Xp11.22
SMC3	structural maintenance of chromosomes 3	10q25.2
SRSF2	serine and arginine rich splicing factor 2	17q25.2
STAG1	stromal antigen 1	3q22.3
STAG2	stromal antigen 2	Xq25
SUZ12	SUZ12 polycomb repressive complex 2 subunit	17q11.2
TET2	tet methylcytosine dioxygenase 2	4q24
TLE1	transducin like enhancer of split 1	9q21.32
TLE4	transducin like enhancer of split 4	9q21.31
TP53	tumor protein p53	17p13.1
U2AF1	U2 small nuclear RNA auxiliary factor 1	21q22.3
WAPL	WAPL cohesin release factor	10q23.2
WT1	Wilms tumor 1	11p13
WTIP	WT1 interacting protein	19q13.11
ZBTB7A	zinc finger and BTB domain containing 7A	19p13.3
ZRSR2	zinc finger CCCH-type, RNA binding motif and serine/arginine rich 2	Xp22.2

**HUGO (human genome organization) Gene Nomenclature Committee*

INTRODUCTION

PARTIE 1 - LES LEUCÉMIES AIGUËS MYÉLOÏDES

1. Définition

Les leucémies aiguës myéloïdes (LAM) sont des affections malignes caractérisées par la prolifération clonale de cellules hématopoïétiques immatures myéloïdes bloquées dans leur différenciation, appelées blastes. Chaque année en France, le nombre de nouveaux cas de LAM est estimé à environ 2 800 avec un sex-ratio de 1,1 (H/F) et un âge médian au diagnostic de 71 ans pour les 2 sexes [1]. L'incidence est relativement basse chez le sujet jeune et augmente significativement avec l'âge, en particulier après 60 ans, avec un maximum au-delà de 85 ans (Figure 1).

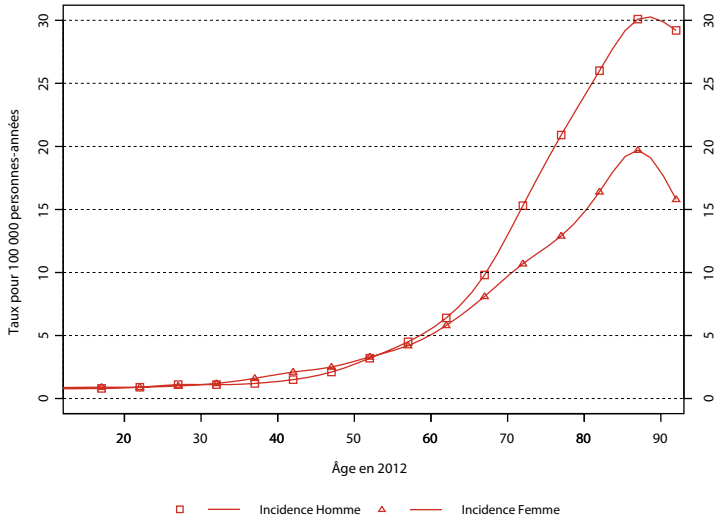

Figure 1 : Incidence des LAM par âge et par sexe (France, InVs).

La cause des LAM reste inconnue dans la majorité des cas bien que des facteurs de risque soient identifiés : exposition à des rayonnements ionisants ou à certains produits chimiques (ex : benzène), antécédents de chimiothérapie (ex : alkylants), maladies hématologiques préexistantes (ex : syndromes myélodysplasiques [SMD] ou syndromes myéloprolifératifs [SMP]) ou enfin certaines anomalies génétiques constitutionnelles syndromiques (ex : syndrome de Down) ou non syndromiques (ex : thrombopénie familiale avec mutation germinale de *RUNX1*).

La présentation clinique est dominée par les signes d'insuffisance médullaire (anémie, thrombopénie, neutropénie), conséquences de l'accumulation de blastes dans la moelle osseuse (éventuellement le sang et/ou d'autres organes) et l'inhibition de l'hématopoïèse physiologique. Les signes cliniques fréquents incluent fatigue, pâleur, essoufflement, susceptibilité aux infections et hémorragies, en particulier cutanéomuqueuses.

Le diagnostic biologique des LAM repose sur l'examen cytologique du sang et de la moelle osseuse montrant une infiltration par des blastes myéloïdes. Il est éventuellement complété par l'immunophénotypage des blastes en cytométrie en flux ainsi que les études cytogénétique et moléculaire utiles à la détermination du pronostic et à la stratification thérapeutique [2].

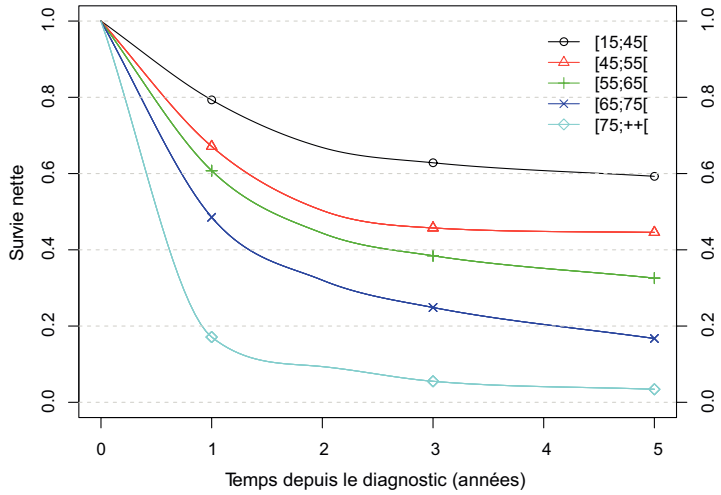

Figure 2 : Survie des patients atteints de LAM par tranches d'âge (France, InVs).

Le traitement des LAM repose avant tout sur la chimiothérapie, éventuellement complétée d'une allogreffe de cellules souches hématopoïétiques (CSH) selon le pronostic de la maladie et la capacité du patient à en supporter la toxicité. En dépit des progrès thérapeutiques, les LAM restent des affections de très mauvais pronostic avec une survie à 5 ans globalement voisine de 20% pour les 2 sexes, mais avec de grandes disparités selon la tranche d'âge (Figure 2). Si une survie plus élevée (d'environ 60%) est observée chez les patients plus jeunes (15-45 ans) – en rapport avec des schémas thérapeutiques spécifiques des LAM avec anomalies génétiques sur-représentées dans cette tranche d'âge – elle s'approche de 30% chez les 55-65 ans et s'effondre à 3% chez les plus de 75 ans [3].

2. Classification des LAM

Deux systèmes de classification des LAM coexistent en pratique. La classification internationale Franco-Américano-Britannique (FAB) établie en 1976 repose sur les caractéristiques morphologiques et cytochimiques (myélopéroxydase, estérases) des cellules leucémiques [4,5]. Elle identifie 8 groupes de LAM, numérotées de M0 à M7 (Table 1) selon leur appartenance à une lignée cellulaire et le stade de maturation. La classification FAB a été utilisée dans le monde entier jusqu'à publication de la classification de l'Organisation Mondiale de la Santé (OMS) dans les années 2000. Elle reste néanmoins très utilisée au laboratoire d'hématologie en l'absence de données cytogénétiques et moléculaires au diagnostic.

Table 1 : Classification FAB des LAM (table adaptée de [5]).

Sous-type FAB	Description	Fréquence
M0	LAM avec différenciation minime	2%
M1	LAM sans maturation	20%
M2	LAM avec maturation	30%
M3	LA promyélocytaire	10%
M4	LA myélomonocytaire	15%
M5	LA monoblastique	15%
M6	Érythroleucémie	5%
M7	LA mégacaryoblastique	2%

La classification OMS complète les critères de la classification FAB en y intégrant les données cytogénétiques et moléculaires (Table 2) connues pour influencer le pronostic de la maladie [6–8]. En outre, elle définit le seuil de blastes médullaires ou sanguins requis pour le diagnostic de LAM à 20% des cellules nucléées (à l'exception de certains cas avec anomalie cytogénétique récurrente).

Table 2 : Classification OMS 2016 des LAM (table adaptée de [9]).

Classification OMS 2016 des LAM
LAM avec anomalies génétiques récurrentes
LAM avec t(8;21)(q22;q22.1) ; *RUNX1-RUNX1T1*
LAM avec inv(16)(p13.1q22) ou t(16;16)(p13.1;q22) ; *CBFB-MYH11*
LA promyélocytaire avec *PML-RARA**
LAM avec t(9;11)(p21.3;q23.3) ; *MLLT3-KMT2A*
LAM avec t(6;9)(p23;q34.1) ; *DEK-NUP214*
LAM avec inv(3)(q21.3q26.2) ou t(3;3)(q21.3;q26.2) ; *GATA2, MECOM*
LAM (mégacaryoblastique) avec t(1;22)(p13.3;q13.3) ; *RBM15-MKL1*
LAM avec *BCR-ABL1* (entité provisoire)
LAM avec mutation de *NPM1*
LAM avec mutations bialléliques de *CEBPA*
LAM avec mutation de *RUNX1* (entité provisoire)
LAM avec anomalies associées aux myélodysplasies
Néoplasies myéloïdes post-chimiothérapie
LAM sans autre spécification (LAM-NOS)
LAM avec différenciation minime
LAM sans maturation
LAM avec maturation
LA myélomonocytaire
LA monoblastique/monocytaire
LA érythroïde pure
LA mégacaryoblastique
LA à basophiles
LA avec myélofibrose (panmyélose aiguë)
Sarcomes myéloïdes
Prolifération myéloïdes associées au syndrome de Down
Néoplasies myéloïdes avec prédispositions génétiques

* Identification de la translocation t(15;17)(q24.1;q21.2) dans plus de 98% des cas.

La révision 2016 de la classification OMS divise les LAM en 4 grandes catégories selon (1) l'existence d'anomalies cytogénétiques ou moléculaires récurrentes ; (2) la présence de signes de myélodysplasie (antécédent de SMD, présence d'une dysplasie multilignée ou d'anomalies cytogénétiques de type SMD) ; (3) la notion de chimiothérapie antérieure (*therapy-related*) et enfin (4) le groupe des LAM ne présentant aucune de ces caractéristiques (*not otherwise specified*, LAM-NOS). Deux groupes mineurs concernent les sarcomes myéloïdes et les prolifération myéloïdes associées au syndrome de Down. A noter depuis 2016 que la classification OMS inclut également le groupe des hémopathies myéloïdes avec prédispositions génétiques telles que les mutations germinales de *RUNX1* (OMIM #601399) ou de *CEBPA* (OMIM #601626).

3. Stratification génétique du risque

La stratification du risque des LAM repose sur l'identification d'anomalies en cytogénétique (caryotype, FISH) et en biologie moléculaire. Le caryotype conventionnel identifie des anomalies dans environ 50% des LAM. Dans la majorité des cas, ces anomalies sont des événements acquis de manière non aléatoire. Elles définissent des entités clinico-biologiques distinctes à la base des classifications utilisées en pratique en raison de leur impact pronostique largement démontré sur l'obtention d'une rémission complète (RC), le risque de rechute et la survie (Figure 3) [10–12].

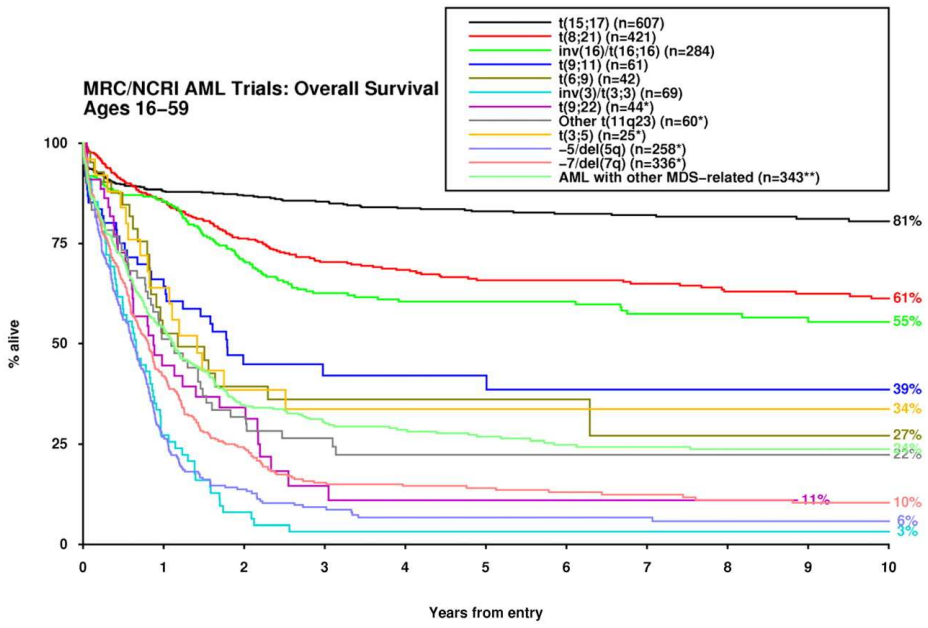

Figure 3 : Impact des anomalies cytogénétiques sur la survie globale dans les LAM [11].

Les leucémies aiguës promyélocytaires (LAP) caractérisées par la t(15;17) sont associées à des taux de guérison importants depuis l'utilisation de l'acide tout-*trans*-rétinoïque et de l'arsenic. De même, les réarrangements du *core binding factor*, t(8;21) et inv(16)/t(16;16), ont une évolution favorable

avec les schémas utilisant la cytarabine à haute dose. A l'inverse, les translocations impliquant le locus 11q23 (ciblant le gène *KMT2A* ou *MLL*), la t(6;9), la t(9;22) ainsi que les inv(3)/t(3;3) restent associées à une évolution péjorative. En l'absence de réarrangement récurrent, il est habituel de distinguer les LAM avec délétions affectant tout ou partie des chromosomes 5, 7 et 17, de même que les LAM à caryotypes complexes ou monosomaux, lesquelles sont associées à un pronostic très défavorable. Environ 50% des LAM ne présentent aucune des anomalies précédentes. Dans ces situations, l'identification de mutations somatiques prend toute son importance et permet l'identification de sous-groupes pronostiques différents. En particulier, les mutations de *NPM1* en l'absence de duplication interne en tandem (ITD) de *FLT3* [13] (ou un ratio faible de *FLT3*-ITD [14]) ainsi que les mutations bialléliques de *CEBPA* [15] ont une valeur pronostique favorable sur la survie sans rechute et la survie globale. En 2017, faisant suite à l'actualisation de la classification de l'OMS et aux progrès dans la description des anomalies moléculaires des leucémies aiguës, l'*European LeukemiaNet* (ELN) a ainsi actualisé ses recommandations pour le diagnostic et la prise en charge des LAM [2]. Le risque génétique y est défini en 3 catégories en prenant en compte à la fois la présence d'anomalies cytogénétiques et l'existence de mutations somatiques (Table 3).

Table 3 : Risque génétique selon l'ELN (table adaptée de [2]).

Risque	Anomalies génétiques
Favorable	t(8;21)(q22;q22.1) ; *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) ou t(16;16)(p13.1;q22) ; *CBFB-MYH11*
	NPM1 muté sans *FLT3*-ITD ou *FLT3*-ITD faible[(a)(b)]
	CEBPA muté biallélique[(b)]
Intermédiaire	*NPM1* muté avec *FLT3*-ITD élevé[(a)(b)]
	NPM1 sauvage sans *FLT3*-ITD ou *FLT3*-ITD faible[(a)(b)]
	t(9;11)(p21.3;q23.3) ; *MLLT3-KMT2A*
	anomalies cytogénétiques non classées dans les autres groupes de risque
Défavorable	t(6;9)(p23;q34.1) ; *DEK-NUP214*
	t(v;11q23.3) ; réarrangement de *KMT2A* sauf t(9;11)(p21.3;q23.3)
	t(9;22)(q34.1;q11.2) ; *BCR-ABL1*
	inv(3)(q21.3q26.2) ou t(3;3)(q21.3;q26.2) ; *GATA2, MECOM*
	-5 ou del(5q) ; -7 ; -17/anomalie 17p
	caryotype complexe[(c)], caryotype monosomal[(d)]
	NPM1 sauvage et *FLT3*-ITD élevé[(a)(b)]
	RUNX1 muté
	ASXL1 muté
	TP53 muté

(**a**) Ratio de l'aire sous la courbe de *FLT3*-ITD/*FLT3* wild-type (faible si < 0.5 ; élevé si ≥ 0.5). (**b**) Quelles que soient les anomalies cytogénétiques associées. (**c**) Défini par 3 anomalies ou plus. (**d**) Défini par la présence d'une monosomie (sauf -X ou -Y) associée à au moins une autre monosomie ou une anomalie structurale.

4. Classes d'anomalies moléculaires des LAM

Les cellules leucémiques à l'origine des LAM sont le résultat d'une combinaison d'anomalies génétiques (amplifications, délétions, réarrangements, mutations ponctuelles) affectant à la fois la prolifération, la survie et la différenciation. Le modèle de leucémogénèse initialement décrit implique la coopération d'une altération conférant un avantage prolifératif (événement de classe I) et d'une altération induisant un blocage de différenciation (événement de classe II) [16]. Bien que biologiquement valable, ce modèle a été régulièrement révisé avec les progrès technologiques dans l'étude des anomalies moléculaires. En particulier, les techniques de séquençage d'exome ou de génome entier ont considérablement contribué à élargir le spectre des mutations en identifiant de nouvelles classes d'altérations [17] et en permettant d'appréhender la complexité de l'architecture clonale de ces pathologies [18,19]. Actuellement, les mutations ou réarrangements identifiés dans les LAM peuvent être classés dans 8 catégories fonctionnelles (Figure 4) [17,20].

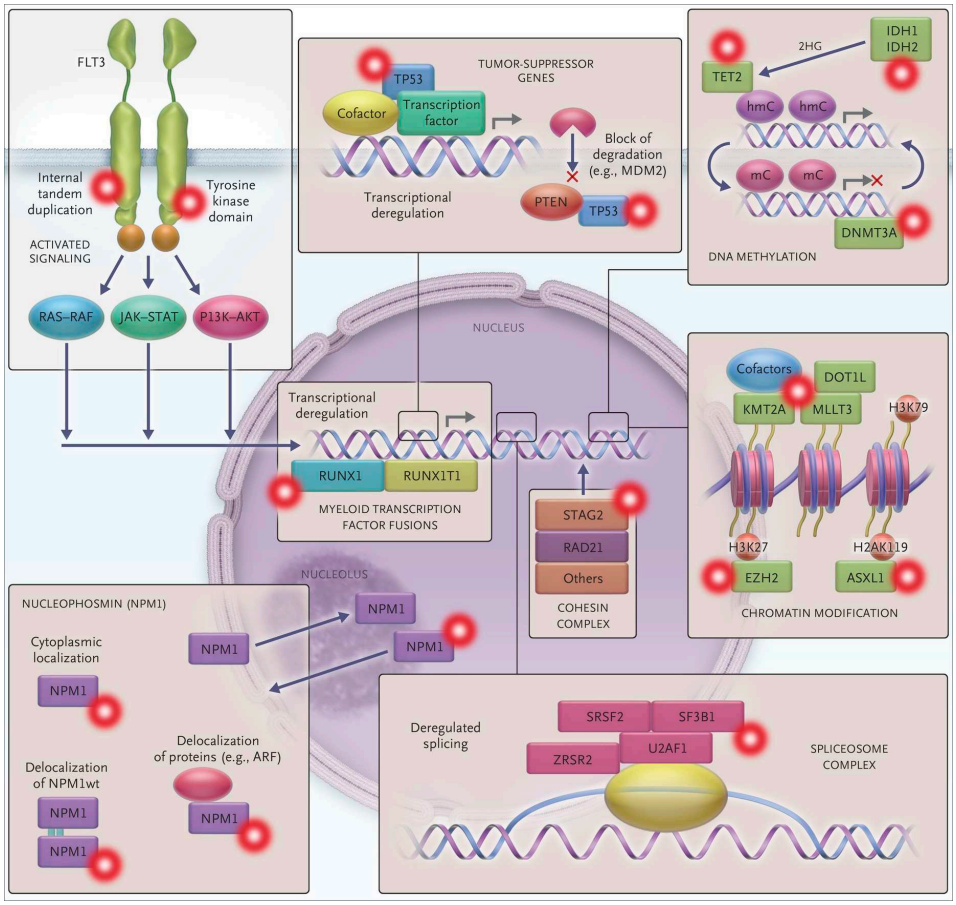

Figure 4 : Représentation des 8 catégories fonctionnelles d'anomalies impliquées dans les LAM [20].

- **Voies de signalisation (59% des cas)**

Ces mutations affectent les gènes codant des récepteurs à activité tyrosine kinase de classe III, tels que *KIT* ou *FLT3*, ainsi que des protéines intracellulaires intervenant dans la transduction du signal (*RAS*, *PTPN11*, *JAK2*). Elles impliquent généralement des acides aminés conservés (*hotspots* mutationnels) conférant un gain de prolifération et/ou de survie des cellules leucémiques (anomalies de classe I) via l'activation constitutive des voies de signalisation *RAS-RAF*, *JAK-STAT* et *PI3K-AKT* [21].

- **Facteurs de transcription (40%)**

Ces anomalies incluent les mutations et réarrangements (fusions) affectant des facteurs de transcription clés de l'hématopoïèse. Elles sont associées à une dérégulation transcriptionnelle et un blocage de différenciation (anomalies de classe II) [21]. On y retrouve les mutations des gènes codant pour les facteurs de transcription *RUNX1* ou *CEBPA* ainsi que les réarrangements du *core binding factor* (fusions *RUNX1-RUNX1T1* et *CBFB-MYH11*) ou de *RARA*, lesquels définissent des entités clinico-biologiques de LAM selon la classification OMS.

- **Nucléophosmine 1 (27%)**

Le gène *NPM1* code pour une phosphoprotéine nucléocytoplasmique multifonctionnelle (synthèse du ribosome, réparation de l'ADN, régulation transcriptionnelle, remodelage de la chromatine). Les mutations de *NPM1* se traduisent par la création d'un signal d'export nucléaire conduisant à sa délocalisation cytoplasmique et la perte de ses fonctions nucléolaires. Les mutations de *NPM1* définissent une entité clinico-biologique de LAM selon la classification OMS.

- **Méthylation de l'ADN (44%)**

Ces mutations agissent en modifiant l'état de méthylation de l'ADN. Elles incluent les mutations des gènes *DNMT3A* et *TET2* codant respectivement pour une DNA méthyl-transférase et une dioxygénase alpha-cétoglutarate-dépendante intervenant dans la méthylation et la déméthylation des cytosines. Les mutations des gènes codant les isocitrate déshydrogénases IDH1 et IDH2 induisent l'inhibition de TET2 (et potentiellement d'autres enzymes) via la production d'un oncométabolite, le 2-hydroxyglutarate, à partir d'alpha-cétoglutarate [22].

- **Modification des histones (30%)**

Ces anomalies incluent notamment les mutations des gènes *ASXL1* et *EZH2* (codant des membres du *polycomb repressive complex 2*) responsables de la perturbation des modifications post-

traductionnelles des histones, en particulier via un défaut de méthylation du résidu K27 de l'histone H3 (H3K27). De même, les très nombreux réarrangements de *KMT2A* (plus de 50 partenaires connus) sont responsables de la perte de son domaine H3K4-méthyltransférase et du recrutement aberrant de la H3K79-méthyltransférase DOT1L [17].

- **Cohésine (13%)**

Le complexe multiprotéique de la cohésine (*SMC1A, SMC3, RAD21, STAG2*) intervient dans la ségrégation des chromosomes au cours de la division cellulaire, la réparation de l'ADN et la régulation transcriptionnelle. Des travaux récents ont montré que les mutations de la cohésine entraînaient un blocage de maturation et une augmentation de l'accessibilité de la chromatine au niveau des sites de liaison de facteurs de transcription clés de l'hématopoïèse [23]. Ces anomalies pourraient donc être rapprochées, du moins en partie, de la classe précédente.

- **Spliceosome (14%)**

Les mutations des gènes du spliceosome (*SRSF2, SF3B1, U2AF1, ZRSR2*) sont associées à des anomalies d'épissage des ARN pré-messagers. De manière intéressante, il a été montré que les mutations de *SRSF2* entraînaient un défaut d'épissage de l'ARN d'*EZH2* et de *BCOR* conduisant à leur dégradation rapide et une altération de la différenciation cellulaire [24].

- **Suppresseurs de tumeurs (16%)**

Cette dernière catégorie inclut le gène *TP53*, jouant un rôle central dans la signalisation des dommages à l'ADN. Les anomalies de *TP53* (mutations, délétions) sont décrites dans la plupart des tumeurs solides mais sont plutôt rares dans les LAM. Les mutations/délétions de *TP53*, souvent bialléliques, sont fortement corrélées à la présence d'un caryotype complexe ou monosomal et confèrent systématiquement un pronostic défavorable [25].

PARTIE 2 - LE CORE BINDING FACTOR (CBF)

1. Du caryotype conventionnel à la découverte du CBF

La translocation équilibrée t(8;21)(q22;q22), décrite initialement par Janet Rowley en 1973 chez un patient atteint de LAM, a été la première translocation chromosomique réciproque identifiée dans les cellules cancéreuses [26] (Figure 5A). En 1991, Hiroyuki Miyoshi et *al* clonent et séquencent un nouveau gène, le gène *AML1* (*acute myeloid leukemia 1*) localisé au point de cassure du chromosome 21, chez les patients atteints de LAM avec t(8;21) [27]. Au niveau moléculaire, il est établi un peu plus tard que la t(8;21) conduit à la fusion d'*AML1* avec le gène *ETO* (*eight-twenty one*) et à la production d'une protéine chimérique [28].

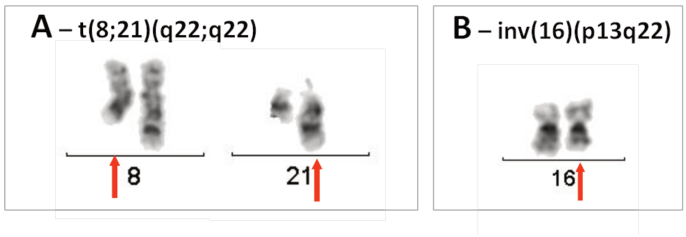

Figure 5 : Réarrangements du CBF par caryotype conventionnel (bandes G).

En 1993, Paul Liu et *al* identifient les gènes *CBFB* et *MYH11* comme partenaires de fusion dans un autre réarrangement chromosomique équilibré récurrent dans les LAM : l'inversion péricentrique du chromosome 16 [inv(16)(p13q22)] (Figure 5B) décrite 10 ans plus tôt et sa variante t(16;16)(p13;q22) [29,30]. Les gènes *AML1* et *CBFB* codent respectivement les sous-unités α et β du *core binding factor* (CBF), facteur de transcription hétérodimérique impliqué dans la régulation de l'hématopoïèse et l'émergence des cellules souches [31]. En 1996, l'utilisation de modèles murins permit de démontrer le rôle fondamental du CBF puisque les souris *knock-out* (KO) pour l'une ou l'autre des 2 sous-unités du CBF mouraient rapidement au cours de l'embryogénèse de troubles hémorragiques disséminés résultant de l'absence d'hématopoïèse définitive [32–34]. Deux autres sous-unités α, AML2 et AML3, capables de s'associer à la sous-unité β commune sont décrites chez l'Homme. AML2 intervient notamment dans la croissance du tissu épithélial gastrique, la neurogénèse et la thymopoïèse [35] tandis qu'AML3 est impliquée dans l'ostéogénèse [31].

En 1999, en raison de l'homologie d'*AML1* avec le gène *runt* décrit chez la drosophile par Peter Gergen dans les années 1980 [36,37], les gènes *AML1, 2 et 3* sont respectivement renommés *RUNX1* (*Runt-related transcription factor 1*), *RUNX3* et *RUNX2* selon la nomenclature de l'Organisation du

Génome Humain (HUGO) (Table 4) [38]. Le gène *ETO* est quant à lui renommé *RUNX1T1* (*RUNX1 translocated to, 1*).

Table 4 : Synonymes utilisés pour décrire les gènes *RUNX* et *RUNX1T1* humains dans la littérature.

Symbole approuvé	Symboles précédents	Locus
RUNX1	*CBFA2 / AML1 / PEBP2alphaB*	21q22
RUNX2	*CBFA1 / AML3 / PEBP2alphaA*	6p21
RUNX3	*CBFA3 / AML2 / PEBP2alphaC*	1p36
RUNX1T1	*CBFA2T1 / ETO / MTG8*	8q22

2. Organisation génomique et domaines fonctionnels de *RUNX1*

Le gène *RUNX1* humain, localisé en 21q22, s'étend sur 260 kb et code pour la sous-unité α du CBF. Son expression est sous la dépendance de 2 promoteurs situés en amont de l'exon 1 (promoteur distal P1) et de l'exon 3 (promoteur proximal P2). Deux isoformes majeures *RUNX1b* (453 acides aminés) et *RUNX1c* (480 acides aminés) sont distinguées selon que la traduction est initiée par P1 ou P2, différant ainsi de 32 acides aminés à leurs extrémités 5' (Figure 6A) [39]. Une troisième isoforme *RUNX1a* plus courte (250 acides aminés) est produite à partir de P2 puis par épissage alternatif au niveau de l'exon 7. Cette dernière isoforme est caractérisée par sa meilleure affinité pour l'ADN et son antagonisme sur l'activité de *RUNX1b* [40]. L'expression de ces 3 isoformes est finement régulée de manière spatio-temporelle au cours de l'embryogénèse : l'isoforme *RUNX1c* est exprimée lors de l'émergence des CSH définitives tandis que *RUNX1b* et dans une moindre mesure *RUNX1a* sont exprimées au cours de la différenciation hématopoïétique [41].

La partie N-terminale des 3 isoformes est caractérisée par la présence du RHD (*runt homology domain*) de 128 acides aminés hautement conservés dans l'évolution (Figure 6B) [39]. Le RHD est impliqué dans l'hétérodimérisation avec la sous-unité β du CBF et la liaison à l'ADN au niveau de sites spécifiques contenant la séquence PyGPyGGTPy (aussi appelée séquence PEBP2) (Figure 6C) [39]. Il intervient également dans les interactions avec d'autres protéines liant l'ADN tels que les facteurs de transcription de la famille Ets (PU.1), MYB ou CEBPα dont les recrutements sont favorisés par la présence de sites de liaison adjacents à la séquence PEBP2 [42]. La liaison entre l'ADN et le CBF est donc le fait de la sous-unité RUNX1. L'interaction avec la sous-unité CBFβ est néanmoins essentielle au fonctionnement *in vivo* de RUNX1 en augmentant son affinité pour l'ADN et en le protégeant de la protéolyse [31]. Ainsi, il est montré que les mutations de *CBFB* phénocopient celles de *RUNX1* [31].

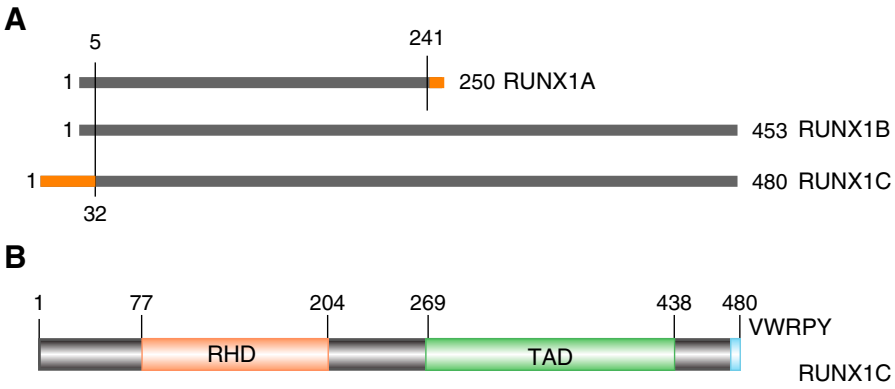

Figure 6 : Représentation schématique de la protéine RUNX1. (A) Isoformes RUNX1a, b et c. Les séquences qui différent entre les isoformes sont signalées en orange (B) Domaines fonctionnels RHD et TAD de RUNX1c. Les acides aminés 77 à 204 de RUNX1c correspondent aux acides aminés 50 à 177 de RUNX1b [39].

A l'inverse de l'isoforme *RUNX1a*, les isoformes *RUNX1b* et *RUNX1c* contiennent un domaine de transactivation (TAD, *transactivation domain*) dans leur partie C-terminale. Le TAD est impliqué dans les interactions avec d'autres protéines co-activatrices (EP300/CREBBP) ou plus rarement co-répressives transcriptionnelles (SIN3A) [42]. Leur recrutement est dépendant de plusieurs facteurs dont l'état de phosphorylation de RUNX1 et la concentration des différents cofacteurs [42]. Enfin les 5 derniers acides aminés des isoformes B et C forment le motif VWRPY interagissant avec le co-répresseur TLE1. Du fait de ses multiples interactions, RUNX1 contrôle l'expression de gènes clés de la différenciation hématopoïétique, parmi lesquels se retrouvent les gènes de l'interleukine-3, du récepteur du *macrophage-colony stimulating factor*, de la myélopéroxydase ou les gènes α du TCR [42]. Par ailleurs, RUNX1 intervient dans la biogénèse du ribosome, la régulation du cycle cellulaire et les voies de signalisation médiées par p53 ou encore le TGFβ (*transforming growth factor β*) [35,43].

3. Dérégulation du CBF

A. Translocations de RUNX1/CBFB

Si le rôle du CBF dans l'hématopoïèse normale est fondamental, il l'est également dans la leucémogénèse puisque *RUNX1* est l'un des gènes les plus fréquemment dérégulés en hématologie maligne. En plus de la **t(8;21)(q22;q22)** concernant à elle seule 5 à 15% des LAM *de novo*, plus de 50 translocations et plus de 20 partenaires différents de *RUNX1* ont pu être identifiés [44]. Parmi les plus fréquentes sont retrouvées la **t(12;21)(p12;q22)** récurrente dans les leucémies aigues lymphoblastiques (LAL) de l'enfant et la **t(3;21)(q26;q22)** décrite dans la leucémie myéloïde chronique en crise blas-

tique et les SMD/LAM chimio-induits générant respectivement les fusions *ETV6-RUNX1* et *RUNX1-MECOM* (Figure 7).

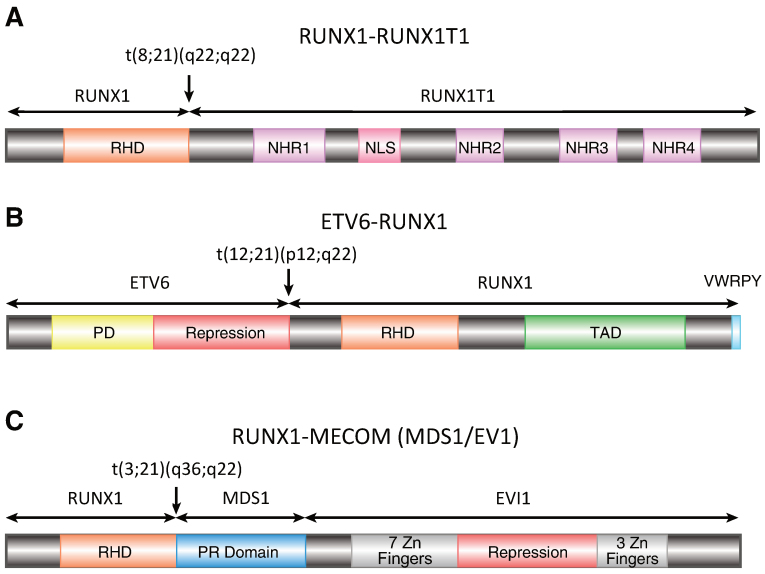

Figure 7 : Représentation schématique des principales protéines de fusion impliquant RUNX1 [39].

Bien qu'exceptionnelle (24 cas rapportés, généralement secondaires), la **t(16;21)(q24;q22)** relève d'un intérêt particulier [45,46]. Elle est responsable de la fusion de *RUNX1* et de *CBFA2T3,* membre de la famille ETO partageant un haut degré d'homologie avec *RUNX1T1*. Dans une étude récente, Lavallée et *al* ont montré que la t(16;21) partageait le profil d'expression génique de la t(8;21) [46]. A cela il faut ajouter la fusion *CBFB-MYH11* résultant de l'**inv(16)(p13q22)** qui perturbe également la protéine RUNX1 normale en la déplaçant de sa liaison avec le CBFβ sauvage [31]. Les caractéristiques des LAM associées à la t(8;21) et l'inv(16) sont détaillées dans la partie 3 (page 26).

La grande majorité des translocations impliquant *RUNX1* conduisent à la formation d'une protéine de fusion conservant la partie 5' de RUNX1 incluant le RHD (mais pas le TAD) fusionnée à la région 3' d'un gène partenaire. Seule la fusion *ETV6-RUNX1* fait exception puisque la partie 5' d'ETV6 y est fusionnée à la quasi totalité de RUNX1 conservant à la fois le RHD et le TAD. Les autres protéines de fusion exercent une partie de leurs effets leucémogènes par effet dominant négatif sur le facteur de transcription RUNX1 normal, en mimant l'effet du variant *RUNX1a* (variant dépourvu de TAD ayant une affinité augmentée pour l'ADN). Ainsi, les modèles murins *knock-in* (KI) pour *Runx1-Runx1t1* meurent rapidement au cours de l'embryogénèse, récapitulant le phénotype des souris KO homozygotes pour *Runx1* ou *Cbfb* [47]. Cependant, si les CSH sont totalement absentes des embryons déficients pour *Runx1* ou *Cbfb*, il est possible d'observer chez les embryons exprimant *Runx1-Runx1t1* la persistance de rares CSH « préleucémiques » aux capacités d'auto-renouvellement augmentées et de différenciation réduites [47,48]. Des données similaires sont générées avec les modèles KI de *Cbfb-*

Myh11 [49] et restent valables dans la moelle osseuse de souris adultes en utilisant des modèles KI inductibles permettant de s'affranchir de la mortalité embryonnaire [50]. De manière intéressante, l'expression de *Runx1-Runx1t1* ou *Cbfb-Myh11* dans ces modèles ne s'accompagne pas d'avantage prolifératif ni du développement d'une leucémie aiguë montrant la nécessité d'anomalies coopératrices [31].

On sait aujourd'hui que le partenaire de fusion contribue également à la leucémogénèse et la variabilité des phénotypes associés aux réarrangements de *RUNX1* [51]. Dans la protéine de fusion RUNX1-RUNX1T1, le TAD de RUNX1 est remplacé par la quasi totalité de RUNX1T1. *RUNX1T1* code pour une protéine nucléaire, homologue de la protéine Nervy de la drosophile, agissant comme répresseur transcriptionnel. La protéine RUNX1T1 est caractérisée par la présence d'un signal de localisation nucléaire (NLS) et de 4 domaines NHR (*nervy homology regions*). RUNX1T1 est impliqué dans le recrutement des histones désacétylases par ses interactions avec les protéines co-répressives NCOR et SIN3A [42]. En conséquence, au lieu d'activer la transcription, la protéine de fusion RUNX1-RUNX1T1 agit essentiellement comme un répresseur transcriptionnel tout en conservant sa capacité de liaison à CBFβ et aux séquences cibles de RUNX1 (Figure 8). De plus, il est démontré que certains effets de l'oncoprotéine RUNX1-RUNX1T1 sont directement supportés par les domaines NHR2 et 4 de RUNX1T1 [52]. Enfin, RUNX1-RUNX1T1 est capable de s'hétérodimériser avec RUNX1T1 et les autres membres de la familles ETO modifiant ainsi leurs fonctions physiologiques [50].

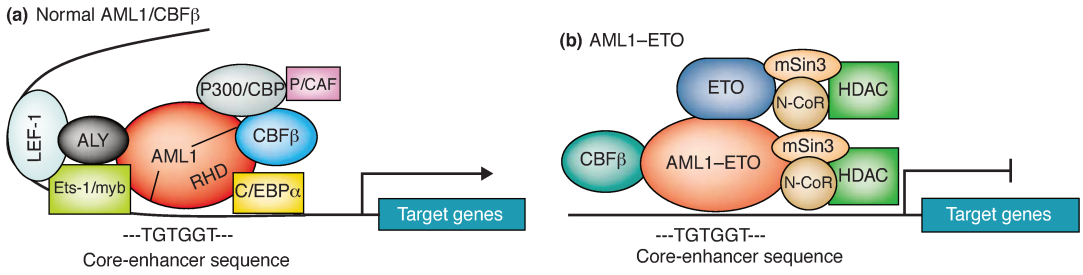

Figure 8 : Complexes protéiques recrutés par (a) l'hétérodimère RUNX1 (AML1)/CBFβ normal et (b) la protéine de fusion RUNX1-RUNX1T1 (AML1-ETO) [50].

Récemment, Justin Loke et *al* ont comparé les signatures moléculaires de *RUNX1-RUNX1T1* et *RUNX1-MECOM* [51]. En effet, si les 2 oncoprotéines partagent le même site de liaison à l'ADN (porté par RUNX1), elles se distinguent par leurs interactions protéiques. L'expression de chacune des protéines de fusion est responsable d'une inhibition du facteur de transcription CEBPα bloquant la différenciation myéloïde [53] mais RUNX1-MECOM s'associe davantage à une signature *CSH-like* dépendante de GATA2 tandis que celle de RUNX1-RUNX1T1 est dépendante de RUNX1 [51]. D'un point de vue clinique, ces différences peuvent expliquer, au moins en partie, le pronostic favorable des LAM

avec t(8;21) en comparaison au pronostic péjoratif des LAM avec t(3;21) dont la survie à 5 ans est inférieure à 15% [54].

B. Mutations/délétions de RUNX1

Les mutations somatiques ponctuelles de *RUNX1*, et plus rarement les délétions, ont été rapportées pour la première fois en 1999 dans les LAM par Motomi Osato et *al*, soit 8 ans après la première mise en évidence de *RUNX1* comme partenaire de translocation [55]. La même année, Woo-Joo Song et *al* identifient des mutations et délétions germinales de *RUNX1* comme responsables de thrombopénies familiales avec prédisposition au développement d'hémopathies malignes (FPD/AML) [56]. A ce jour, plus de 70 familles ont été décrites [39]. Une description est donnée dans la partie 4 (page 60).

Les mutations de *RUNX1* sont schématiquement divisées en 2 catégories : les mutations faux-sens touchent essentiellement le domaine Runt (exons 3 à 5) et interfèrent avec la liaison à l'ADN tandis que les mutations non-sens et frameshift conduisant à la formation d'une protéine tronquée sont distribuées sur tout le gène (Figure 9) [57]. L'étude de modèles murins présentant une haploinsuffisance de *RUNX1* met en évidence un effet dose sur l'hématopoïèse avec une modification de la balance entre CSH et progéniteurs engagés [58].

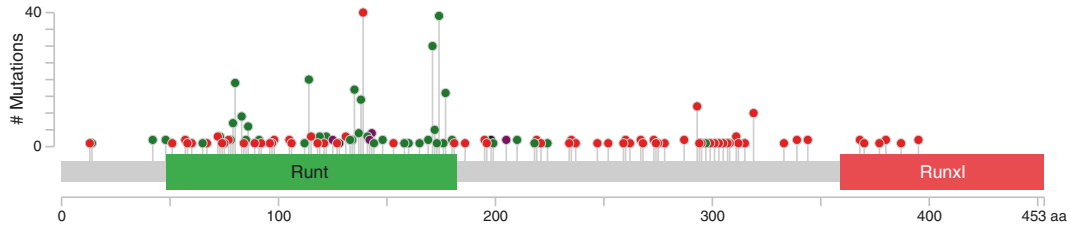

Figure 9 : Spectre des mutations de *RUNX1* dans les LAM (isoforme RUNX1b). Les mutations faux-sens sont en vert. Les mutations non-sens ou frameshift sont en rouge. Données extraites de COSMIC (Catalogue of Somatic Mutations in Cancer).

L'incidence des mutations de *RUNX1* dans les LAM varie largement d'une étude à l'autre, allant de 3% dans une série de LAM pédiatriques [59] à 33% dans une cohorte d'adultes atteints de LAM à caryotype non complexe [60]. Il est probable que ces larges variations soient le reflet des caractéristiques différentes des patients étudiés (en termes d'âges, de groupes cytogénétiques et du caractère primaire ou secondaire des LAM), de la méthode utilisée (Sanger *vs*. Séquençage à haut débit) et de la région du gène étudiée (les études les plus anciennes s'étant généralement limitées au domaine *runt*). Plus récemment, l'étude de larges cohortes de LAM rapportait une fréquence de mutations somatiques de 5 à 15% [61–64] avec un association significative aux LAM0 de la classification FAB et aux LAM avec trisomie 21 ou trisomie 13 [61,65,66] bien que le caryotype soit normal dans environ la

moitié des cas. D'un point de vue moléculaire, les mutations de *RUNX1* sont significativement associées aux mutations d'*ASXL1* et exclusives de celles de *NPM1* [67,68].

En 2016, l'association de certaines caractéristiques clinico-biologiques (âge avancé, prédominance masculine, cytologie immature, anomalies cytogénétiques et moléculaires associées), d'un profil d'expression génique particulier [68] et d'un pronostic globalement défavorable [61] valent aux LAM avec mutations de *RUNX1* de figurer comme une entité génétique distincte des classifications de l'OMS et de l'ELN [2,8]. Outre les LAM, on sait aujourd'hui que les mutations de *RUNX1* sont également impliquées dans la physiopathologie des SMD, de la leucémie myélomonocytaire chronique (LMMC), des SMD/LAM chimio-induites ou encore de certaines LAL [69–72].

C. *Amplification intrachromosomique du chromosome 21 (iAMP21)*

Le gène *RUNX1* est plus rarement impliqué dans les amplifications intrachromosomiques du chromosome 21 (iAMP21). L'iAMP21 concerne environ 2% des LAL-B et définit un sous-groupe de LAL-B de pronostic défavorable en l'absence d'intensification thérapeutique [73]. Il est constamment retrouvé dans la région la plus amplifiée, permettant de l'utiliser comme marqueur en FISH (5 copies de *RUNX1* ou plus définissant l'iAMP21 ; Figure 10) [74]. Cependant, si *RUNX1* constitue un bon candidat, aucune preuve ne permet d'affirmer son rôle initiateur dans l'iAMP21 et d'autres gènes cibles pourraient être impliqués [73].

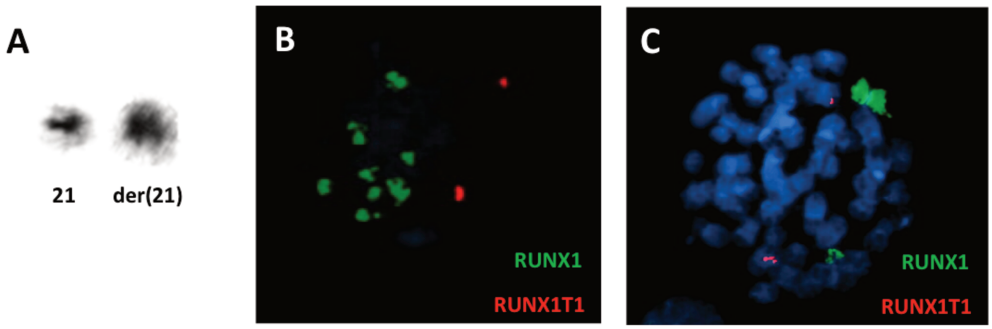

Figure 10 : Caractérisation de l'amplification intrachromosomique du chromosome 21 (iAMP21) dans un cas de LAL-B. (A) Chromosomes 21 au caryotype conventionnel. (B) FISH interphasique et (C) FISH métaphasique avec marquage de *RUNX1* (vert) et *RUNX1T1* (rouge) [74].

PARTIE 3 - LES LAM À CORE BINDING FACTOR (CBF)

1. Epidémiologie des LAM CBF

Les LAM CBF sont des affections du sujet jeune. Leur incidence diminue avec l'âge, inversement aux caryotypes normaux et aux cytogénétiques défavorables (Figure 11) [75,76]. La fréquence de la t(8;21)/fusion *RUNX1-RUNX1T1* dans les LAM de l'adulte est estimée à 7-8% [10–12]. Elle est exceptionnelle chez le nourrisson mais représente 11 à 14% des LAM de l'enfant et de l'adolescent, ce qui en fait l'anomalie chromosomique récurrente la plus fréquente dans cette classe d'âge [77–79]. La fréquence de l'inv(16) ou t(16;16)/fusion *CBFB-MYH11* est quant à elle estimée à 5-9% dans les LAM de l'adulte et 6-8% dans les LAM de l'enfant et de l'adolescent [10–12].

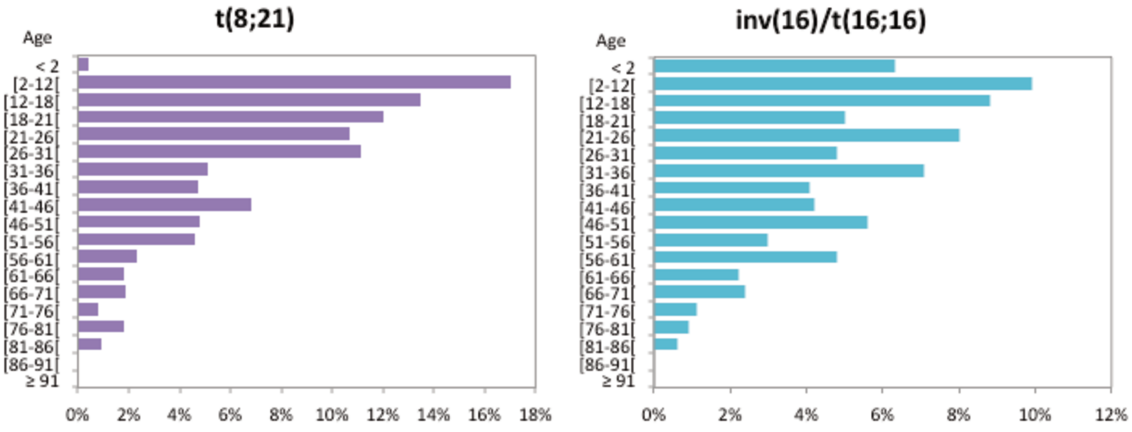

Figure 11 : Proportions de patients atteints de LAM présentant une t(8;21) ou une inv(16)/t(16;16) selon l'âge (figure adaptée de [76]).

2. Présentation clinico-biologique

La présentation clinique des LAM CBF est analogue à celles des autres LAM, dominée par les signes d'insuffisance médullaire avec néanmoins une fréquence plus élevée de sarcomes granulocytaires, plutôt en association avec la t(8;21) [80,81]. Les localisations extramédullaires sont plus fréquentes dans les LAM avec inv(16), le plus souvent sous la forme d'infiltrations gingivales, d'hématodermies et d'adénopathies [82] (Table 5). La leucocytose est variable avec une médiane au diagnostic d'environ 10 G/l dans la LAM avec t(8;21) et de 40 G/l dans les LAM avec inv(16) (Table 5). La blastose sanguine et médullaire est variable, parfois inférieure à 20% pouvant faire discuter le diagnostic différentiel de myélodysplasie. A ce stade, il est important de rappeler que l'identification d'une t(8;21) ou d'une inv(16) pose le diagnostic de LAM quel que soit le pourcentage de blastes selon les

critères de l'OMS [8]. D'un point de vue morphologique, les LAM avec t(8;21) se présentent typiquement sous forme de LAM2 (LAM avec maturation), rarement LAM1 ou LAM4. Les LAM avec inv(16) sont associées aux LAM4 (LA myélomonocytaires) avec éosinophiles médullaires anormaux (LAM4Eo) ou plus rarement LAM2Eo lorsque le contingent monocytaire est réduit.

Table 5 : Caractéristiques clinico-biologiques des LAM CBF au diagnostic (table adaptée de [83]).

	LAM avec t(8;21)	LAM avec inv(16)	p-value
Hémoglobine *(median, range)*	8.6 g/dl (3.5-13.4)	9.2 g/dl (3.1-14.8)	0.03
Leucocytes *(median, range)*	10.5 G/l (0.9-252)	37.7 G/l (2.2-500)	<0.001
Plaquettes *(median, range)*	40 G/l (6-311)	42 G/l (5-272)	0.72
Blastose sanguine *(median, range)*	41% (0-96)	56% (0-97)	<0.001
Blastose médullaire *(median, range)*	54% (20-95)	60% (14-93)	0.005
Type FAB			<0.001
LAM1	6%	0%	
LAM2	91%	12%	
LAM4/M4Eo	3%	88%	
Manifestations extramédullaires	22%	41%	<0.001
Envahissement du SNC	2%	1%	
Hépatomégalie	8%	13%	
Splénomégalie	4%	16%	
Adénopathies	7%	22%	
Localisations cutanées	6%	11%	
Hypertrophie gingivale	4%	15%	

Les blastes de LAM avec t(8;21) sont classiquement de grande taille avec un cytoplasme basophile abondant contenant de nombreuses granulations azurophiles et une large zone claire périnucléaire (correspondant à l'appareil de Golgi). La présence d'un corps d'Auer unique de grande taille se disposant en travers de l'appareil de Golgi (aspect en « boussole ») est caractéristique (Figure 12A). Il existe parfois de volumineuses granulations de type pseudo-Chediak. Les cellules de la lignée granulocytaire neutrophile montrent généralement une dysgranulopoïèse particulière associant des défauts de segmentation du noyau et des anomalies de coloration ou de vacuolisation du cytoplasme (Figure 12B) [84,85]. Les éosinophiles médullaires sont souvent augmentés sans toutefois présenter d'atypies morphologiques. L'immunophénotypage des blastes montre une expression forte des antigènes myéloïdes ainsi qu'une expression aberrante du CD19 et/ou du CD56 dans 60 à 80% des cas [86,87]. A l'inverse, les LAM avec inv(16) ou t(16;16) sont associées à une maturation éosinophile anormale. Les cellules leucémiques associent typiquement des blastes myéloïdes ainsi qu'une composante monocytaire (Figure 12C). La moelle osseuse montre des éosinophiles habituellement augmentés, à tous stades de maturation et caractérisés par la présence de grosses granulations pourpre-violet surtout visibles aux stades promyélocytes et myélocytes (Figure 12D) [84,88]. Ils sont exceptionnels sur le frottis sanguin. L'immunophénotypage des blastes montre souvent l'expression aber-

rante (mais non spécifique) du CD2 associée aux marqueurs granulocytaires et monocytaires habituels [84].

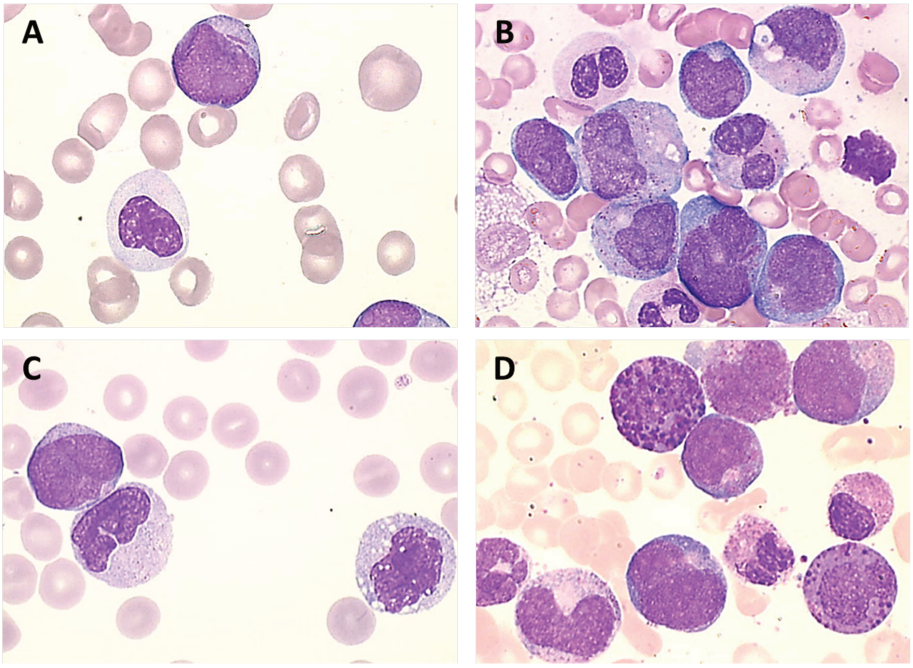

Figure 12 : Aspects cytologiques classiques des LAM CBF. (A) LAM avec t(8;21), sang ; (B) LAM avec t(8;21), moelle ; (C) LAM avec inv(16), sang ; (D) LAM avec inv(16), moelle.

Le diagnostic évoqué par la morphologie et l'immunophénotypage doit être confirmé par la cytogénétique conventionnelle et la biologie moléculaire. La t(8;21) est facilement visualisée au caryotype. Les variantes cytogénétiques sont possibles mais rares. L'inv(16) est d'identification plus délicate et nécessite parfois d'avoir recours à la FISH. La RT-PCR réalisée sur le sang et/ou la moelle permet de mettre en évidence les transcrits de fusion *RUNX1-RUNX1T1* ou *CBFB-MYH11*. Les points de cassure de *RUNX1* sont toujours situés dans l'intron 5 et ceux de RUNX1T1 se localisent dans les introns 1a et 1b donnant lieu à un seul transcrit de fusion possible juxtaposant l'exon 5 de *RUNX1* et l'exon 2 de *RUNX1T1* [89]. A l'inverse, plus de 10 transcrits de fusion *CBFB-MYH11* différents sont décrits. Le transcrit de type A (juxtaposant l'exon 5 de *CBFB* et l'exon 12 de *MYH11*) concerne 85% des cas, suivi des transcrits de type D (*CBFB* exon 5 et *MYH11* exon 8) et de type E (*CBFB* exon 5 et *MYH11* exon 7) chacun identifié dans environ 5% des cas, les autres étant sporadiques [90]. L'identification du type de transcrit est primordiale puisque les fusions *RUNX1-RUNX1T1* et *CBFB-MYH11* (formes A, D et E) peuvent être quantifiées au cours du traitement afin d'évaluer la maladie résiduelle (cf. page 35). Ces taux sont généralement interprétés en log-réduction par rapport au diagnostic et sont prédictifs du risque de rechute [91].

3. Anomalies cytogénétiques secondaires des LAM CBF

Des anomalies cytogénétiques additionnelles sont détectées au caryotype dans environ 70% des LAM avec t(8;21) et 40% des LAM avec inv(16) (Figure 13) [83,92,93]. Certaines de ces anomalies sont exceptionnelles en dehors des LAM CBF et diffèrent d'une entité à l'autre, suggérant un rôle leucémogène particulier coopérant avec le réarrangement du CBF.

La perte d'un chromosome sexuel est de loin l'anomalie la plus fréquemment rencontrée dans les LAM avec t(8;21). La perte des chromosomes X et Y concernent respectivement 30 à 40% des femmes et 50 à 60% des hommes. La seconde anomalie par ordre de fréquence correspond à la délétion du bras long du chromosome 9 [del(9q)] rapportée dans 15 à 20% des LAM avec t(8;21) [83,92,93]. Ces deux anomalies sont par ailleurs très rarement retrouvées dans les autres sous-types de LAM, y compris les LAM avec inv(16).

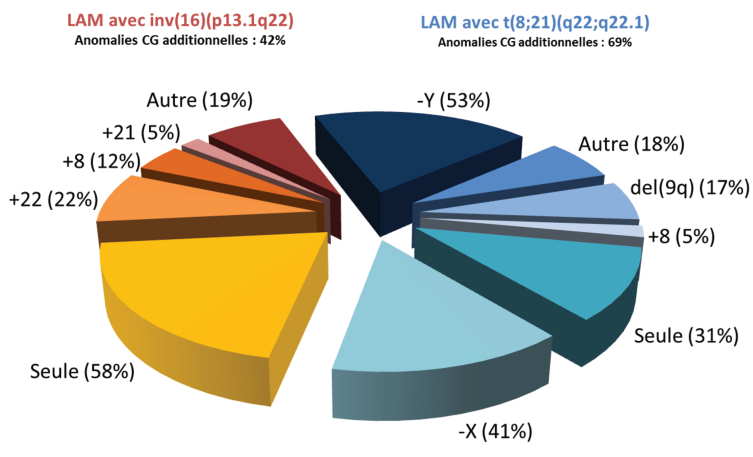

Figure 13 : Anomalies cytogénétiques additionnelles rencontrées dans les LAM CBF (figure adaptée de [92]).

A l'inverse, la trisomie 22 est rapportée dans 15 à 20% des LAM avec inv(16), constituant l'anomalie chromosomique additionnelle la plus fréquente dans ce sous-groupe tandis qu'elle n'est jamais retrouvée dans d'autres sous-types de LAM [94]. Ainsi, l'identification d'une trisomie 22, facilement visible au caryotype, impose la recherche d'un réarrangement cryptique des gènes *CBFB* et *MYH11* par FISH ou RT-PCR. La trisomie 21 est quant à elle observée dans 5% des LAM avec inv(16) alors qu'elle est très rare dans les LAM avec t(8;21).

Les autres anomalies fréquentes incluent la trisomie 8 et la délétion du bras long du chromosome 7 [del(7q)] rencontrées dans les 2 sous-groupes de LAM CBF. La trisomie 8 est identifiée dans 5-7% et 10-15% des LAM avec inv(16) et LAM avec t(8;21) respectivement. La del(7q) est rapportée dans 5-10% des LAM avec t(8;21) et LAM avec inv(16). Cependant, près de la moitié des del(7q) ne seraient

pas identifiées par cytogénétique conventionnelle en raison de leur petite taille, nécessitant l'utilisation de techniques plus résolutives (FISH, CGH-array, SNP-array) [95]. Enfin, la trisomie 4 a été exceptionnellement observée, conduisant à la duplication des mutations de *KIT* (en 4q12), lesquelles sont très fréquentes dans les LAM CBF [96].

L'impact pronostique de ces anomalies a été rapporté et débattu par différentes études, parfois contradictoires. Dans les séries plus récentes, aucune anomalie cytogénétique additionnelle ne semble modifier significativement le pronostic de la maladie à l'exception de la trisomie 22 qui influence positivement celui des LAM avec inv(16). Ces données sont discutées dans la revue « *Prognosis and monitoring of core binding factor acute myeloid leukemia: current and emerging factors* » (page 36).

4. Anomalies moléculaires secondaires des LAM CBF

Les LAM CBF sont considérées comme un modèle de leucémogénèse. Elles illustrent parfaitement la théorie « multi-étapes » énoncée par Gary Gilliland au début des années 2000 dans laquelle la LAM est le résultat d'une coopération entre une anomalie conférant un avantage prolifératif et une anomalie ciblant un facteur de transcription clé (tel que le CBF) responsable d'un blocage de différenciation [16,21]. La validation *in vivo* de ce modèle est venue de l'observation de la haute fréquence des mutations activant de manière constitutive des récepteurs à activité tyrosine kinase (RTK) ou leurs voies de signalisation en aval dans les LAM CBF.

Deux gènes codant pour des RTK de type III, les gènes *KIT* et *FLT3*, sont particulièrement impliqués dans la physiopathologie des LAM CBF. Les mutations du gène *KIT* sont retrouvées dans 30 à 40% des LAM CBF [97–101] tandis qu'elles concernent moins de 2% des autres sous-types cytogénétiques. Il s'agit de mutations activatrices affectant le domaine extracellulaire du récepteur (exon 8), le domaine tyrosine kinase (exon 17) ou plus rarement le domaine juxta-membranaire (exon 11). Les duplications internes en tandem de *FLT3* (*FLT3*-ITD) sont plutôt rares dans les LAM CBF. Elles ne concernent que 5 à 10% des cas tandis que leur fréquence approche 40% dans les LAM à caryotype normal et les LAP [102]. En revanche, les mutations ponctuelles du domaine kinase (*FLT3*-TKD) sont rapportées dans environ 20% des LAM avec inv(16) mais moins de 10% des LAM avec t(8;21) [97–101]. Les mutations de *KIT* et *FLT3* ont été parmi les plus étudiées dans les LAM CBF. Elles sont globalement associées à une leucocytose plus importante au diagnostic et à un risque de rechute supérieur, bien que cet impact ne soit pas constamment retrouvé [103]. Certains auteurs ont suggéré que le pronostic défavorable des mutations des RTK était corrélé à des ratios mutationnels élevés [104]. Ces résul-

tats sont discutés dans la revue « *Prognosis and monitoring of core binding factor acute myeloid leukemia: current and emerging factors* » (page 36).

Les mutations des gènes *RAS* (*NRAS*, *KRAS*, *HRAS*), codant pour des protéines intracellulaires intervenant dans la transduction du signal, sont rapportées avec une haute fréquence dans les LAM CBF. En particulier, on les retrouve dans 30 à 50% des LAM avec inv(16) et dans 10 à 20% des LAM avec t(8;21) [97–101]. Enfin, d'autres altérations moins fréquemment retrouvées incluent les mutations de *JAK2*, *CBL* ou encore *PTPN11.* Avec l'essor des techniques de séquençage à haut débit, il est devenu possible d'apprécier en routine la charge allélique des différentes mutations. Les mutations de type tyrosine kinase apparaissent comme des événements tardifs et il est fréquent d'observer la coexistence de plusieurs mutants chez un même patient reflétant différents sous-clones de la maladie. Aussi, il n'est pas rare d'observer l'émergence ou la disparition de certaines de ces mutations lors des rechutes.

5. Traitement des LAM CBF

A. Données générales

Comme dans les autres sous-groupes de LAM, la chimiothérapie d'induction repose essentiellement sur un schéma « 3+7 » associant une anthracycline et la cytarabine. La daunorubicine à la dose de 60 mg/m² est la plus souvent utilisée. Dans une large étude randomisée du groupe anglais, l'augmentation de dose à 90 mg/m² ne montrait pas de différence significative sur la survie globale [105]. De même, une analyse rétrospective française ne montrait pas de différence significative entre les 2 doses en termes de survie globale bien que la dose de 90 mg/m² soit associée à une diminution du risque de rechute [106]. Le protocole français CBF2006 (cf. page 33) a randomisé un schéma d'induction traditionnel « 3+7 » (renforcé par une 2ème cure en cas de blastose persistante à J15), et un schéma plus intensif d'induction séquentielle systématique, sans démontrer d'avantage en termes d'incidence cumulée de rechute et de survie globale [91]. De façon remarquable, les LAM CBF sont caractérisées par des taux de RC de l'ordre de 90 à 95%, supérieurs à ceux généralement observés dans les LAM [107,92,83,108,109,93,91]. Les taux de survie à 5 ans oscillent entre 50 et 70% [107,92,83,108,109,93,91], la récidive de la maladie étant la principale cause de décès.

Bien que peu d'études randomisées aient posé la question, il est admis que les LAM CBF bénéficient de la cytarabine à hautes doses (HDAC) en consolidation [110–113]. Dès la fin des années 1990, le groupe américain (CALGB) montrait qu'une phase de consolidation incluant la cytarabine à hautes

doses (3000 mg/m²/12h à J1, J3 et J5) prolongeait particulièrement la survie sans maladie des patients atteints de LAM CBF en comparaison à des schémas comprenant de la cytarabine à des doses plus faibles (100 à 400 mg/m²/j pendant 5 jours) [110]. Le même groupe démontrait peu de temps après l'impact favorable de la multiplication des cycles de HDAC (en règle 3 à 4) par rapport à un seul cycle chez ces patients [111,112]. Enfin, l'impact favorable sur la survie sans maladie était confirmé par une étude japonaise randomisée comparant l'administration de 3 cycles de HDAC à 4 cycles de polychimiothérapie (incluant la cytarabine à doses plus faibles) [113].

L'allogreffe de CSH n'a quant à elle montré aucune supériorité dans la prise en charge des patients en première RC (RC1). Il n'est donc pas justifié de prendre le risque de mortalité et d'effets secondaires liés à une transplantation en RC1. En situation de rechute, le pronostic des LAM CBF reste globalement favorable avec des taux de deuxième RC (RC2) satisfaisants de l'ordre de 85 à 90% [114]. Comme dans les autres sous-groupes de LAM, l'allogreffe de CSH reste indiquée en RC2 [115]. Néanmoins, la persistance d'une bonne survie à 5 ans en RC2, y compris chez les patients ne recevant pas d'allogreffe, fait discuter son indication par certaines équipes. Notamment, si le protocole CBF2006 prévoyait l'indication d'allogreffe de CSH chez les patients « mauvais répondeurs moléculaires », c'est-à-dire dont la diminution de la maladie résiduelle (MRD) était inférieure à 3 logs avant la 2ème cure de consolidation, seuls 12 des 52 patients à risque furent allogreffés [91].

Plus récemment, l'essai AML-15 du groupe anglais (MRC) a montré un bénéfice, en termes d'amélioration de la survie globale, de l'adjonction du gemtuzumab ozogamicin (GO : anticorps anti-CD33 couplé à la calichéamycine) dans les LAM CBF (survie à 5 ans : 79% *vs.* 51% chez les patients recevant du GO et ne recevant pas de GO respectivement ; p=0.0003) [116]. L'intérêt du GO dans les LAM CBF en rechute a également été suggéré par une étude rétrospective française. Dans cette série de 145 patients, l'utilisation du GO n'avait pas d'incidence sur les taux de RC2 mais s'associait à une amélioration de la survie sans maladie (68% *vs.* 42% ; p=0.05) et de la survie globale (65% *vs.* 44% ; p=0.02) [114]. Enfin, étant donné la forte prévalence des mutations de *KIT* dans les LAM CBF, l'utilisation des inhibiteurs de tyrosine kinase (ITK) constitue une piste intéressante pour diminuer le risque de rechute. Récemment le protocole français, DasaCBF n'a cependant pas permis de mettre en évidence d'amélioration de la survie sans récidive dans un sous-groupe de patients « mauvais répondeurs moléculaires » traité en entretien par dasatinib [117]. L'utilisation plus précoce des ITK, en association à la chimiothérapie d'induction et/ou de consolidation, nécessite par ailleurs d'être évaluée [118].

B. *Protocole ELAM02*

L'essai clinique ELAM02 (ClinicalTrials.gov NCT00149162) est un protocole multicentrique, prospectif et randomisé, évaluant l'intérêt d'un traitement d'entretien par interleukine-2 (IL2) dans les LAM de l'enfant et de l'adolescent. Au total, 438 patients de moins de 18 ans ont été inclus entre mars 2005 et décembre 2011 par 28 centres français. Parmi eux, 98 (22%) étaient atteints d'une LAM CBF.

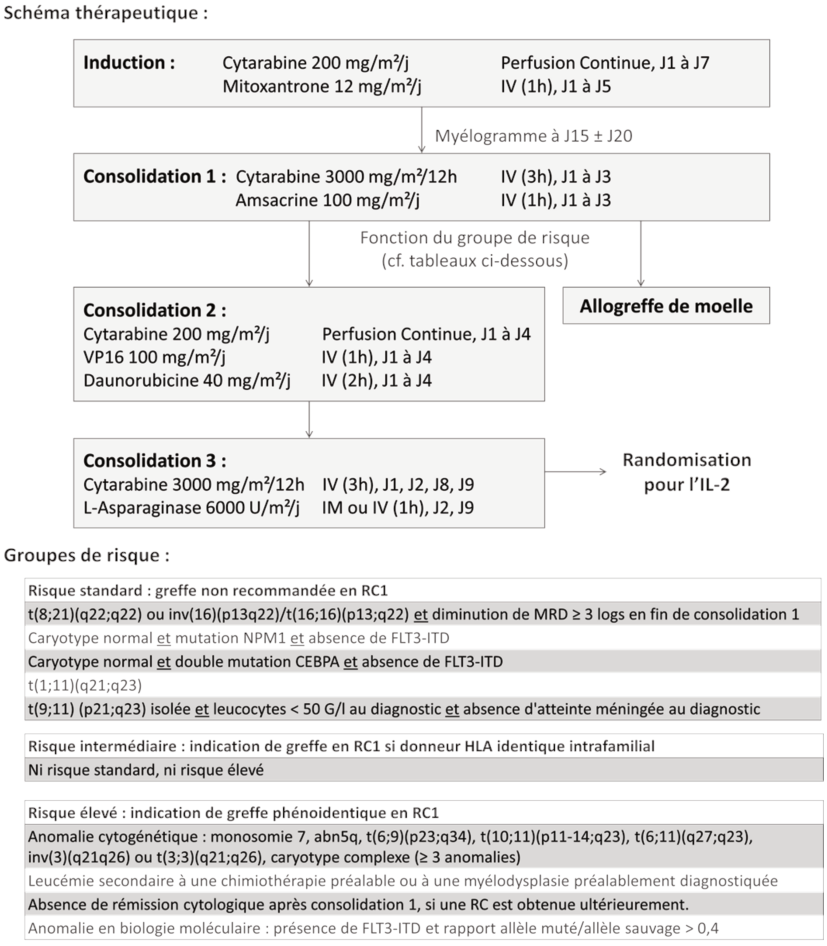

Figure 14 : Schéma thérapeutique et groupes de risque du protocole ELAM02.

Les patients recevaient d'abord un traitement d'induction associant cytarabine et mitoxantrone avec évaluation de la chimiosensibilité par un myélogramme à J15, éventuellement répété au J20 (Figure 14). La 1ère cure de consolidation associait HDAC et amsacrine. Deux autres cures de consolidation étaient réalisées chez les patients de risque standard (translocation t(8;21) ou autres anomalies cyto-génétiques et moléculaires de pronostic favorable[1]) ainsi que chez les patients de risque intermédiaire ne disposant pas de donneur familial HLA identique (Figure 14). Une injection intrathécale

[1] Actualisation en 2010 : ne concernait que les LAM avec t(8;21) lors de la mise en place du protocole.

triple était réalisée chez tous les patients à J1 du traitement d'induction et 3 autres injections étaient réalisées à titre prophylactique pour les LAM à composante monocytaire et les formes hyperleucocytaires > 50 G/l. Une allogreffe de moelle géno-identique en RC1 était recommandée pour les patients de risque intermédiaire ayant un donneur familial HLA identique, après la 1ère cure de consolidation. Dans le groupe des patients de risque élevé, la greffe en RC1 était indiquée en présence d'un donneur HLA identique intrafamilial mais également avec un donneur non apparenté ou un greffon de sang placentaire.

Le traitement d'entretien par IL2 était randomisé pour les patients en RC persistante à l'issue de l'ensemble de la chimiothérapie. L'IL2 était administrée en cures de 5 jours par mois pendant 1 an.

C. Protocole CBF2006

Le protocole CBF2006 (EudraCT #2006-005163-26; ClinicalTrials.gov ID #NCT00428558) est une étude française de phase III, multicentrique, prospective et randomisée, comparant une chimiothérapie d'induction séquentielle systématique à une chimiothérapie d'induction séquentielle conditionnelle chez des patients atteints d'une LAM du groupe CBF. Les patients âgés de 18 à 60 ans ont été inclus entre juillet 2007 et novembre 2010 par 46 centres des groupes ALFA et GOELAMS/FILO. Au total, 198 patients randomisés dans deux bras (Figure 15) étaient évaluables.

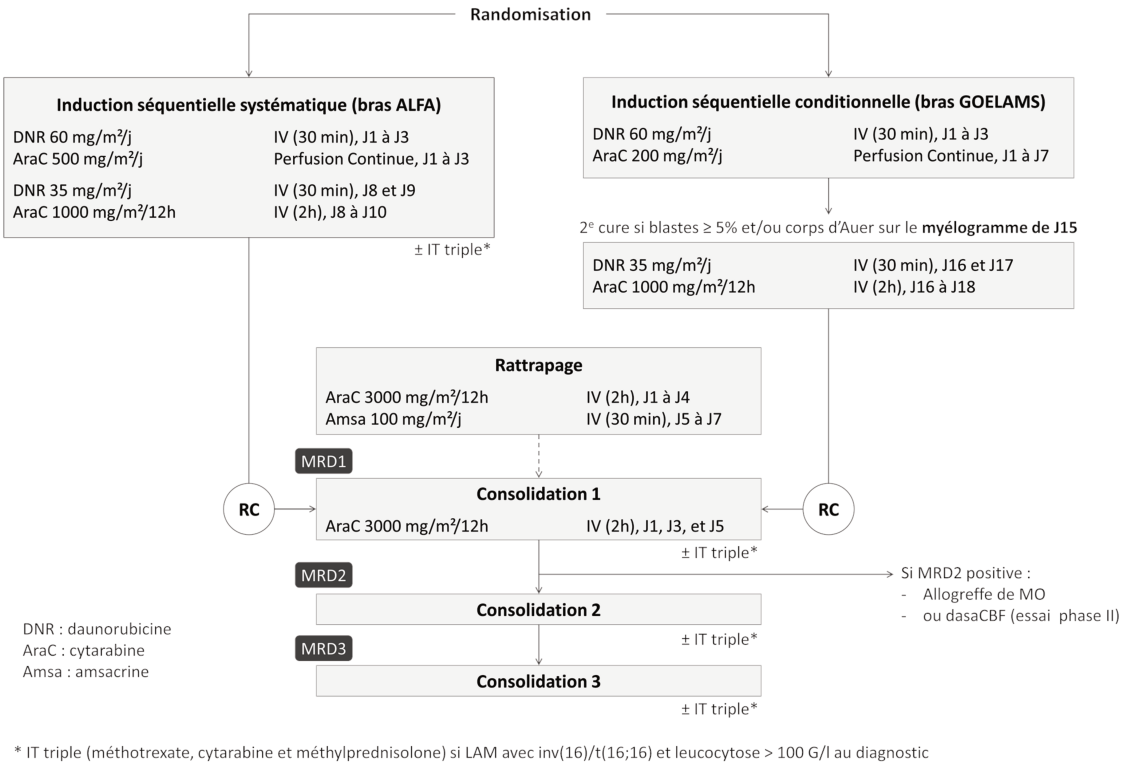

Figure 15 : Schéma thérapeutique du protocole CBF2006.

Les patients du bras ALFA recevaient une 1ère cure de J1 à J3 comprenant daunorubicine et cytarabine suivie systématiquement d'une 2ème cure à J8 comprenant les mêmes drogues (les doses et modalités d'administration apparaissent dans la Figure 15). Les patients du bras GOELAMS recevaient une 1ère cure de daunorubicine (J1 à J3) et cytarabine (J1 à J7). La 2ème cure (identique au bras ALFA) était administrée à J16 seulement chez les patients dont le myélogramme de J15 montrait une blastose ≥ 5 % ou la présence de corps d'Auer. En cas d'échec d'induction séquentielle évalué par le myélogramme en sortie d'aplasie, les patients recevaient une cure de rattrapage par HDAC puis amsacrine. Les patients atteignant la RC à la fin de l'induction ou de la cure de rattrapage recevaient 3 cures mensuelles de consolidation par HDAC. Ceux présentant une LAM avec inv(16)/t(16;16) et une leucocytose > 100 G/l recevaient en plus 4 injections intrathécales triples (méthotrexate, cytarabine et méthylprednisolone à l'induction et à chaque cure de consolidation) à titre prophylactique.

La MRD était évaluée dans le sang et la moelle osseuse, par quantification du transcrit *RUNX1-RUNX1T1* ou *CBFB-MYH11* en RQ-PCR au moment de la RC (après l'induction séquentielle ± la cure de rattrapage ; MRD1) et après chaque cure de consolidation (MRD2, MRD3 et MRD4). Les patients n'atteignant pas une réduction de la MRD de plus de 3 logs avant la 2ème cure de consolidation (MRD2) étaient candidats à l'allogreffe de CSH s'ils avaient un donneur HLA identique ou non apparenté compatible 10/10. En cas d'impossibilité de greffe allogénique, ils pouvaient être orientés vers l'essai DasaCBF (étude de phase II testant le dasatinib) après les 3 cures de consolidation.

6. Pronostic et suivi des LAM CBF

Depuis la fin des années 1990, les LAM CBF sont reconnues par l'ensemble des grands groupes coopérateurs [107,92,83,108,109,93,91] comme un groupe cytogénétique de pronostic favorable associé à une bonne réponse à la chimiothérapie. Malheureusement, leur évolution reste marquée par un taux de rechutes de 30 à 40%. L'identification de facteurs pronostiques est donc essentielle dans ce sous-groupe où, du fait du pronostic favorable, l'intensification du traitement n'est pas envisagée en 1ère intention. A côté des paramètres classiques liés au patient (âge, comorbidités) et à la maladie (leucocytose, caractère secondaire de l'hémopathie, facteurs cytogénétiques), les anomalies moléculaires (en particulier les mutations activatrices des récepteurs à activité tyrosine kinase *KIT* ou *FLT3*) et l'utilisation des transcrits de fusion *RUNX1-RUNX1T1* et *CBFB-MYH11* comme marqueurs de maladie résiduelle (MRD) ont fait et font toujours l'objet d'investigations en recherche clinique.

Cette partie de l'introduction est discutée dans la revue « *Prognosis and monitoring of core-binding factor acute myeloid leukemia: current and emerging factors »* présentée ci-après (page 36).

Prognosis and monitoring of core binding factor acute myeloid leukemia: current and emerging factors

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Abstract: Core binding factor acute myeloid leukemia (CBF-AML) – including AML with t(8;21) and AML with inv(16) – accounts for about 15% of adult AML and is associated with a relatively favorable prognosis. Nonetheless, relapse incidence may reach 40% in these patients. In this context, identification of prognostic markers is considered of great interest. Due to similarities between their molecular and prognostic features, t(8;21) and inv(16)-AML are usually grouped and reported together in clinical studies. However, considerable experimental evidences highlighted they represent two distinct entities and should be considered separately for further studies. This review summarizes recent laboratory and clinical findings in this particular subset of AML and how they could be used to improve management of patients in routine practice.

Keywords: Core binding factor, *RUNX1-RUNX1T1*, *CBFB-MYH11*, acute myeloid leukemia, minimal residual disease, mutational analysis, prognostic markers.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous group of hematological malignancies characterized by clonal accumulation and expansion of immature myeloid cells within the bone marrow. Among various clinical and biological aspects of AML, cytogenetic and molecular features are the most discriminating parameters to define specific diseases entities and are the basis of current classifications. Core binding factor (CBF)-AML includes two major AML subtypes defined by the presence of t(8;21)(q22;q22) chromosomal translocation or inv(16)(p13q22)/t(16;16)(p13;q22) chromosomal rearrangement [abbreviated t(8;21) and inv(16)], leading respectively to the *RUNX1-RUNX1T1* (*AML1-ETO*) and *CBFB-MYH11* fusion genes. CBF-AML is among the most common cytogenetic subtype of AML since t(8;21) and inv(16) account together for about 15-20% of adult de novo AML cases, predominantly in younger patients. Their identification is critical as it significantly impacts on their

subsequent management. Importantly, according to the World Health Organization (WHO) classification of hematological neoplasms, patients with t(8;21) or inv(16) should be considered to have AML regardless the percentage of blasts [1].

Since their first description, respectively in 1973 [2] and 1983 [3], numerous experimental evidences have revealed the molecular basis of the t(8;21) and inv(16) rearrangements. In t(8;21), *RUNX1* breakpoints are located within intron 5, while *RUNX1T1* breakpoints occur upstream of exon 2, giving rise to a single type of *RUNX1-RUNX1T1* fusion transcript (**figure 1a**) [4]. On the other hand, more than 10 differently sized *CBFB-MYH11* fusion transcript variants have been reported (**figure 1b**) [5,6]. More than 85% of fusions are type A. Type D and E transcripts each represent 5-10%, while other types occur in sporadic cases. Both recurrent genetic abnormalities result in disruption of genes encoding subunits of the CBF, a heterodimeric transcription factor involved in expression of various genes specific to normal hematopoiesis including M-CSF receptor, IL-3, myeloperoxidase or TCR genes [7,8]. The CBF complex consists of three distinct DNA-binding CBFα proteins (*RUNX1*, *RUNX2* and *RUNX3*) and a common non-DNA-binding CBFβ protein (encoded by *CBFB*). The α subunits share, in the N-terminal part, a highly evolutionary conserved domain of 128 amino acids called the RUNT homology domain which is essential for binding to the DNA targets and heterodimerization with the β subunit, and a transactivation domain in the C-terminal part [9,10]. *RUNX1* was the first CBF mammalian gene to be isolated and has been known by a number of names including *AML1*, *CBFA2* or *PEBPA2B*. It was renamed by the Human Genome Organization (HUGO) in 1999 and is now formally known as Runt-related transcription factor 1 (RUNX1). The β subunit does not contact DNA directly but significantly increases RUNX1 binding affinity for DNA and protects it from proteolysis. The RUNX1/CBFβ complex is required for hematopoietic stem cell emergence during development and is a key regulator of hematopoiesis [7,8]. Thus, homozygous disruption of *RUNX1* or *CBFB* in mice results in identical developmental defects. These include failure to develop definitive hematopoiesis and embryonic death [11–13].

Importantly, patients with t(8;21) or inv(16)-AML seem to differ with respect to several biologic and clinical features [14]. Gene expression profiling studies showed that they segregated into distinct subgroups according to their gene expression patterns [15,16], that could reflect the apparent clinical and biological differences at diagnosis of such AML. Patients with t(8;21)-AML frequently present with the French-American-British (FAB) morphologic subtype M2 or AML with maturation, while patients with inv(16) more often are diagnosed with the FAB subtype M4Eo or acute myelomonocytic leukemia with abnormal marrow eosinophils [17–20]. Patients exhibiting inv(16) usually show a significantly higher leukocytosis at diagnosis than t(8;21)-AML patients (median about 35.10^9/l versus 10.10^9/l respectively) [21–25]. Bone marrow involvement is variable, and sometimes it may show a

misleading low number of blasts cells. Extramedullary involvement at diagnosis is found to be more frequent in inv(16) than in t(8;21)-AML, including lymphadenopathy, skin infiltrates and gingival hypertrophy [22,26]. In contrast, myeloid sarcoma appear to be more frequent in t(8;21) than in inv(16)-AML [21,27].

CBF-AML belongs to the favorable-risk AML subset with usually a high sensitivity to standard chemotherapeutic agents used for AML treatment allowing high complete remission (CR) rates and a relatively favorable prognosis when treated with intensive strategies, including the administration of repeated courses of high-dose cytarabine (HDAC) in younger patients (**table 1**). Nonetheless, about 30-40% of patients with CBF-AML relapse and not all may be cured. In this context, prognostic markers are needed to identify CBF-AML patients unlikely to respond optimally to current therapies and who could benefit from more intensive or novel therapies. This review summarizes recent findings in these particular subsets of AML and how they could be used to improve management of patients in routine practice.

INFLUENCE OF AGE

With aging, both the nature of AML and the health of the patient change [28]. Therefore, age is known to confer a negative impact on disease-free survival (DFS) and overall survival (OS) [25,29–31]. Analysis of survival data revealed that age remains a highly significant prognostic factor, even with hierarchical cytogenetic risk subgroups [25,30]. The management of older patients is usually more difficult since they are more likely to have comorbidities and a poorer performance status, which is correlated with higher therapy-related mortality (TRM). Moreover, when treated with the same regimens, elderly patients more often fail to clear leukemic blasts. These results suggest increased drug resistance in such patients which could be due to modifications in pharmacokinetics and/or intrinsic properties of older hematopoietic stem cells [25,28,32].

The proportion of CBF-AML decreases with age, accounting for 5-8% of all AML over 60-70 years [30,33]. In elderly patients, CBF-AML retains to have a better prognosis than other AML subtypes [30,34]. Nonetheless, some studies reported a worse outcome among elderly (usually over 60 years) compared to younger patients [25,29–31,33,35]. Indeed, Appelbaum et *al*. reported a 5-year probability of OS of 48% in the whole population compared with 22% for patients older than 65 years. CR rate was lower in older patients due to increased resistant disease and early deaths [25]. A recent study based on the Surveillance Epidemiology and End Results (SEER) database showed that CBF-AML outcome worsened with increased age since the 3-year OS decreased from 68.7% in patients aged 15-44 years to 9.3% in patients aged 75-94 years [29]. However, in most studies, very few older patients with CBF-AML received repeated courses of intermediate to HDAC, which probably accounts

for survival differences. Overall, poor outcome can be explained, at least in part, by the inability of older patients to tolerate intensive treatments.

The French CBF-AML Intergroup conducted a retrospective study of 147 CBF-AML patients over age 60 treated with conventional induction regimens [33]. Post-remission therapy consisted of low-dose maintenance chemotherapy or intensive consolidation. Despite a CR rate comparable to younger patients, the 5-year probability of DFS was only 27%. Moreover, the early death rate reached 10% and morbidity was high with 17% of patients who required transfer to an intensive care unit. The patients who survived had a poor outcome because administration of an appropriate post-remission therapy was often compromised. Intensive post-remission therapy was associated with a trend to a better DFS, especially in t(8;21)-AML (median not reached at 5 years vs 10 months). This highlighted the need to evaluate post-remission strategies in elderly patients with CBF-AML. In patients younger than 60 years, some studies suggest that age does not influence prognosis of CBF-AML [30]. Nonetheless, Delaunay et *al*. showed that patients with inv(16)-AML older than 35 years old had a worse outcome [24] whereas this was not observed in t(8;21)-AML patients [23]. This results was in accordance with recent report by Cairoli et *al*. who found an optimal cut-point at 43 years in inv(16)-AML patients [36].

The t(8;21) and inv(16) rearrangements occur in a higher percentage in children (although rarely in infants) than in adults [37,38]. CBF-AML accounts for up to 25-30% of AML in children. Among pediatric patients, the excellent outcome conferred by the t(8;21) or inv(16) has been established by several studies with CR rates of 90 to 100% among series [39–42]. The BFM study group reported a 5-year OS of 91% and 87% and 5-year event-free survival (EFS) of 84% and 70% for children younger than 18 years with t(8;21) or inv(16) respectively [41]. Similarly, a 3-year OS of 91% and a 3-year EFS of 86% were found in the AML02 trial [42]. Consequently, allogeneic hematopoietic stem cell transplantation is not indicated in first CR [43]. Overall, CBF-AML in pediatric patients does not really differ from CBF-AML in young adults. Biological and clinical aspects of the disease are quite similar, although patients remain different, which could explain subtle differences on prognosis.

LEUKOCYTE COUNT

A high leukocyte count at presentation is usually considered as a negative prognostic factor in AML. Notably, a high leukocytosis has been associated with an increased induction mortality and poor results in a number of other survival measures [44–46].

In t(8;21)-AML, several studies showed a high white blood cell (WBC) count or absolute granulocyte count had a prognostic significance [21,23,35,47,48]. The German AML intergroup reported a WBC

count higher than 25.10^9/l as a negative prognostic variable both on DFS and OS in t(8;21)-AML patients [21]. After 3 years, DFS and OS were 35% and 38% in patients with a WBC count greater than 25.10^9/l versus 60% and 65% in the whole cohort. In accordance with those results, a cut-point of 30.10^9/l was relevant in the French AML intergroup study [23]. Moreover, to further take into account the spontaneous differentiation potential of the leukemic clone, which is known to be highly variable at the individual level, the French AML Intergroup proposed a WBC index derived as the product of WBC by the ratio of marrow blast [23]. In their study, the WBC index appeared to have more potent prognostic impact on DFS than non-adjusted WBC count, whereas it did not add prognostic information in the German cohort [21].

In inv(16)-AML patients, impact of leukocytosis on outcome is less clearly established. Some results have suggested that inv(16)-AML patients with high initial WBC count are not at higher risk for relapse and would not thus have to be differently managed once the CR is achieved [21,24,35] but recent studies found that WBC count adversely affect relapse-free survival (RFS) in inv(16) patients [36,49]. On the other hand, marked hyperleukocytosis has been reported to correlate with induction failure and early death [21,24,50]. Martin et *al*. found a significantly worse prognosis for the inv(16) patients with leukocytes above 100.10^9/l [50]. The French AML intergroup defined an optimal WBC cut-point at 120.10^9/l as predictive of induction failure, whereas it did not affect outcome of patients who obtained CR. Accordingly, CR rate was 68% in patients with a WBC count of 120.10^9/l or more compared with 98% in the remaining patients, suggesting evaluation of more careful approaches to induce remission in such situations [24].

Interestingly, prognostic impact of leukocytosis may reflect the molecular/cytogenetic perturbation driving the proliferation, such as *FLT3* or *KIT* mutations, that will be discussed in later sections.

THERAPY-RELATED CBF-AML

Rarely, CBF-AML may occur as a late complication of cytotoxic chemotherapy and/or radiation therapy administered for a prior neoplastic or non-neoplastic disorder [51–55]. Therapy-related secondary AML (t-AML) with balanced translocations are usually characterized by a short latency period, absence of preleukemic phase, and are especially associated with prior exposure to topoisomerase II (topo II) inhibitors [56–58]. The mechanisms responsible for such rearrangements are largely unknown but may involve several chromatin structural elements such as topo II cleavage sites are found to co-localize with preferential breakage sites after exposure to damage, including topo II inhibitors [59].

Regarding presentation at the time of diagnosis, studies showed that patients with secondary CBF-AML were usually older than those with de novo diseases and had a lower WBC count [60–62]. For AML with t(8;21), blasts of de novo and t-AML cases share morphologic, immunophenotypic, cytogenetic and molecular features [60,61], although t-AML with t(8;21) seem to have more dysplastic changes than their de novo counterpart [61,63]. At the molecular level, breakpoints in the *RUNX1* and *RUNX1T1* genes occur in the same regions in both de novo and t-AML patients [64]. In AML with inv(16), Schnittger et *al*. have linked the presence of unusual *CBFB-MYH11* transcripts with t-AML cases. These cases were found to correlate with an atypical morphology not primarily suggestive for the FAB subtype M4Eo but did not constitute an independent prognostic parameter [62]. Thus, they suggested that the molecular mechanisms of the inv(16) rearrangement differ in de novo AML and in t-AML in contrast with the t(8;21).

Reports of the long-term outcome of patients with secondary CBF-AML are rare because most reports do not distinguish between patients with de novo and those with t-AML associated with CBF abnormalities. Although first reports suggested that the outcome of patients with secondary CBF-AML was similar to their de novo counterparts [65,66], Borthakur et *al*. have suggested in a single center study, that patients with secondary CBF-AML had worse OS and EFS compared with de novo CBF-AML [60]. The authors analyzed 188 CBF-AML patients, including 17 (9%) secondary cases. Interestingly, patients with secondary CBF-AML were found to have a worse OS compared with their de novo counterpart (100 weeks versus more than 600 weeks), even in matched analysis by age, performans status and additional cytogenetic abnormalities (100 weeks versus 376 weeks). In another study focused on t(8;21)-AML patients, Gustafson et *al*. [61] found that patients with secondary AML had a comparable initial response to induction chemotherapy than their de novo counterpart but had a shorter OS (19 months versus not reached), in line with the study by Slovak et *al*. [67]. Regarding only inv(16)-AML, the German-Austrian AML Study Group found that patients with t-AML with inv(16) had inferior OS compared with those with de novo disease [49,68].

Finally, several factors could explain this poorer outcome, such as older age and comorbidities, injury to organs from prior therapy, depletion of normal hematopoietic stem cells, damage to marrow stroma (particularly with radiation therapy), chronic immunosuppression leading to colonization with pathogenic or antibiotic-resistant bacteria and fungi, refractoriness to transfusion support and eventually persistence of active primary cancer or co-occurrence of another cancer at the time of t-AML diagnosis, but cryptic alterations not detected with conventional methods could not be excluded [60,61,69].

ADDITIONAL CYTOGENETIC ABNORMALITIES

Secondary chromosome aberrations are detected at diagnosis in approximately 70% of patients with t(8;21)-AML and 40% of patients with inv(16)-AML [21,22,25,70]. Interestingly, secondary aberrations are nonrandom and some of them are extremely rare in non-CBF-AML signifying their importance for CBF-AML leukemogenesis [71]. Moreover, while CBF-AML are supposed to share a common physiopathology pathway, i.e. the disruption of the CBF complex, the patterns of secondary aberrations differ between inv(16) ant t(8;21)-AML patients.

In the t(8;21)-AML subset, loss of a sex chromosome (LOS) is by far the most frequently secondary abnormality, since loss of the X chromosome accounts for 30-40% of female patients and loss of the Y chromosome accounts for 50-60% of male patients [21,22,25,70], in contrast to other types of AML [including inv(16)-AML] in which LOS occurs in less than 5% of patients [72]. Impact of LOS on outcome remains debated since conflicting data have been reported. Schlenk et *al*. found that loss of Y in male patients was prognostic for shorter OS [21], while Grimwade et *al*. reported a trend for better OS in such patients, in accordance with other studies [73]. Loss of X had consistently no impact on outcome in those studies. The second most frequent additional aberration in t(8;21)-AML corresponds to the deletion of the long arm of chromosome 9 [del(9q)] which accounts for 15-20% of t(8;21)-AML cases [21,22,25,74], whereas it is relatively rare in other AML [75,76]. Previous reports have suggested that del(9q) associated with t(8;21) carried a relatively poor prognosis [33,77]. However, more recent studies have shown that del(9q) did not influence CR rates, DFS nor OS in this subset of AML [25,74,78]. Overall, neither secondary abnormalities nor the complexity of karyotype adversely affect the outcome of t(8;21)-AML patients [78–80], although some results may be debated.

By contrast, trisomy 22 appears to be specific of the inv(16)-AML and represents the most frequent secondary aberration in this subset of AML in which it accounts for 15-20% of cases [21,22,25,49,74]. Thus, presence of trisomy 22 as sole karyotype abnormality in AML imposes to search cryptic rearrangements of the *CBFB* and *MYH11* genes as the presence of trisomy 22 without inv(16) or its equivalent is debated [81,82]. In accordance with previous studies [21,22], the German-Austrian AML study Group (AMLSG) recently confirmed significantly superior DFS in inv(16)-AML patients with trisomy 22 compared with patients without trisomy 22 [49]. Moreover, trisomy 22 was associated with a significantly better OS in the study of Grimwade et *al*. [78] and a trend of better OS in the AMLSG study [49]. Interestingly, patients with additional chromosome 22 have a significantly lower presenting WBC count compared with those with inv(16) alone [21,49,78] and preferentially exhibit a type A *CBFB-MYH11* transcript [6,62]. Trisomy 21 is also reported in about 5% of inv(16)-AML, whereas it is

extremely rare in t(8;21)-AML patients, but its prognostic significance has never been established [21,22,25].

Others abnormalities frequently involved in CBF-AML include trisomy 8 and deletion of the long arm of the chromosome 7 [del(7q)] in both t(8;21) and inv(16)-AML. Trisomy 8 is present in approximately 5-7% and 10-16% for t(8;21) and inv(16)-AML respectively. The prognostic significance of trisomy 8 in CBF-AML is not well established [83]. Although it does not seem to affect outcome in previous studies [21,22,74], the AMLSG recently found that trisomy 8 was associated with an inferior OS in inv(16)-AML patients, whereas it did not impact on DFS [49]. On the other hand, Krauth et *al*. reported an adverse effect on EFS in the t(8;21)-AML group [84]. Del(7q) is reported in 5% to 10% of both t(8;21)-AML and inv(16)-AML but a prognostic significance has never been shown in these particular subsets of AML [22,49,74]. Importantly, up to one half of del(7q) is not detected by conventional cytogenetics but only using high-resolution genetic profiling because of their limited size [85]. Particular findings concern trisomy 4, although it is an uncommon aberration in CBF-AML [22,86]. It has been suggested a relatively poor prognosis in AML patients with t(8;21) and trisomy 4 [87] which could be supported by the increase in mutated *KIT* allele burden (*KIT* is located in 4q11-q12) in such cases [88], despite the small number of cases described in the literature.

Overall, little is known about how such abnormalities may cooperate in CBF-AML. Secondary aberrations are likely to involve multiple genes and mechanisms of these associations have not yet been elucidated, although few studies have started to explore interesting hypothesis [72,89–91].

MUTATIONAL ANALYSIS

Tyrosine kinase signaling

CBF-AML is considered as a model of leukemogenesis. It perfectly illustrates the multistep pathogenesis of leukemia developed by Gilliland et *al*. [7,92], in which leukemia results from the cooperation between an aberration targeting hematopoietic transcription factors (such as CBF) that impairs differentiation and an activating mutation that increases proliferation. Indeed, evidences supporting this model have been generated by the observation in CBF-AML of frequent mutations in genes encoding protein effectors which control cell proliferation and survival, such as *KIT* (*v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog*), *FLT3* (FMS-like tyrosine kinase) or *RAS* (rat sarcoma viral oncogene homolog).

The most common mutations are those in the *KIT* gene which encodes a transmembrane glycoprotein belonging to the type III receptor tyrosine kinase (RTK) family (as well as FLT3) [8]. They affect either the extracellular domain of the KIT receptor (correspond to in-frame insertions/deletions in

exon 8 resulting in loss of the acid aspartic residue at amino acid 419), the tyrosine kinase domain (TKD) (mostly mutations affecting amino acid residues at codon 816 or 822), or more rarely the juxtamembrane domain (JMD) (such as internal tandem duplication in exon 11) [8]. The overall frequency of *KIT* mutations in non-CBF-AML is approximately 1-2% [93–95]. In contrast, they occur in 17% to 38% of CBF-AML [36,49,74,84,96–101]. In the recent MRC study focused on a large series of 354 adults with CBF-AML [199 t(8;21)-AML and 155 inv(16)-AML], the incidence of *KIT* mutations was 28%. There was a significantly higher frequency of mutations in inv(16)-AML compared with t(8;21)-AML (35% versus 23%), related to a higher frequency of mutations in exon 8 (20% versus 3%), while there was no difference within exon 17 (17% and 18%) and exon 11 (3% and 4%) [102]. Lück et *al*. showed that *KIT* mutations conferred a distinct gene expression profiling in CBF-AML compared with non-mutated cases characterized by deregulation of genes belonging to the NFkB (nuclear factor kappa B) signaling complex suggesting impaired control of apoptosis [99]. Regarding clinical behavior, *KIT* mutations have been associated with a higher WBC count at diagnosis [74,98,99,103–105]. In t(8;21)-AML, most published reports indicate that *KIT* mutations confer an adverse prognosis with a higher incidence of relapse and shorter DFS, especially for *KIT*-D816 mutations [74,94,98,100,101,104,105]. However, results differ concerning OS : some authors found a worse prognostic value of *KIT* mutation [94,96,100,104,105] while the others did not [74,84,98,106]. In their study focused on 139 t(8;21)-positive cases, Krauth et *al*. found an impact on OS only when restricted the analysis to *KIT*-D816 mutations whereas there was no impact when then considered all *KIT* mutations [84]. In inv(16)-AML patients, prognostic significance of *KIT* mutations is less well established. In the recent study of the AMLSG, including a large cohort of 176 inv(16)-positive cases, *KIT* mutations were associated with lower DFS but did not impact on OS. Impact of mutated *KIT* on DFS was mainly attributed to mutations in exon 8 [49], in accordance with other studies [36,106]. However, in the CALGB study, the negative impact on relapse rate was mainly attributed to mutations in exon 17 [98]. In most studies, no effect on OS was observed [36,49,74,97,104–106] which could be explained by the high sensitivity of salvage therapy in inv(16)-AML patients [21,22]. Interestingly, in a study by Schwind et *al*., *KIT* mutations were encountered in 48/178 (27%) patients with type A *CBFB-MYH11* fusion transcript whereas no mutation was detected in the 24 remaining patients analyzed with non-type A transcripts. When restricted the analysis to patients exhibiting a type A transcript, those with mutated *KIT* had a shorter OS and EFS than those without *KIT* mutation [6]. Finally, the discrepancies among the studies may reflect differences in treatment regimens, including salvage therapy [98], selection of the study cohort, small numbers of patients studied or the wide age range of some cohorts. Notably, the impact of mutations may be different in pediatric patients. Pollard et *al*. analyzed a large cohort of 203 children with CBF-AML but *KIT* mutations lacked prognostic significance [97], in accordance with previous studies [107,108]. In contrast, two studies reported worse outcome in *KIT*-

mutated pediatric AML with t(8;21) [96,100]. Allen et *al*. suggested differences may also relate to the *KIT*-mutated allele burden. Indeed, in their study, *KIT* mutations were associated with a significantly increased relapse rate in multivariate analysis only for cases with a high mutant level of 25% or greater [cumulative incidence of relapse (CIR) of 52%, 44% and 35 for patients with high-level *KIT* mutant, low-level *KIT* mutant and *KIT* wild-type respectively] [102]. Moreover, it may reflect variations in exons studied and the mutations investigated or the sensitivity range of methods used in the different studies, since mutant level can be very low [102]. Because of their high frequency in CBF-AML and their gain-of-function nature leading to constitutive activation of the KIT receptor, *KIT* mutations could be interesting targets for tyrosine kinase inhibitors (TKI). Imatinib is active against exon 8 mutations and exon 17 mutations involving codon N822 but not D816 which can be successfully targeted with dasatinib [109–112].

FLT3 mutations are among the most frequent genetic alterations in AML [113,114]. Nonetheless, *FLT3* internal tandem duplication (*FLT3-ITD*) is relatively uncommon in CBF-AML, being detected in 2-9% and 0-6% of t(8;21)-AML and inv(16)-AML patients respectively [49,74,84,102,105,106,113]. Point mutations within the tyrosine kinase domain (*FLT3-TKD*), mostly at codon D835, occur in 2-7% of t(8;21)-AML patients and 6-24% of inv(16)-AML patients [49,74,84,102,105,115]. Using exome sequencing, Opatz et al. recently increased the spectrum of *FLT3* mutations in CBF-AML. The authors identified a recurrent *FLT3 N676K* mutation in 5/84 inv(16)-AML and 1/36 t(8;21)-AML [116], while Allen et *al*. found it in 4/276 CBF-AML (all of them were inv(16)-AML) [102]. Overall, the prognostic significance of *FLT3* mutations remains to be established in CBF-AML, especially because they constitute potential targets for therapy. Boissel et *al*. reported a worse OS in CBF-AML patients with *FLT3* mutations [105]. Although there was no effect on DFS, the recent study of the AMLSG reported an inferior OS in inv(16)-positive patients with *FLT3* mutations compared with those without *FLT3* mutations [49]. This adverse effect was mainly attributed to *FLT3-TKD* rather than *FLT3-ITD* mutations. These results were in contrast with one MRC study which reported favorable OS associated with *FLT3-TKD* mutations, including CBF-AML [115,117]. Also, in the more recent study by Allen et *al*., a high *FLT3-TKD* mutant level retained a favorable prognostic factor for OS whereas a high *FLT3-ITD* mutant level was a significant adverse factor for OS [102].

Mutations in the *RAS* genes, *NRAS* and less often *KRAS*, are reported with particularly high frequency in CBF-AML. Together, *RAS* mutations are found in 8-18% of t(8;21)-AML patients and in 17-53% of inv(16)-AML patients [74,84,98,102,105,118]. Thus far, *RAS* mutations have never been correlated with clinical outcome. Other mutations reported in CBF-AML include mutations in the *JAK2* and *CBL* genes, both implicated in the tyrosine kinase pathways [119,120]. *JAK2* mutations appear to be more frequent in CBF-AML, in which they occur in 3-6% of cases, compared with other AML subtypes [120–

125]. They seem to be more frequent in t(8;21)-AML than in inv(16)-AML [49,84,123,125]. Although *JAK2* mutations are infrequent events, they could constitute an adverse factor with a higher relapse rate in CBF-AML [120,121] but further studies are needed to confirm. The recent MRC study reported *CBL* mutations in 6% of CBF-AML with no difference between the incidence in t(8;21)-AML and inv(16)-AML. *CBL* mutations had no impact on CR and DFS but a high mutant level was a favorable factor for OS [102] although these results also require confirmation.

Importantly, additional molecular mutations, especially *KIT* and *FLT3* mutations, are highly dynamic at relapse, since more than one half of patients gain, lose or change their mutations at relapse, which could limit the interest of targeted therapy in practice [84,102]. Deciding how to incorporate coexisting abnormalities, especially *KIT* mutations, which are of prognostic significance and could provide targets for therapy, will likely be important in future risk classification of AML. Accordingly, guidelines published by the National Comprehensive Cancer Network recommend that patients with CBF-AML and *KIT* mutations should be treated as intermediate-risk rather than favorable-risk [126].

ASXL mutations

Although the "two-hit model" of leukemogenesis described by Gilliland is biologically relevant, it is currently impossible to ignore the multitude of genetic and epigenetic aberrations described in human leukemia [127]. In 2014, Krauth et *al*. identified that *ASXL1* mutations occur in 11.5% of t(8;21)-adult AML with a significant negative impact on EFS [84]. The *ASXL* genes family (*ASXL1*, *ASXL2* and *ASXL3*) are mammalian homologs of the Drosophila Additional sex combs (Asx) gene. Somatic mutations involving *ASXL1* are described in all myeloid malignancies, especially in myelodysplastic syndromes and chronic myelomonocytic leukemia, mostly associated with worsened OS [128]. Despite this association, alterations in other ASXL family members were not described in malignant hematopoietic diseases. More recently, we reported *ASXL2* mutations in about 23% (25/110) of t(8;21)-AML, both in adults and children [129]. As described for the well-known *ASXL1* gene [128], mutations in the *ASXL2* gene are frameshift or nonsense mutations that result in C-terminal truncation of the protein upstream of its Plant homeodomain (PHD). Interestingly, these mutations are mutually exclusive with *ASXL1* mutations, suggesting a common pathway. Intriguingly, *ASXL2* mutations were not identified in prior whole exome/genome sequencing studies in AML. Moreover, *ASXL* mutations were not found in 60 inv(16)-AML patients, arguing that they should be considered separately from t(8;21)-AML in future molecular studies [129]. Together, *ASXL1* and *ASXL2* mutations occur in about 35% of t(8;21)-AML cases, making *ASXL* mutations among the most common genetic alterations in t(8;21)-AML patients [84,129]. Moreover, in our study, *ASXL1* and *ASXL2* mutations were associated with a trend of higher cumulative incidence of relapse (54% and 36% respectively at 3 years compared with

25% in *ASXL1/2* wildtype counterpart), which suggests the need for further studies addressing their prognostic relevance. On the other hand, understanding the functional basis for the frequency of *ASXL1/2* mutations and the apparent specificity of *ASXL2* mutations in t(8;21)-AML will be critical to promote our knowledge of CBF-AML pathogenesis and to develop novel therapies.

MINIMAL RESIDUAL DISEASE

Both the specificity of the fusion transcripts and the high sensitivity of real time-reverse transcriptase polymerase chain reaction (RT-qPCR) techniques have made *RUNX1-RUNX1T1* and *CBFB-MYH11* ideal markers for diagnosis and follow-up in t(8;21)-AML and inv(16)-AML respectively [130]. Several studies have reported promising results about the monitoring of minimal residual disease (MRD), allowing to assess treatment response, to detect patients at high risk of relapse or to guide therapeutic decisions.

Schnittger et *al*. showed fusion transcript levels at diagnosis were correlated with a significant impact on OS as well as on EFS in CBF-AML patients [131]. Accordingly, Leroy et *al*. reported a trend of a higher relapse rate in patients with high pretreatment *RUNX1-RUNX1T1* expression levels [132]. On the other hand, the reduction of initial fusion transcript level measured by RT-qPCR after induction and consolidation therapies was shown to correlate with prognosis [131–141]. After induction therapy, Leroy et *al*. reported that transcript level below 10^{-3} or a greater than 3 log decrease by comparison with diagnosis levels were significant predictors of the absence of relapse in t(8;21)-AML patients [132]. MRD levels were also significant after consolidation therapy, since only one of 15 patients with transcript levels below 10^{-5} relapsed, compared with 5 of the 6 patients with higher levels. In inv(16)-AML patients, Guièze et *al*. reported that molecular response assessed by reduction of more than 3 log in *CBFB-MYH11* transcript level after the first consolidation was a strong prognostic factor with a 3-year DFS of 85% in patients with a decrease greater than 3 log compared with 25% in the others.

The United Kingdom MRC AML-15 trial prospectively assessed the MRD monitoring in bone marrow (BM) and peripheral blood (PB) in a large cohort of 278 CBF-AML patients [163 with t(8;21)-AML and 115 with inv(16)-AML] [142]. At remission after the induction therapy, *RUNX1-RUNX1T1* transcripts log reduction in the BM and absolute copy numbers in the BM (>500) or in the PB (>1000) were predictive of relapse in t(8;21)-AML patients. A reduction greater than 3 log in *RUNX1-RUNX1T1* transcript level in the BM was the strongest predictive factor for relapse risk with a 5-year CIR of only 4% in the patients achieving it, compared with more than 30% for those who did not reach this threshold. In inv(16)-AML patients, absolute copy numbers of *CBFB-MYH11* transcripts after remission in the BM (>100) or in the PB (>10) were predictive of relapse and the PB transcript level was the strongest prognostic variable. Moreover, higher PB copy numbers had a significant adverse impact

on OS after CR. Monitoring of MRD after 2 consolidation courses was also informative. In t(8;21)-AML, a reduction greater than 4 log in *RUNX1-RUNX1T1* transcripts in the BM was associated with a 5-year CIR of only 10% whereas an absolute copy number exceeding 500 copies invariably predicted relapse. In inv(16)-AML, an absolute copy number of *CBFB-MYH11* transcripts in the PB higher than 10 remained the most predictive of relapse after 2 consolidation courses and was associated with a 5-year CIR of 78%. During follow-up, the authors identified cut-off MRD thresholds associated with a 100% relapse rate (>500 copies in the BM or >100 copies in the PB for t(8;21)-AML patients and >50 copies in the BM or >10 copies in the PB for inv(16)-AML patients). Consistent with this study, the French AML Intergroup showed that MRD levels should be used for treatment stratification [74]. The authors prospectively evaluated gene mutations and MRD in a cohort of 198 CBF-AML [96 t(8;21)-AML and 102 inv(16)-AML]. MRD response appeared to be the sole prognostic factor in multivariate analysis. At 3 years, CIR and RFS were 22% and 73% in patients who achieved a 3-log reduction at the MRD2 time-point (before initiation of the second consolidation course) compared with 54% and 44% in the others. OS from CR was estimated at 90% in patients who achieved a 3-log reduction at the MRD2 time-point compared with 71% in the others. Similar results were observed when using an absolute level lower than 0.1% rather than log-reduction at MRD2 time-point. Interestingly, the 3-log MRD2 reduction was less frequently achieved in patients with *KIT* mutations (54% versus 75%), whereas no differences were observed according to the *FLT3* or *RAS* status. Another study by Zhu et *al*., including a cohort of 116 t(8;21)-AML patients, reported that MRD status after the second consolidation course may be the best timing treatment stratification, allowing identification of patients who could benefit from allogeneic hematopoietic stem cell transplantation [143]. The same group recently showed that MRD monitoring early after allogeneic transplantation in t(8;21)-AML patients allowed rapid identification of patients at risk of relapse [144].

Finally, quantification of fusion transcript levels is a powerful tool for prognostic prediction that is independent of pretreatment risk factors, and may be helpful to guide therapeutic decisions in the future. Combination of MRD levels and MRD kinetics allow early and accurate prediction of relapse. Ommen et *al*. reported interesting findings about the kinetics of relapse in CBF-AML patients [145]. They estimated that the median doubling time of *CBFB-MYH11* leukemic clones was significantly longer than that of *RUNX1-RUNX1T1* leukemic clones (36 days versus 14 days). The authors also observed that 50% of inv(16)-AML patients were positive by RT-qPCR 8 months before relapse and, by comparison, 50% of t(8;21)-AML patients were positive 3 months before relapse. Interestingly PB and BM sampling seemed to be equally useful. Thus, PB sampling every 6 months in patients with *CBFB-MYH11* resulted in a relapse detection fraction of 90% and a median time from molecular positivity to hematological relapse of 180 days. In *RUNX1-RUNX1T1*-positive patients, PB sampling every 4

months resulted in a relapse detection fraction of 75% and a median time from molecular positivity to hematological relapse of 55 days while BM sampling every 4 months showed better results with a relapse detection fraction of 95% and a median time from molecular positivity to hematological relapse of 85 days.

In laboratories in which molecular biology is not currently available, MRD monitoring by flow cytometry (FC) could be a useful alternative and remains of great interest for inv(16)-AML patients with rare *CBFB-MYH11* fusion transcripts (i.e. non A/D/E fusion transcripts). In a recent study, Inaba et *al*. have compared MRD monitoring by RT-qPCR and FC in 55 CBF-AML [31 t(8;21)-AML and 24 inv(16)-AML] enrolled in the AML02 trial [146]. Only 3 patients relapsed. MRD was considered positive by FC when cells with a leukemia-associated aberrant immunophenotype were above 0.1%. Interestingly, among the 179 tested samples that were positive by RT-qPCR, only 13 were also positive by FC. Among the 69 samples with MRD levels ≥ 0.1% by RT-qPCR, only 8 had detectable AML cells by FC. It is likely that these discrepancies reflect the higher sensitivity of the RT-qPCR assay. Nonetheless, it could also reflect changes in AML cells immunophenotype or persistence of partially differentiated cells. Spontaneous differentiation of the leukemic cells occur in CBF-AML, allowing detection of t(8;21) or inv(16) not only in blast cells but also in differentiated cells. Likewise, it has been shown that acquisition of these rearrangements occurs in stem cells capable of differentiating into erythroid, granulocyte-macrophage and megakaryocytic lineages as well as lymphoid cells [147]. However, the interest to detect low level of MRD remains debated since it seems to be compatible with durable remission. Inaba et *al*. detected persistent *RUNX1-RUNX1T1* and *CBFB-MYH11* fusion transcripts in 24 patients after completion of therapy who remained in long-term remission, as reported by others [142]. These observations may be related to the ability of the immune system to control residual tumor burden. According to the cancer immunoediting hypothesis, an equilibrium phase could precede leukemic cells escapement. Anyway, this justifies an increased sequential surveillance in such patients, to detect escapement as soon as possible [130].

EXPERT COMMENTARY

Identification the t(8;21) or inv(16) rearrangements is critical in patients with AML as it significantly impacts on subsequent management. Indeed, CBF-AML is considered to have a good prognosis compared with other AML subtypes and treatments using high-dose cytarabine based chemotherapy in younger patients have resulted in markedly improved outcome. However, relapse incidence may reach 40% in such patients. Although CBF-AML is recognized as a unique entity, it is important to note that additional cytogenetic or molecular abnormalities may coexist and influence its physiopathology as well as its clinical and biologic behavior. In this context, many prognostic factors have

been studied to allow early detection of patients at high risk of relapse who could be candidate for a more intensive treatment or allogeneic stem cell transplantation. Current reports focus on activating mutations of tyrosine kinase genes and MRD monitoring. It is likely that the future classifications will incorporate secondary molecular aberrations that are of prognostic significance. Notably, *KIT* mutations are now associated in most published reports with a higher relapse rate in CBF-AML patients. Also, the specificity of the fusion transcripts and the high sensitivity of current technologies allow the early detection of a molecular relapse before its hematological translation providing a time interval which could be used for an early therapeutic intervention. Although, optimal cutoffs and time-points as well as interventional therapeutic guidelines remain to be firmly established, MRD monitoring by RT-qPCR, or to a less extent by flow cytometry, is a sensitive and specific tool and should be generalized to assess treatment response of CBF-AML patients. Particularly, this approach may be very informative with sequential measurements. Overall, these prognostic markers will likely take a rising place in patient management, allowing targeted therapies or serving as tools for treatment stratification.

FIVE-YEAR VIEW

Because of similarities between their molecular and prognostic features, t(8;21) and inv(16)-AML are usually grouped and reported together in clinical studies. However, several reports have demonstrated differences in genetic, molecular, clinical and prognostic features supporting the notion they should be reported separately in future studies. Notably, although t(8;21)-AML and inv(16)-AML share mutations in genes involved in tyrosine kinase pathways (*KIT*, *RAS*, *FLT3*), the frequent *ASXL1* and *ASXL2* mutations have been recently reported to occur only in t(8;21)-AML patients. Also, it has been suggested for future studies that relative mutant levels should be taken into account when evaluating the impact of mutations in CBF-AML and it is likely that this information will become more readily available in the future with the introduction of next-generation sequencing platforms into routine diagnostic practice. Moreover, MRD monitoring which is used today to assess response to therapy, will probably become a guide for therapeutic stratification. Finally, translational research integrating these molecular findings with clinical trials will probably improve treatment of patients with CBF-AML.

KEY ISSUES

1. Patients with CBF-AML are considered to have a favorable prognosis but constitute in fact a heterogeneous population: relapse incidence may reach 40% and not all may be cured.
2. Recurrent molecular abnormalities, especially *KIT* mutations, could improve risk stratification and define therapeutic targets.
3. Minimal residual disease monitoring on therapy has been shown to be a significant prognostic factor and could be used for treatment stratification.
4. AML with t(8;21) and those with inv(16) should be reported separately in futures studies.
5. Participation in clinical trials and the collection of samples for correlative science analysis is recommended for all patients with newly diagnosed CBF-AML.

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FIGURES AND TABLES

Figure 1: Schematic diagram of (a) the *RUNX1-RUNX1T1* fusion transcript and (b) the most frequent *CBFB-MYH11* fusion transcripts.

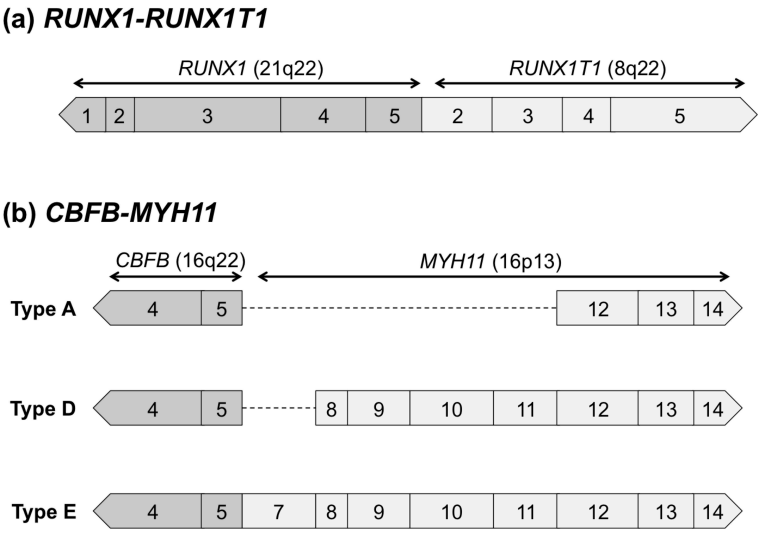

Table 1: CR, DFS and OS in CBF-AML trials.

	French AML inter-group [23,24]	**German AML intergroup** [21]	**MRC (UK)** [28]	**CALGB (US)** [22]	**SWOG/ECOG/ MDA (US)** [25]
AML with t(8;21)					
Patients (n)	161	191	122	144	174
CR rate	96%	87%	98%	89%	89%
DFS (3/5 years)	-/52%	60%/-	-	-	-/45%
OS (3/5 years)	-/59%	65%/-	-/69%	-/46%	-/45%
AML with inv(16)/t(16;16)					
Patients (n)	110	201	57	168	196
CR rate	93%	89%	88%	87%	85%
DFS (3/5 years)	48%/-	58%/-	-	-	-/44%
OS (3/5 years)	58%/-	74%/-	-/61%	-/54%	-/50%

CALGB: Cancer and Leukemia Group B; MRC: Medical Research Council; SWOG: Southwest Oncology Group; ECOG: Eastern Oncology Group; MDA: MD Anderson Cancer Center.

PARTIE 4 - LAM FAMILIALES AVEC MUTATION DE *RUNX1*

Les hémopathies familiales sont rapportées depuis le début du XX^{e} siècle [119]. Le premier gène de prédisposition identifié est le gène *RUNX1* en 1999 dans la thrombopénie familiale avec prédisposition au développement d'hémopathies malignes (FPD/AML) (OMIM#601399) [56]. En quelques années, les progrès technologiques spectaculaires dans l'étude du génome humain et la coopération internationale ont conduit à une augmentation considérable du nombre de familles rapportées et des gènes identifiés. En 2016, les néoplasies avec prédisposition génétique sont intégrées dans la classification des hémopathies proposée par l'OMS [8]. Bien que rares, il devient évident que les prédispositions génétiques aux hémopathies malignes sont sous-estimées. Leur diagnostic reste difficile en raison de la grande variation des pathologies développées, de l'âge de survenue variable affectant à la fois enfants et adultes, de la pénétrance incomplète et parfois du manque d'histoire familiale. Les mutations de *RUNX1* partagent toutes ces caractéristiques et sont, depuis leur découverte, considérées comme un modèle *in vivo* de la leucémogénèse.

Cette partie de l'introduction est discutée dans la revue « *Myelodysplastic syndromes and acute leukemia with genetic predispositions: a new challenge for hematologists »* (page 61). Cette revue concerne les mutations germinales de *RUNX1* ainsi que celles de *CEBPA*, *GATA2*, *ANKRD26*, *SRP72*, *PAX5*, *DDX41*, *ETV6, TERT* et *TERC* dont certaines participent au diagnostic différentiel des thrombopénies familiales avec prédisposition aux hémopathies malignes (*ANKRD26* et *ETV6* en particulier). Ce travail de thèse portant uniquement sur le CBF, seule la partie qui concerne les mutations germinales de *RUNX1* est présentée ci-après.

Myelodysplastic syndromes and acute leukemia with genetic predispositions: a new challenge for hematologists

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Abstract: *Introduction:* The determination of an underlying genetic predisposition is not automatically part of the diagnosis of hematological malignancies (HM) in routine practice. However, it is assumed that genetic predispositions to HM are currently underestimated due to great variations in disease phenotype, variable latency and incomplete penetrance. Most of patients do not display any biological or clinical signs besides the overt hematological disease and many of them have a lack of personal or family history of malignancies. *Areas covered:* Collaborative studies and important advances in molecular testing have led to the discovery of several genes recurrently deregulated in familial HM including *RUNX1*, *CEBPA*, *GATA2*, *ANKRD26*, *SRP72*, *PAX5*, *DDX41*, *ETV6, ATG2B/GSKIP* and *TERT/TERC*. This review summarizes biological and clinical findings encountered within these disorders. *Expert Commentary:* Identify and manage individuals with genetic predisposition is a current challenge for hematologists. Their identification has immediate implications for hematopoietic stem cell transplantation including donor selection and conditioning regimen. Importantly, several features, including familial and personal history as well as molecular and cytogenetic findings, may help clinicians to suspect an underlying genetic predisposition.

Keywords: *ANKRD26, ATG2B/GSKIP, CEBPA, DDX41, ETV6, GATA2, GSKIP, PAX5, RUNX1, SRP72, TERC, TERT,* genetic predisposition, acute leukemia, myelodysplastic syndrome, bone marrow failure.

[…]

GERMLINE *RUNX1* MUTATIONS ASSOCIATED THROMBOCYTOPENIA

Familial platelet disorder with propensity to myeloid malignancies (FPD/AML) was the first inherited disorder to be defined. The association between platelet disorder and HM was first noticed in 1978 in a family in which 3 siblings died from leukemia while the mother and at least 3 other siblings had chronic thrombocytopenia and laboratory evidences of platelet dysfunction [19]. In 1985, Dowton et *al*. described a large pedigree (192 individuals) in which at least 29 members had a moderate bleeding tendency, variable thrombocytopenia and aspirin-like platelet dysfunction with autosomal dominant (AD) inheritance. Six individuals had developed HM [20]. A decade later, Ho et *al*. determined by

linkage analysis that the gene responsible for the disorder in this family was located in a critical region in 21q22 containing 5 candidate genes [21] including *RUNX1*. In 1999, Song et *al*. finally identified germline *RUNX1* mutations (more rarely deletions) as causative of the FPD/AML phenotype in 6 unrelated pedigrees [3]. The *RUNX1* gene (runt related transcription factor 1), located at 21q22, encodes the alpha subunit of the core binding factor (CBF), a heterodimeric transcription factor involved in the expression of various genes specific to normal hematopoiesis including MCSF receptor, IL-3, MPO or TCR genes [22]. *RUNX1* has been shown to play a key role in megakaryocytes (MKs) maturation and differentiation but also in ploidization and proplatelet production [23–29]. The RUNX1 protein contains a highly conserved Runt homology domain (RHD) located in the N-terminal part which is responsible for both DNA binding to target genes and heterodimerization with CBF beta-subunit [30]. Among sporadic HM, *RUNX1* is one of the most frequently deregulated genes through several mechanisms including translocations, point mutations, deletions and amplifications [31–33]. Overall, somatic *RUNX1* mutations have been described in about 6 to 20% of sporadic AML [34], CMML [35] and MDS [36].

More than 40 FPD/AML pedigrees have been reported so far [3,37–48]. Most characterized germline *RUNX1* mutations include nonsense and frameshift mutations throughout the gene resulting in a truncated protein as well as missense mutations usually clustered in the RHD. More complex aberrations such as partial deletions and tandem-duplications, possibly overlooked by DNA sequencing, are also described, requiring the use of alternative methods [43,49]. Moreover, FPD/AML can be part of a contiguous gene syndrome due to large genomic deletions of the long arm of chromosome 21 including the *RUNX1* gene [39,50–52]. Large deletions usually result in a more complex phenotype, depending of the genes involved, with dysmorphism, cardiac abnormalities and mental retardation. FPD/AML patients classically exhibit a mild to moderate thrombocytopenia (typically 80 to $100x10^9$/L) with normal platelet size, aspirin-like platelet dysfunction and dense-granule storage pool deficiency [37,41,52,53]. Affected individuals do not usually require treatment in the absence of clinical bleeding or surgical procedures. Bone marrow (BM) examination prior to the development of HM, may show increased proportion of immature MKs and/or dysplastic features including small size hypolobulated MKs [37,52]. Germline *RUNX1* mutations have been linked to a variety of dysregulated genes including the myosin heavy polypeptide 10 for which persistence in platelets has been proposed as a detection tool to differentiate FPD/AML from other inherited forms of thrombocytopenia [23,54]. On the other hand, FPD/AML predisposes to the development of a variety of myeloid diseases, especially MDS/AML [41,42] and CMML [47] but also T-ALL [45,48] while malignancies of B-lineage origin appear very uncommon [55]. In a report considering 11 families (114 affected pa-

tients), the median risk of HM occurrence in FPD/AML patients was estimated to be 35% varying from 20 to 60% between the different families with an age of onset ranging from 6 to 75 years [37].

Importantly, the clinical phenotype appears highly heterogeneous between affected families but also within affected individuals from the same family. Some families only display mild thrombocytopenia without HM while others have been reported with pure familial leukemia rather than platelet disorder [3,41,52]. Such heterogeneity could be explained, at least in part, by different types of germline *RUNX1* mutations, with dominant negative (DN) mutants conferring a higher risk of HM than loss-of-function (LOF) mutants [56]. Thus, while nonsense/frameshift mutants that lead to the truncation of a substantial part of the RHD are likely to be LOF alleles, mutants displaying an intact RHD usually retain the ability to heterodimerize with CBF-beta and inhibit wild-type CBF in a DN-fashion [56]. Using induced pluripotent stem cells (iPSCs) from FPD/AML patients, Antony-Debré et *al.* showed that both DN and LOF mutants demonstrated profound defects in megakaryopoiesis while DN mutants also led to the amplification of the granulomonocytic compartment and a higher genomic instability [57]. In line with this hypothesis, a recent review of 9 families with FPD/AML reported HM incidence of 55% when the mutated allele was likely to cause a DN effect compared with 33% when it was a LOF allele [52].

Interestingly, the incomplete penetrance, the latency period before onset of HM as well as the variety of HM encountered suggest that secondary acquired events are necessary to the development of HM and may influence HM phenotype. To date, the most frequent additional event implicates the acquisition of a second *RUNX1* alteration [42] although this does not occur in all patients [44,58].

[…]

In absence of hematopoietic stem cell transplantation (HSCT), occurrence of several HM and rapid MDS progression to AML are common [42,52]. When HSCT is considered, related donors must be screened for the germline mutation. Adverse outcome including graft failures and donor-derived leukemia have been reported when a carrier sibling was used as a donor [41,52,61].

[…]

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PARTIE 5 - CBF ET ÉPIGÉNÉTIQUE : MUTATIONS DES GÈNES *ASXL*

1. Structure et fonctions des gènes *ASXL*

Les gènes *ASXL1* (20q11.21), *ASXL2* (2p23.3) et *ASXL3* (18q12.1) sont les homologues humains du gène *Asx* de la drosophile contrôlant l'expression des gènes homéotiques et initialement identifié chez un phénotype mutant de drosophile caractérisé par l'apparition de peignes sexuels supplémentaires sur les pattes (*additional sex combs*) [120]. Les protéines ASXL sont des régulateurs épigénétiques majeurs activant ou réprimant la transcription selon le contexte cellulaire en modifiant la méthylation des histones [121]. Les membres de la famille ASXL sont définis par la présence de plusieurs domaines conservés ASXN, ASXH, ASXM1 et ASXM2 impliqués dans les interactions protéine-protéine et un domaine PHD (*plant homeodomain*) C-terminal (Figure 16A).

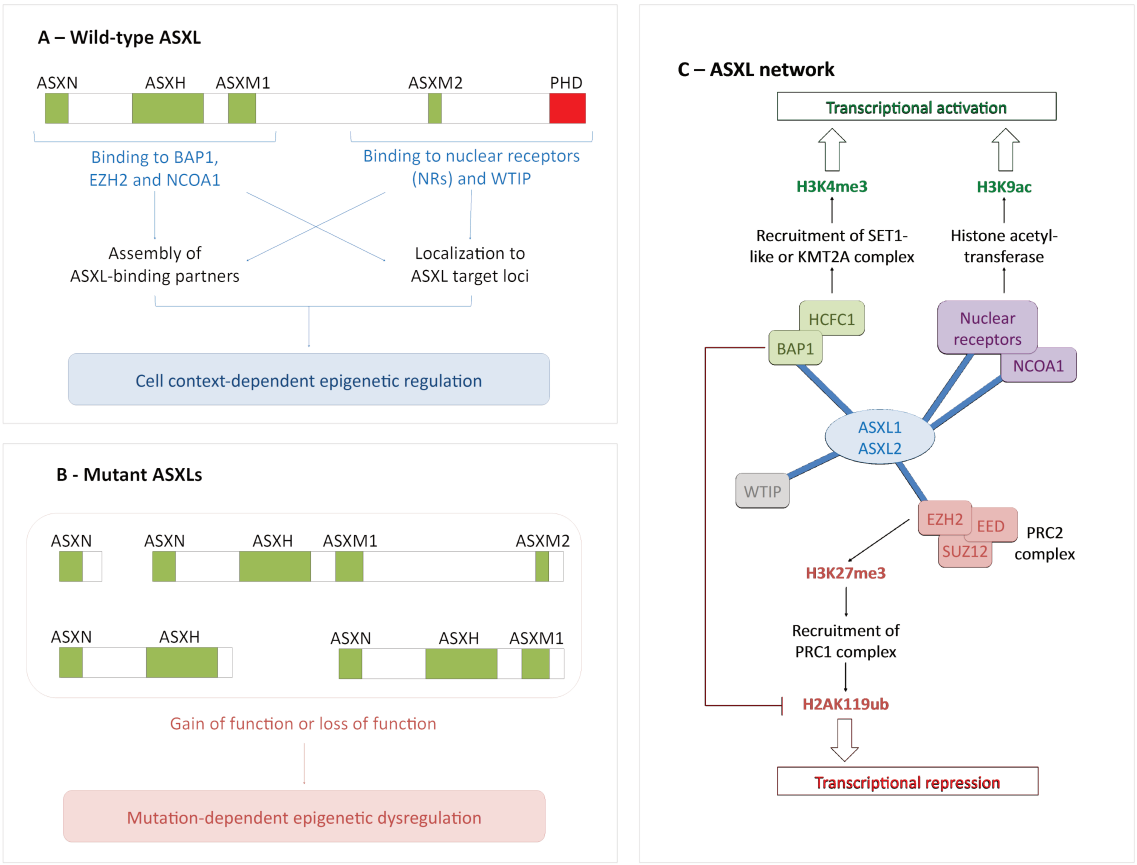


Figure 16 : Protéines ASXL. (A) Domaines conservés des protéines ASXL. (B) Mutants non-sens ou frameshift des protéines ASXL conduisant toujours à la perte du domaine PHD +/- ASXM2. (C) Principales interactions protéiques et régulation épigénétique médiée par ASXL1 et ASXL2 (adaptée de [121,122]).

Le PHD est une structure conservée en doigts de zinc, définie par un motif Cys4-His-Cys3, retrouvée dans de nombreuses protéines impliquées dans la régulation de la structure chromatinienne et la

transcription génique. Des études récentes montrent que les doigts PHD constituent un module de lecture spécifique des séquences d'histones et de leurs modifications post-traductionnelles (en particulier le statut de méthylation de l'histone H3). Ils permettent ainsi le contrôle de l'expression des gènes via le recrutement de complexes multiprotéiques régulateurs de la chromatine et des facteurs de transcription [123]. La région N-terminale contenant les domaines ASXN, ASXH et ASXM1 est impliquée dans les interactions avec l'histone-deubiquitinase BAP1, l'histone-méthyltransférase EZH2 (composant du PRC2 : *polycomb repressor complex 2*) et le coactivateur des récepteurs nucléaires NCOA1. La région autour de ASXM2 et du PHD est impliquée dans la liaison aux récepteurs nucléaires (RARα, PPARγ, LXRα…) et à la protéine *WTIP (WT1 interacting protein*) [124–126].

Le rôle des protéines ASXL est donc double. Le PRC2 catalyse la triméthylation de la lysine 27 de l'histone H3 (H3K27me3), marque permettant à son tour le recrutement du PRC1 et l'ubiquitination de la lysine 119 de l'histone H2A (H2AK119ub). H3K27me3 et H2AK119ub jouent un rôle synergique dans l'établissement et le maintien de la répression transcriptionnelle [127]. De l'autre côté, BAP1 catalyse la déubiquitination de H2AK119 et permet, via HCFC1, l'activation de la transcription par la triméthylation de la lysine 4 de l'histone H3 (H3K4me3) [128]. Enfin, NCOA1 permet le recrutement d'histone-acétyltransférase et favorise ainsi la transcription dépendante des facteurs nucléaires (Figure 16C)[121]. Certaines interactions apparaissent néanmoins plus spécifiques de certaines protéines ASXL. Ainsi, la protéine CBX5 (HP1A) interagit avec la région située entre les domaines ASXN et ASXH d'ASXL1 et d'ASXL3, mais pas avec ASXL2 pouvant expliquer certaines divergences fonctionnelles entre ASXL1 et ASXL2 [124].

2. Mutations somatiques des gènes *ASXL*

Les mutations somatiques d'*ASXL1* ont été décrites pour la première fois en 2009 chez des patients atteints de SMD et de LMMC [129]. Il s'agit de mutations hétérozygotes tronquantes (non-sens ou frameshift) dans l'exon 12 (plus rarement l'exon 11) conduisant à la production d'une protéine raccourcie ayant perdu son PHD C-terminal (Figure 16B). On les retrouve dans tous les types d'hémopathies myéloïdes où elles s'associent globalement à un pronostic défavorable [130]. Elles concernent environ 15% des SMD, 45% des LMMC, 35% des myélofibroses primitives, 35% des LAM secondaires et 6.5% des LAM *de novo*. A noter qu'elles sont également rapportées dans certaines tumeurs solides (utérus, colon, foie) [122]. Dans les LAM, les mutations d'*ASXL1* sont exclusives des mutations de *NPM1* et significativement associées aux altérations de *RUNX1* (mutations et fusion *RUNX1-RUNX1T1*) [131]. En effet, environ 30% des LAM avec mutation d'*ASXL1* présentent égale-

ment une mutation de *RUNX1* contre 10% des LAM non mutées pour *ASXL1* [131]. Inversement, environ 35% des LAM avec mutation de *RUNX1* présentent une mutation d'*ASXL1* contre seulement 6% des LAM non mutées pour *RUNX1* [68]. Les mutations d'*ASXL1* sont également rapportées dans 10-12% des LAM avec t(8;21) de l'adulte et de l'enfant [131–133].

En 2013, les mutations somatiques d'*ASXL2* sont identifiées dans des leucémies pédiatriques. Cette étude du *St Jude's Research Hospital* portait sur le séquençage de 633 gènes régulateurs épigénétiques chez plus de 1000 enfants atteints de 21 types de cancers différents [134]. En 2014, l'intergroupe français identifiait, par séquençage *whole exome*, une mutation d'*ASXL2* chez un enfant atteint de LAM avec t(8;21). Le séquençage ciblé des gènes *ASXL1* et *ASXL2* dans 170 LAM CBF (adultes et enfants) confirmait alors l'association spécifique des mutations d'*ASXL2* et de la t(8;21) [133]. Les mutations d'*ASXL1* et *ASXL2* étaient mutuellement exclusives et concernaient 10% et 23% des LAM avec t(8;21) tandis qu'elles n'étaient pas retrouvées dans les LAM avec inv(16). Comme décrit précedemment pour *ASXL1*, les mutations d'*ASXL2* étaient exclusivement retrouvées dans la région 3' du gène, au niveau des exons 11 et 12. Toutes les mutations entraînaient l'apparition prématurée d'un codon STOP et la perte du domaine PHD. De manière surprenante, aucune mutation d'*ASXL2* n'était identifiée au sein des LAM avec mutation ponctuelle de *RUNX1* pourtant caractérisées par la haute fréquence des mutations de son paralogue *ASXL1* (Figure 17)[133].

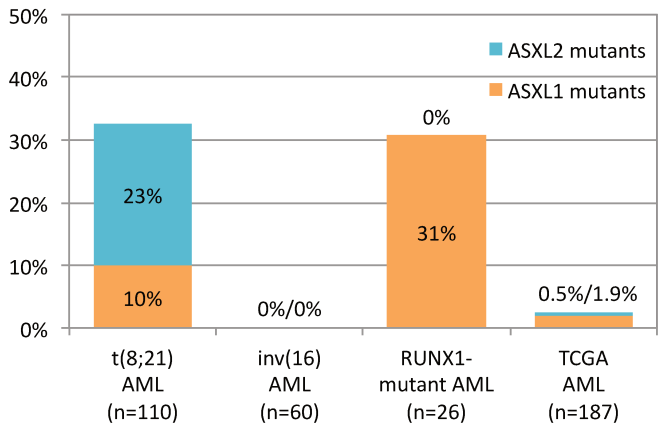


Figure 17 : Fréquences des mutations d'*ASXL1/2* dans les LAM CBF et LAM avec mutations de *RUNX1* [133]. Les fréquences des mutations *ASXL1/2* de l'étude TCGA [incluant 19 LAM CBF] sont aussi représentées [17].

Globalement, l'impact pronostique des mutations des gènes *ASXL1* et *ASXL2* dans les LAM avec t(8;21) reste controversé. Dans l'étude de l'intergroupe français, les mutations d'*ASXL1* et *ASXL2* n'avaient pas d'impact sur la survie globale mais une tendance à l'augmentation du risque cumulé de rechute était observée (55% et 36% à 3 ans pour les patients mutés *ASXL1* et *ASXL2* respectivement, contre 25% pour les patients non mutés ; p=0.226) [133]. D'autre part, l'étude de 204 LAM de

l'adulte avec t(8;21) du groupe austro-allemand AMLSG montrait une absence d'effet sur la survie globale ou la survie sans rechute [135]. Les fréquences des mutations d'*ASXL1* et *ASXL2* rapportées dans cette étude étaient de 17% et 8% respectivement. L'étude du *Munich Leukemia Laboratory* portant sur 139 LAM de l'adulte avec t(8;21) montrait une association entre mutations d'*ASXL1* (11.5% de la cohorte) et diminution de la survie dans événement (29% *vs.* 57% à 2 ans ; p=0.021), sans modification de la survie globale [132]. Les mutations d'*ASXL2* n'y étaient pas explorées. Enfin, une étude japonaise sur 107 LAM pédiatriques avec t(8;21) ne retrouvait aucune association des mutations d'*ASXL1* ou *ASXL2* avec la survie globale ou la survie sans événement [136].

Au-delà de l'impact pronostique, la découverte de ces mutations découvre un pan non exploré de la physiopathologie des LAM CBF : la coopération des anomalies épigénétiques. Le fait que les mutations d'*ASXL2* n'aient pas été identifiées lors d'études précédentes de séquençage *whole genome* ou *whole exome* de LAM suggère en effet une coopération spécifique entre mutations d'*ASXL2* et fusion *RUNX1-RUNX1T1* [17,137–139]. Récemment, Jean-Baptiste Micol et *al.* ont démontré le rôle d'ASXL2 dans l'hématopoïèse et la leucémogénèse dépendante de *RUNX1-RUNX1T1*. Le résumé de ces travaux est présenté en annexe de cette thèse (*ASXL2 is essential for hematopoiesis and acts as a haploinsufficient tumor suppressor in leukemia. Nat Commun, 2017*).

OBJECTIFS

Les LAM CBF (*i.e.* présentant une fusion *RUNX1-RUNX1T1* ou *CBFB-MYH11*) et les LAM familiales associées aux mutations de *RUNX1* sont considérées comme des modèles de leucémogénèse multi-étape. De nombreux travaux ont démontré que si la désorganisation du CBF constitue l'anomalie initiatrice de la leucémie, elle est insuffisante à son développement [50].

Ainsi, l'expression seule de *RUNX1-RUNX1T1* ou *CBFB-MYH11* dans des modèles murins perturbe la différenciation myéloïde et augmente les capacités d'autorenouvellement des CSH mais reste sans effet sur la prolifération cellulaire. En revanche, le développement d'une LAM est rapide lorsque ces modèles sont exposés à un mutagène [140,141] ou coexpriment un RTK constitutivement activé [142]. Chez l'Homme, ces travaux sont supportés par l'observation *in vivo* des transcrits de fusion chez des patients en RC prolongée [143] ou encore dans des tests de Guthrie de nouveau-nés ayant développé une LAM CBF jusqu'à 12 ans après la naissance [144]. De plus, si le « two-hit model » [21] associant désorganisation du CBF et activation d'une tyrosine kinase reste biologiquement valable, la découverte récente des mutations d'*ASXL1* et *ASXL2* dans les LAM avec t(8;21) traduit la plus grande complexité, notamment épigénétique, de la physiopathologie de ces leucémies [132,133]. Enfin, il faut noter à l'heure actuelle qu'aucune mutation de type tyrosine kinase n'est identifiée chez 30 à 40% des patients atteints de LAM CBF.

De la même manière, les mutations germinales de *RUNX1* prédisposent aux LAM avec une pénétrance incomplète (20-60% selon les familles) et un temps de latence très variable (6-75 ans) [39]. L'étude génétique de ces patients a montré que la progression vers le diagnostic de LAM était concomitante de l'acquisition d'anomalies génétiques impliquant le plus souvent le second allèle de *RUNX1* [145]. Cependant, à l'inverse des réarrangements du CBF, les mutations germinales de *RUNX1* s'associent à une grande variété de phénotypes leucémiques pouvant refléter des anomalies acquises de différentes natures [39].

En conséquence, ce travail a pour objectifs l'identification des anomalies additionnelles acquises dans les LAM avec réarrangement du CBF (de type *RUNX1-RUNX1T1* et *CBFB-MYH11*) et les LAM avec mutation germinale de *RUNX1* ainsi que l'établissement de corrélations avec les caractéristiques clinico-biologiques et pronostiques de ces différentes entités.

RÉSULTATS

ARTICLE1 : ÉTUDE DU GÈNE *ASXL3*

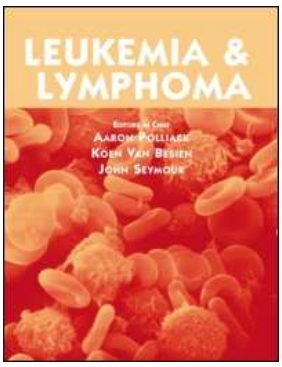
Unlike *ASXL1* and *ASXL2* mutations, *ASXL3* mutations are rare events in acute myeloid leukemia with t(8;21)

Nicolas Duployez, Jean-Baptiste Micol, Nicolas Boissel, Arnaud Petit, Sandrine Geffroy, Maxime Bucci, Hélène Lapillonne, Aline Renneville, Guy Leverger, Norbert Ifrah, Hervé Dombret, Omar Abdel-Wahab, Eric Jourdan & Claude Preudhomme

Résumé : Les gènes de la famille *ASXL* (*ASXL1*, *ASXL2* et *ASXL3*) sont des régulateurs épigénétiques majeurs participant au remodelage de la chromatine et intervenant dans la physiopathologie de nombreux cancers solides et hématologiques. Ils sont caractérisés par une forte homologie de séquence suggérant des interactions et effets cellulaires redondants. Récemment, les mutations d'*ASXL1* ou d'*ASXL2* ont été rapportées dans environ un tiers des LAM avec t(8;21) supposant une coopération spécifique entre les protéines ASXL et la fusion *RUNX1-RUNX1T1*. Le gène *ASXL3* n'a par ailleurs jamais été étudié dans ce groupe de LAM.

Nous avons donc séquencé *ASXL3* dans les prélèvements diagnostiques de 110 LAM avec t(8;21) dont 36 présentaient une mutation d'*ASXL1* ou *ASXL*2. Une seule mutation frameshift d'*ASXL3* était finalement identifiée dans la cohorte, contrastant avec la haute fréquence des mutations des 2 autres homologues. L'élucidation des mécanismes expliquant la haute fréquence des mutations d'*ASXL1/2*, la spécificité des mutations d'*ASXL2* et la quasi-absence des mutations d'*ASXL3* dans ce groupe de LAM permettra vraisemblablement de mieux comprendre la physiopathologie des LAM avec t(8;21).

Unlike *ASXL1* and *ASXL2* mutations, *ASXL3* mutations are rare events in acute myeloid leukemia with t(8;21)



Nicolas Duployez[1], Jean-Baptiste Micol[2,3], Nicolas Boissel[4], Arnaud Petit[5], Sandrine Geffroy[1], Maxime Bucci[1], Hélène Lapillonne[6], Aline Renneville[1], Guy Leverger[5], Norbert Ifrah[7], Hervé Dombret[4], Omar Abdel-Wahab[3], Eric Jourdan[8] and Claude Preudhomme[1].

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LETTER TO THE EDITOR:

Additional sex combs-like (*ASXL*) genes are human homologues of the Drosophila-*Asx* gene encoding proteins that control gene expression through the regulation and the recruitment of epigenetic regulator complexes and transcription factors to specific genomic loci with histone modifications. The human ASXL family consists of three members ASXL1, ASXL2 and ASXL3 that share a common domain architecture, including highly conserved ASXN and ASXH domains in the amino-terminal region and a carboxy-terminal plant homeo-domain (PHD)[1]. Bioinformatics analysis predicted that the ASXN domain is a DNA-binding region. The ASXH domain is known to be involved in protein-protein interactions with several ASXL partners such as BAP1 or KDM1A[1,2]. Finally, the PHD domain represents a protein-protein interaction domain which may recognize histone modifications.

In 2009, somatic *ASXL1* mutations were first identified in 10-20% of myeloid neoplasms[3], mostly associated with adverse features[4]. Despite this association, alterations in other *ASXL* family members were not described in hematological malignancies. In a recent report, we performed next-generation sequencing (NGS) for *ASXL1* and *ASXL2* in 170 adult and pediatric acute myeloid leukemia (AML) with disruption of the core-binding factor (CBF), including 110 t(8;21)-AML and 60 inv(16)-AML patients[5]. *ASXL1* mutations were found in 10% of t(8;21)-AML patients, in line with other reports[6]. Surprisingly, *ASXL2* mutations were found in 23% of t(8;21)-AML patients, making *ASXL2* the second most commonly mutated gene in this cytogenetic subgroup, behind *KIT*. As described for its paralog *ASXL1*, *ASXL2* mutations were frameshift and nonsense mutations that are predicted to result in either nonsense-mediated decay of the mutated transcript or production of a stable protein with truncation of

the C-terminal PHD domain. Interestingly, no *ASXL2* mutations were found in inv(16)-AML patients. The fact that recurrent *ASXL2* mutations were not identified in previous whole genome/exome sequencing studies in AML[7], together with our results, suggests there is a functional connection between the *RUNX1-RUNX1T1* fusion protein and *ASXL1/2* mutations[8]. In addition, these findings could be of clinical importance since cumulative incidence of relapse appeared to be higher in patients with *ASXL* mutations (although this did not reach statistical significance). We hypothesized that lack of statistical significance could be related, at least in part, to undefined gene alterations involving the ASXL pathway in *ASXL1/2*-wild type patients, such as mutations of the third *ASXL* family member *ASXL3*. This hypothesis is supported by the identification of a single *ASXL3* mutant AML sample in the AML TCGA study[7] as well as the presence of *ASXL3* mutations in >10% of patients with melanoma and lung adenocarcionoma[9]. Moreover, it has been shown that *ASXL3* was expressed in similar tissues to *ASXL1* including bone marrow, although at a lower level[10]. Interestingly, germ-line *ASXL3* mutations have been reported in a syndrome with phenotypic overlap with Bohring-Opitz syndrome, a disease associated with germ-line *ASXL1* mutations, arguing that these mutations may have redundant effects[10].

We then performed NGS of all coding exons of *ASXL3* in 74 *ASXL1/2*-wild type t(8;21)-AML patients and 36 *ASXL1* or *ASXL2* mutated patients from the CBF-2006 (ClinicalTrials.gov NCT00428558) and ELAM02 (ClinicalTrials.gov NCT00149162) trials. Patient characteristics are included in **table S1 (supplemental data)**. Library preparation was performed using the Ion Ampliseq™ Library kit 2.0-384 LV (Life Technologies®). Template-positive Ion Sphere Particles™ were prepared using the Ion OneTouch™ 200 Template Kit v2 (Life Technologies®). Sequencing was performed with the Ion PGM™ 200 Sequencing Kit on the Personal Genome Machine® platform (Life Technologies®). Mean depth of coverage was 1780 reads allowing detections of small clones. No mutation was found in *ASXL1/2*-wild type t(8;21)-AML patients excepted for a p.M1989I variant. Although germline material was not available, this variant was considered as a heterozygous polymorphism because it was not predicted to be pathogenic according to bioinformatics tools[11] and was found with a variant allele frequency (VAF) of 50%. Surprisingly, an *ASXL3* mutation was found in an *ASXL1*-mutated patient with a VAF of 29%. As described for *ASXL1* and *ASXL2* in AML, it was a frameshift mutation (c.1842_1855del(14bp):p.S615GfsX27) leading to a premature stop codon.

Extensive mutational analysis (MiSeq, Illumina®) found concomitant mutations in *ASXL1* (c.1934dupG:p.G646WfsX12) and *KIT* exon 8 with VAF of 30% and 34% respectively. All mutations were validated by Sanger sequencing. The patient was a 7-year old girl diagnosed with t(8;21)-AML and an extramedullary orbital granulocytic sarcoma. She was enrolled in the ELAM02 trial and achieved complete remission (CR) after the induction course. Unfortunately, she relapsed 13 months

later and then received placental cord blood transplantation in second CR. She is still in CR after 5 year post-transplant and is considered to be cured.

In conclusion, we showed that, in contrast with *ASXL1* and *ASXL2* mutations, *ASXL3* mutations were rare events within t(8;21)-AML patients. The fact that *ASXL* mutations are not randomly distributed among the 3 ASXL members suggests additional mechanisms that could not be explained by the conserved domains. For example, a CBX5-binding motif is located in the region located between the ASXN and ASXH domains of ASXL1 and ASXL3 but is absent in ASXL2, which could potentially explain divergent effects of disrupting *ASXL1/3* versus *ASXL2*[12]. Moreover, we found concomitant *ASXL1* and *ASXL3* mutations in a same patient suggesting theses abnormalities could cooperate, although we cannot exclude they happen in different clones. Overall, understanding the functional basis for the high frequency of *ASXL1/2* mutations, the apparent specificity of *ASXL2* mutations and the virtual absence of *ASXL3* mutations in t(8;21)-AML will be critical to promote our knowledge of t(8;21)-AML pathogenesis and remain an exciting challenge for future studies.

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CONFLICT-OF-INTEREST DISCLOSURE: The authors declare no competing financial interests.

SUPPLEMENTAL DATA

Supplemental Table S1: Patient characteristics.

Patient characteristics	All patients (n = 110)	Adults (CBF-2006 trial) (n = 76)	Children (ELAM-02 trial) (n = 34)
Age, median [range] (years)	31 [4-60]	40 [18-60]	10 [4-17]
Sex (M/F)	59/51	44/32	16/18
WBC, median [range] (G/l)	12.8 [1.3-163]	11 [2.2-94.5]	14 [1.3-163]
Blasts [range] (%)	55 [17-98]	53 [17-98]	60 [30-93]
Additional cytogenetic abnormalities			
Loss of Y	36/109 (33%)	28/75 (37%)	8/34 (24%)
Del(9q)	16/109 (15%)	11/75 (15%)	5/34 (15%)
+8	6/109 (6%)	3/75 (4%)	3/34 (9%)
Gene mutations			
FLT3-TKD	4/110 (4%)	3/76 (4%)	1/34 (3%)
FLT3-ITD	10/110 (9%)	5/76 (7%)	5/34 (15%)
KIT	35/110 (32%)	20/76 (26%)	15/34 (44%)
N-RAS* or *K-RAS	20/110 (18%)	12/76 (16%)	8/34 (23%)
ASXL1	11/110 (10%)	9/76 (12%)	2/34 (6%)
ASXL2	25/110 (23%)	16/76 (21%)	9/34 (26%)
ASXL3	1/110 (0.9%)	0/76 (0)	1/34 (3%)

ARTICLE 2 : PROFIL MUTATIONNEL ÉTENDU DES LAM CBF

Comprehensive mutational profiling of core binding factor acute myeloid leukemia

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Résumé : Les LAM CBF sont caractérisées par la présence d'une t(8;21) ou d'une inv(16) conduisant à la perturbation du *core binding factor*. Elles sont associées à une bonne réponse à la chimiothérapie mais leur évolution reste hétérogène, marquée par un taux de rechute allant jusqu'à 40%. La perturbation du CBF constitue bien le *primum movens* du processus leucémogène mais de nombreux travaux ont démontré que la transformation leucémique nécessitait l'intervention d'anomalies secondaires. Si certaines anomalies moléculaires impliquant les voies tyrosine kinase (TK) sont aujourd'hui bien connues, le spectre complet de mutations coopératrices n'a pas encore été identifié.

Nous avons étudié les prélèvements au diagnostic de 215 LAM CBF inclus dans les protocoles CBF2006 (adultes) et ELAM02 (enfants) par séquençage haut débit (validation croisée par séquençage double sur MiSeq [Illumina] et PGM [Life Technologies]) sur un panel ciblé de 40 gènes. Les mutations des voies TK (*KIT, N/KRAS, FLT3*) étaient les plus fréquentes quel que soit le sous-type de LAM. En revanche, les mutations des gènes de la cohésine (*SMC1A, SMC3, RAD21, STAG2*) ou du remodelage chromatinien (*ASXL1/2, EZH2, KDM6A*) étaient observées dans 18% et 41% des LAM avec t(8;21) tandis qu'elles étaient absentes dans les LAM avec inv(16). Un ratio allélique élevé de *KIT* muté définissait un sous-groupe de LAM avec t(8;21) de pronostic défavorable tandis que des ratios élevés de *N/KRAS* mutés étaient associés à un pronostic favorable et à l'absence de mutations de *KIT* ou *FLT3*. Enfin, les mutations des gènes du remodelage chromatinien ou de la cohésine étaient associées à un mauvais pronostic chez les patients avec des mutations de type TK.

Ces résultats suggèrent que différents événements coopérateurs sont capables d'influencer la physiopathologie des LAM CBF ainsi que leurs caractéristiques cliniques et biologiques. De plus, la découverte de profils mutationnels distincts entre LAM avec t(8;21) et LAM avec inv(16) fait discuter l'apparente homogénéité des LAM CBF et met en avant une voie pathologique potentielle unique aux LAM avec t(8;21).

Comprehensive mutational profiling of core binding factor acute myeloid leukemia

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Abstract: Acute myeloid leukemia (AML) with t(8;21) or inv(16) have been recognized as unique entities within AML and are usually reported together as core binding factor AML (CBF-AML). However, there is considerable clinical and biological heterogeneity within this group of diseases and relapse incidence reach up to 40%. Moreover, translocations involving CBFs are not sufficient to induce AML on its own and the full spectrum of mutations coexisting with CBF translocations has not been elucidated. In order to address these issues we performed extensive mutational analysis by high-throughput sequencing in 215 patients with CBF-AML enrolled in the CBF2006 and ELAM02 trials (aged from 1 to 60 years). Mutations in genes activating tyrosine kinase (TK) signaling (including *KIT*, *N/KRAS*, *FLT3*) were frequent in both subtypes of CBF-AML. In contrast, mutations in genes that regulate chromatin conformation or encode members of the cohesin complex were observed with high frequencies in t(8;21) AML (42% and 18%, respectively) while they were nearly absent in inv(16) AML. High *KIT* mutant allele ratios defined a group of t(8;21) AML patients with poor prognosis while high *N/KRAS* mutant allele ratios were associated with the lack of *KIT* or *FLT3* mutations and a favorable outcome. In addition, mutations in epigenetic modifying or cohesin genes were associated with a poor prognosis in patients with TK pathway mutations suggesting synergic cooperation between these events. These data suggest that diverse cooperating mutations may influence CBF-AML pathophysiology as well as clinical behavior and point to potential unique pathogenesis of t(8;21) versus inv(16) AML.

Keywords: Acute myeloid leukemia, core binding factor, t(8;21), inv(16), mutation analysis, high-throughput sequencing, cohesin, epigenetics, polycomb, tyrosine kinase, prognosis.

Key points:

1. Recurrent mutations in chromatin modifiers and cohesin were observed in t(8;21) AML but not inv(16) AML.
2. t(8;21) AML patients with mutations in kinase signaling plus chromatin modifiers or cohesin members had the highest risk of relapse.

INTRODUCTION

Core-binding factor (CBF) acute myeloid leukemia (AML) includes AML with t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16)(p13;q22) chromosomal rearrangements (abbreviated t(8;21) as inv(16) respectively) leading to the *RUNX1-RUNX1T1* and *CBFB-MYH11* fusion genes respectively. CBF AML is among the most common cytogenetic subtypes of AML as t(8;21) and inv(16) together account for approximately 25% of pediatric and 15% of adult *de novo* AML patients [1]. Their identification is critical in routine practice as the presence of these alterations significantly impacts clinical management of AML [2]. CBF AML is considered to have a good prognosis relative to other AML subtypes and treatments using high-dose cytarabine-based chemotherapy have resulted in markedly improved outcome. Nonetheless, relapse occurs up to 40% in such patients indicating clinical heterogeneity amongst CBF AML patients [3–6].

Since the first description of t(8;21) and inv(16) AML in 1973 [7] and 1983 [8] respectively, a great deal has been learned about the molecular consequences of both rearrangements. Both alterations result in disruption of genes encoding subunits of the CBF complex (i.e. *RUNX1* and *CBFB*), a heterodimeric transcription factor complex which regulates the expression of genes required for normal hematopoiesis [9,10]. Homozygous disruption of *Runx1* or *Cbfb* in mice results in identical developmental defects, including failure to develop definitive hematopoiesis and embryonic death [11]. At the same time, experience from murine models has demonstrated that the expression of the *RUNX1-RUNX1T1* or *CBFB-MYH11* fusion proteins alone induces aberrant self-renewal but is insufficient to induce fulminant leukemia [11]. Consistent with this, preleukemic cells harboring *RUNX1-RUNX1T1* or *CBFB-MYH11* fusion genes have been identified for >10 years before clinical development of AML as well as following long-term clinical remission of AML [12,13]. CBF AML is therefore considered to be a model for the multistep pathogenesis of leukemia in which AML development requires the cooperation occurring from disruption of a transcription factor (such as the CBF complex) that impairs differentiation plus an activating mutations that increases proliferation [14]. Further evidence supporting this model comes from the fact that frequent mutations activating tyrosine kinase (TK) signal-

ing (including *KIT*, *FLT3* and *N/KRAS* family genes) are frequently observed in both CBF AML subtypes [3].

Given the similarities in prognostic features and involvement of CBF transcription factors in their pathogenesis, t(8;21) and inv(16) AML have been recognized as a unique entity within AML and are usually grouped and reported together in clinical studies. However, patients with t(8;21) or inv (16) AML differ with respect to several biological and clinical features [15]. Morphologically, patients with t(8;21) AML frequently present with the French-American-British (FAB) morphological subtype M2 or AML with maturation, while patients with inv(16) more often are diagnosed with the FAB subtype M4Eo or acute myelomonocytic leukemia with abnormal marrow eosinophils [16]. Moreover, gene expression profiling of CBF AML segregate t(8;21) and inv(16) patients into distinct subgroups [17] reflecting different pathways activated in each subtype of CBF AML [18]. While a genetic basis for morphologic and transcriptional differences between t(8;21) and inv(16) AML were previously unknown, frequent mutations in *ASXL1* and *ASXL2* were recently described specifically in t(8;21) AML patients. *ASXL1/2* mutations have been described in ~35% of t(8;21) AML but are absent in inv(16)AML [19,20]. Interestingly, *ASXL2* mutations are not recurrent in subsets of AML other than t(8;21) AML suggesting an important potential functional intersection between *ASXL2* mutations and the *RUNX1-RUNX1T1* fusion specifically [21]. Besides *ASXL1/2* mutations, however, no other recurrent mutations specific to one or both CBF AML subsets are currently known.

Given that (1) up to 40% of patients with CBF AML relapse and that (2) CBF disruption is not sufficient to induce AML on its own, we hypothesized that additional recurrent genetic abnormalities may be enriched in one or more subsets of CBF AML patients. Through extensive mutational analysis of a large and well annotated cohort of with CBF AML patients we identified a series of recurrent genetic alterations in genes encoding epigenetic modifiers and cohesin members with direct relevance specifically in t(8;21) AML. Moreover, we identified additional importance of allelic ratios of mutations affecting TK signaling across CBF AML. These data suggest that t(8;21) and inv(16) AML may have distinct pathophysiology and that comprehensive genetic analysis may be utilized to refine prognostication in CBF AML.

METHODS

Patients and treatments

This study included 215 patients with CBF AML including 106 with t(8;21) and 109 with inv(16) AML. The cohort included 142 adults treated in the CBF2006 trial [3] (a phase 3 trial of systematic versus

response-adapted timed-sequential induction in patients with CBF AML; EudraCT 2006 005163-26; ClinicalTrials.gov NCT00428558) as well as 73 children treated in the ELAM02 trial (treating patients with childhood AML with Interleukin-2; ClinicalTrials.gov NCT00149162) (**Supplementary Figure 1**). Studies were approved by the Ethics Committee of Nimes University Hospital and by the Institutional Review Board of the French Regulatory Agency and were conducted in accordance with the Declaration of Helsinki protocol.

Mutational analysis by High-Throughput Sequencing

Bone marrow samples from CBF AML patients at diagnosis were studied by high-throughput sequencing (HTS) of 40 genes recurrently mutated in myeloid malignancies. This included genes encoding proteins involved in signal transduction (*CBL* [exons 8-9], *CSF3R* [exons 3-18], *FLT3* [exons 14-15 + 20], *JAK2* [exons 12 + 14], *KIT* [exons 8-13 + 17], *KRAS* [exons 2-3], *MPL* [exon 10], *NRAS* [exons 2-3], *PTPN11* [exons 3 + 13]), transcription (*BCOR* [exons 2-15], *BCORL1* [exons 1-12], *CEBPA* [exon 1], *ETV6* [exons 1-8], *GATA1* [exon 2], *GATA2* [exons 2-6], *IKZF1* [exons 1-8] *RUNX1* [exons 1-6]), chromatin modification (*ASXL1* [exon 12], *ASXL2* [exons 1 to 12], *EZH2* [exons 2-20], *KDM6A* [exons 1-29], *KMT2A* [exons 5-8]), DNA methylation (*DNMT3A* [exons 2-23], *IDH1* [exon 4], *IDH2* [exon 4], *TET2* [exons 3-11]), RNA splicing (*SF3B1* [exons 13-18], *SRSF2* [exon 1], *U2AF1* [exons 2 + 6], *ZRSR2* [exons 1-11]), cohesin complex (*RAD21* [exons 2-14], *SMC1A* [exons 1-25], *SMC3* [exons 1-29], *STAG2* [exons 3-35]) tumor suppression (*PHF6* [exons 2-10], *TP53* [exons 2-11], *WT1* [exons 7 + 9]] and other pathways [*CALR* [exon 9], *NPM1* [exon 11], *SETBP1* [exon 4]). For all but *ASXL2*, libraries were prepared using Haloplex™ Target Enrichment System (Agilent Technologies®) according to the manufacturer's instructions and run on MiSeq® (Illumina®). A high depth of coverage (>2000X) was obtained for all genes (**Supplementary Figure 2**), allowing detection of mutations with a variant allele frequency (VAF) until 1%. Raw HTS data were analyzed with 2 distinct softwares: SureCall™ (Agilent Technologies®) and SeqNext (JSI Medical System®). Frameshift and nonsense variants were always considered as relevant mutations. Single nucleotide variants were retained in the absence of description into public databases of human polymorphisms and effects on protein function were predicted with six established prediction tools: SIFT, PolyPhen-1, PolyPhen-2, MAPP, PhD-SNP and SNAP [22]. All variants were validated with another HTS technology with library preparation using Ampliseq™ System and sequencing on Personal Genome Machine® (PGM, Life Technologies®). Data from PGM sequencing were processed by Torrent Browser (Life Technologies®) and SeqNext (JSI Medical System®). *ASXL2* sequencing was performed as previously described [19]. Notably, because of technical limitations, the mutation c.1934dupG in *ASXL1* cannot be detected with PGM sequencing justifying its systematic validation by Sanger sequencing as previously described [19]. In one case, this *ASXL1* variant

could not be verified by Sanger sequencing because of a low variant allele frequency (VAF: 4%) but was retained by visual check of the reads (UPN 53).

Other cytogenetic and molecular analyses

The presence of the t(8;21) or the inv(16)/t(16;16) rearrangements were determined by karyotype (as well as additional cytogenetic abnormalities) and/or fluorescence in situ hybridization and/or evidence of *RUNX1-RUNX1T1* or *CBFB-MYH11* fusion transcripts as previously described [3]. The presence of the *FLT3*-internal tandem duplication (ITD) was not determined by HTS but systematic screening was performed for all patients as previously described [23]. Minimal residual disease (MRD) was evaluated with *RUNX1-RUNX1T1* or *CBFB-MYH11* real-time quantitative PCR analysis as previously described [3].

Statistical methods

Failure time data were analyzed and compared after censoring at transplant for patients who received allogeneic stem cell transplantation (SCT) in first complete remission (CR). Cumulative incidence of relapse (CIR) and overall survival (OS) were estimated by the Kaplan-Meier method. Cumulative incidence of relapse (CIR) was estimated taking into account death in first complete remission (CR) for competing risk. CIR and OS were compared by cause-specific hazard Cox models after stratification on the trial (CBF2006 versus ELAM02). Comparisons between patient subgroups were performed by the Mann-Whitney test for continuous variables and by Fisher's exact test for categorical variables. Optimal cut-points for allelic ratios were determined by maximally selected log-rank statistics [24]. Specific hazards of relapse (SHRs) and HRs are given with 95% confidence interval (CI). Univariate and multivariate analysis assessing the impact of categorical and continuous variables were performed with a Cox model. Proportional-hazards assumption was checked before conducting multivariate analyses [25]. Covariates with a p-value less than 0.1 in univariate analysis were included in the multivariable models. All statistical tests were performed with the Stata/IC 12.1 software (StataCorp, College Station, TX).

RESULTS

Patient's characteristics at diagnosis

Patient's characteristics as well as additional molecular and cytogenetic aberrations are shown in **Table 1** according to CBF subtype. Median age was 32 years (patients were aged from 1 to 17 years in the ELAM02 trial and from 18 to 60 years in the CBF2006 trial). The median follow-up was 5.3 years.

Adult and pediatric patients were grouped together for further investigations since CIR was similar regardless of the trial (**Supplementary Figure 3**). Patients with inv(16) AML were younger (median age: 25 vs 37; p=0.004) and had a higher WBC count (median 34.4 vs 11.5; p<0.001) than those with t(8;21) AML.

Additional aberrations are found in more than 90% of CBF AML

Mutation frequencies are reported in **Table 1**. All identified variants with their VAFs are reported in **Supplementary Table 1**. Among the 215 patients analyzed, 182 (85%) had at least one mutation. Among them, 72 patients had 1 alteration, 51 had 2, 41 had 3, 13 had 4, 4 had 5 and 1 had 6. Considering the most common cytogenetic abnormalities in CBF AML together with mutations, additional aberrations were found in 93% of CBF AML (respectively 96% and 90% of t(8;21) AML and inv(16) AML).The **Figure 1** depicts the identified mutations as well as additional cytogenetic aberrations according to CBF AML subtype. Notably, mutational patterns appeared to be quite similar regardless of age group (**Supplementary Table 2**).

Mutations disrupting tyrosine kinase signaling are the most frequent events in CBF AML

Amongst all genes sequenced, the most common mutations involved genes affecting TK signaling (especially *KIT*, *FLT3* and *N/KRAS* mutations). A higher mutation incidence in these genes was identified here compared with prior studies using standard PCR and direct sequencing [3,23,26], likely due to the fact that HTS and cross-validation allowed for detection of mutations with very low VAFs (**Supplementary Figure 4**).

KIT mutations were found in 40% of AML with t(8;21) and 33% of AML with inv(16) (p=0.345). Most of *KIT* mutations were small deletions and or insertions in exon 8 leading to replacement of codon D419 or point mutations in exon 17. All but one exon 17 mutation involved codons D816 and N822 in the activation loop of the kinase domain. *KIT* exon 11 mutations were only detected in 2 patients. Two patients harbored rare *KIT* variants K509I in exon 9 (UPN206) and N655K in exon 13 (UPN3), previously reported in pediatric mastocytosis [27] and gastrointestinal stromal tumor [28], respectively, and demonstrated to cause constitutive ligand-independent activation of KIT (**Supplementary Figure 5A**).

FLT3-TKD mutations (especially at codon D835) were involved in 22% of inv(16) AML but only 4% of t(8;21) AML (p<0.001). On the other hand, *FLT3*-ITD was present in only 3% of inv(16) AML while they occurred in 10% of t(8;21) AML (p=0.028).

Notably, adult and pediatric patients with inv(16) AML differ by the pattern of mutations in genes coding for receptor tyrosine kinases (RTK) *KIT* and *FLT3*, a feature not shared by t(8;21) AML patients. Indeed, *FLT3*-TKD mutations have an incidence of 28% in adult inv(16) AML while they account for 7% of pediatric cases (p=0.019). On the other hand, *KIT* mutations are found in 50% of pediatric inv(16) AML but were present in 27% of adult cases (p=0.024) (**Supplementary Figure 6**).

As previously reported [29], *RAS* (*NRAS* or *KRAS*) mutations were the most frequent mutations in inv(16) AML. *RAS* mutations were found in 54% of inv(16) AML and 26% of t(8;21) AML (p<0.001). All but 4 *RAS* mutations involved the hotspots at codons G12, G13 and Q61 (**Supplementary Figure 5B**).

Other events in TK pathway included rare mutations in *JAK2* (V617F) *CBL* and *PTPN11*.

Co-occuring mutations in TK pathway gene members are frequent in CBF-AML

Seventy-five CBF AML patients (35%) had 2 or more mutations in genes coding for TK pathway effectors (involving the same gene or different genes, especially *KIT*, *FLT3* and *RAS* genes). Among them, 6 patients had 2 or more *KIT* mutations, 25 patients had 2 or more *RAS* mutations and 8 patients had 2 or more *FLT3*-ITD or TKD mutations. These findings highlight the multi-clonality of CBF AML (**Supplementary Figure 7**). In all but 6 CBF AML patients, the total of VAFs for TK pathway mutations was less than 50% (corresponding to 1 heterozygous mutation per cell). In the 6 remaining patients, 2 had mutations in the *CBL* gene which is known to be involved in uniparental disomies [30] and 1 patient (UPN 71) had a *KIT* mutation with a VAF at 62%. In this patient, review of the karyotype showed duplication of the long arm of the chromosome 4 (probably containing the mutated *KIT* gene). Considering the redundant effects of these mutations, it is likely they occur in distinct clones although further investigations are needed to study clonal architecture and extend those findings.

Mutations of epigenetic regulators and cohesin complex are common in t(8;21) but rare in inv(16) CBF AML

Mutations in genes encoding epigenetic regulators that control chromatin conformation were found in 42% of t(8;21) AML but only in 6% of inv(16) AML (p<0.001). These mutations were largely mutually exclusive with one another (**Supplementary Figure 8**). Amongst t(8;21) AML patients, *ASXL1* or *ASXL2* mutations occurred together in 32% of cases (all mutations were frameshift and nonsense mutations). *EZH2* mutations were identified in 7% of t(8;21) AML. All but one were located within the post-SET domain, normal expression of which is essential for the conformation of the protein and its subsequent catalytic activity [31]. Other alterations within chromatin modifiers included *KDM6A*, *BCOR* and *BCORL1* mutations in 6%, 1% and 3% of patients with t(8;21) AML.

Likewise, mutations in genes encoding members of the cohesin complex were identified specifically in patients in t(8;21) AML but not in inv(16) AML. Cohesin mutations were present in 18% of t(8;21) AML but in none of inv(16) AML patients (p<0.001). All cohesin mutations were mutually exclusive among each other (**Supplementary Figure 9**). All *RAD21* and *STAG2* mutations were nonsense or out-of-frame frameshift mutations. *SMC1A* and *SMC3* mutations were missense mutations and involved functional domains (mostly hinge domain and ATPase heads). Two patients harbored the same *SMC1A* variant R96H, previously reported in AML [32]. We found a hotspot in *SMC3* since 3 out of 5 mutations involved the codon R661 in the hinge domain of the protein. Mutations involving this co-don have been previously described in cancer as well as in the leukemia-derived cell line MOLM-7 [33].

Finally, mutations in effectors that control DNA methylation (*TET2*, *IDH1* R132H/L or *IDH2* R140) were identified in 8% t(8;21) AML and 2% of inv(16) AML with mutual exclusivity (p=0.57).

Outcome

The complete remission rate in this cohort of 215 patients was 98.1% (211/215). The 5-year CIR was 33.2% (95% CI 27.2%-40.2%) with no difference between the CBF2006 and ELAM02 (35.2% [95% CI 27.9%-43.9%] versus 29.3% [95% CI 19.8%-42.1%], p=.415, **Supplementary Figure 3**). The 5-years OS was 83.6% [95% CI: 77.7%-88.0%]. A total of 14 patients received allogeneic SCT in first CR and were censored at SCT time for prognostic analyses. 5-year CIR was estimated at 31% (95% CI 23%-41%) in patients with inv(16) AML and 35% (95% CI 27%-46%) in patients with t(8;21) AML. All but 1 patient, who died during induction course, entered CR. Thirty-four patients died during follow-up, 9 of them died in first CR and 19 died after hematological relapse; 6 adults died from allogeneic SCT complica-tions. Seventy patients had hematological relapse.

Importance of VAFs for evaluating impact of TK pathway mutations on outcome

Univariate prognosis analyses for CIR are summarized in **Supplementary Table 3**. Altogether, muta-tions in genes activating TK signaling were associated with a higher cumulative incidence of relapse (SHR 2.81 [95% CI 1.39-5.69]; p=0.004) especially in t(8;21) AML patients (SHR 5.22 [95% CI 1.82-14.96]; p=0.002). This was also evident across RTK mutations (*KIT* and/or *FLT3* mutations) (SHR 1.72 [95% CI 1.05-2.81]; p=0.031).

Given the range in VAFs identified in mutations activating TK signaling, we determined optimal cut-points for VAFs in the most frequent mutations, i.e. *KIT*, *FLT3*-TKD, *NRAS* and *KRAS* mutations, by maximally selected log-rank statistics [24]. *KIT* mutations were associated with a significant higher CIR for t(8;21) AML patients with a mutant allelic ratio of 35% or greater ($KIT^{\geq 35\%}$). Patients in this

particular subgroup had a particularly adverse outcome with a 5-year CIR of 69.4% versus 30.7% and 31.9%% for *KIT*$^{<35\%}$ and *KIT*^{wildtype} respectively (p=0.008) (**Supplementary Figure 10**). In patients with t(8;21) and *KIT* mutation, the only baseline characteristic that was associated with a higher incidence of relapse was logWBC (SHR 3.72 [95%CI 0.97-14.28]; p=0.056). In bivariate analysis, both logWBC and KIT$^{\geq 35\%}$remained significantly associated with a poorer prognosis (logWBC: SHR 8.45 [95%CI 1.93-36.90], p=.005; KIT$^{\geq 35\%}$: SHR 8.83 [95%CI 2.45-31.76], p=0.001).

Similarly, *FLT3*-TKD mutations were associated with a higher CIR in CBF AML patients with a mutant allelic ratio of 10% or greater (*FLT3*-TKD$^{\geq 10\%}$) when compared to lower ratio and non-mutated patients (SHR 2.28 [95% CI 1.16-4.50]; p=0.018). The 5-year CIR was 58.8% (95% CI 37.4%-81.4%) in patients with *FLT3*-TKD$^{\geq 10\%}$, 20.0% (95% CI 5.41%-59.1%) in patients with *FLT3*-TKD$^{<10\%}$, and 31.5% (95% CI 25.2%-39.0%) in patients without *FLT3*-TKD. Conversely, high *NRAS* and *KRAS* mutant allelic ratios (over 35%) were both favorable factors for CIR in CBF AML (5y-CIR was 13% for *NRAS*$^{\geq 35\%}$ versus 30.2% for *NRAS*^{wt} [p=0.033] and 0% for *KRAS*$^{\geq 35\%}$ versus 31.9% for *KRAS*^{wt} respectively [p=0.008]). Patients with high *NRAS* or *KRAS* mutant allelic ratios were characterized by the lack of RTK mutations, which could explain this favorable outcome (**Supplementary Figure 11**).

Mutations in epigenetic modifying or cohesin genes are associated with a poor prognosis in t(8;21) AML patients with TK pathway mutations

Besides mutations in genes affecting TK signaling, as previously reported, we observed a trend toward a higher SHR for *ASXL1/2* mutations (*ASXL1* or *ASXL2*) in patients with t(8;21) AML (SHR 1.71, [95% CI 0.88-3.33];p=0.113) which did not reach statistical significance. Interestingly, in this CBF AML subtype, patients who had mutations in both TK pathways and chromatin modifiers and/or cohesin genes had the worst prognosis with a 5-year CIR of 54.1% (95% CI 39.7%-69.9%) when compared to patients with TK mutations without chromatin modifiers and/or cohesin gene mutations (5y-CIR 33.9% [95% CI 25.8%-43.8%]), or to patients without TK mutations (5y-CIR 16.3% [95% CI 8.9%-29.1%]; **Figure 2**). In multivariate analysis (**Table 2**), the association of mutations in TK pathway genes and chromatin modifiers or cohesin genes remained associated with the highest hazard of relapse in patients with t(8;21) AML (SHR 5.44 [95% CI 1.82-16.27]; p=0.002).

DISCUSSION

AML with t(8;21) and inv(16), collectively referred to as CBF AML, represent two of the most common genetic abnormalities in AML. Both entities disrupt the normal function of the heterodimeric transcription factor CBF complex and have similar clinical outcomes. However, the molecular genetic

abnormalities potentially explaining differences between these two subtypes of AML have not been explored in detail. While implication of TK pathways in CBF AML leukemogenesis has been widely studied [34], only few reports have identified cooperating mutations in CBF AML outside of TK pathway alterations. Although the "two-hit model" of leukemogenesis mentioned earlier is biologically relevant, it is impossible to ignore the multitude of genetic and epigenetic aberrations that have recently been described in human leukemia [35]. Within CBF AML, *ASXL1* and *ASXL2* mutations were recently reported to occur exclusively in AML with t(8;21) but not in AML with inv(16) [19,20,36]. The present study extends those findings. We performed extensive mutational analysis by HTS in 215 patients with CBF AML from 1 to 60 years. As expected, mutations in TK pathways were the most frequent aberrations in both subtypes (65% and 80% of t(8;21) AML and inv(16) AML respectively; p=0.021). Mutations in TK pathway, especially RTK mutations (*KIT* and *FLT3* mutations) were associated with a higher SHR and higher CIR. *KIT* mutations with high mutant allelic ratio appeared to significantly impact prognosis of t(8;21) AML patients, in accordance with a previous report by Allen et *al* [37]. Conversely, high *NRAS* and *KRAS* mutant allelic ratios were associated with the lack of RTK mutations and a favorable outcome. *FLT3*-TKD mutations were associated with a higher CIR in the present study, especially in inv(16) AML. These results are in accordance with previous studies showing inferior OS and PFS in inv(16) AML [29,38] although it remains controversial [39,40].

However, TK pathways mutations appear to constitute only a portion of the genetic landscape of CBF AML. Interestingly, the two CBF AML subtypes showed greatly distinct mutational profiles. Mutations in genes that regulate chromatin conformation (*ASXL1/2*, *EZH2*, *KDM6A, BCOR/BCORL1*) or implicated in the cohesin complex (*RAD21*, *SMC1A*, *SMC3*, *STAG2*) were observed almost exclusively in t(8;21) AML. In line with those findings, using whole exome sequencing in 13 CBF AML patients, Sood et *al* recently identified cohesin and chromatin modifiers mutations in t(8;21) but not in inv(16) patients [41].

Functionally, ASXL1, ASXL2 and EZH2 are Polycomb group-associated proteins that influence chromatin configuration and thus gene transcription by directing modifications at specific chromatin marks. EZH2 is the catalytic component of Polycomb Repressive Complex 2 (PRC2) and serves as a H3K27 methyltransferase activity. ASXL1 and ASXL2 are part of the Polycomb repressive deubiquitylase complex (PR-DUB) which removes an ubiquitin from H2AK119 marks [42] but studies have shown that ASXL proteins may also function in recruitment and/or stabilization of the PRC2 complex to specific loci [43]. KDM6A (also known as UTX) is an H3K27 demethylase that counters the enzymatic activity of PRC2. On the other hand, BCOR and BCORL1 are part of a complex similar to the PRC1 [44].

Cohesin is a multimeric protein complex that is conserved across species and is composed of 4 core subunits (SMC1A, SMC3, RAD21 and STAG proteins) together with a number of regulatory proteins such as NIPBL, PDS5 or ESCO proteins [33]. The four subunits form a ring structure that regulates chromosome segregation during meiosis and mitosis but recent data suggests additional functions such as double-strand DNA repair and regulation of transcription [42,45]. In a previous study by Thol et *al*, recurrent cohesin mutations have been reported in about 6% of AML patients [45]. However, cohesin mutations concerned less than 2% of CBF AML in this study. Interestingly, it has been shown that the cohesin complex could functionally interacts with polycomb group proteins to control gene transcription [46]. It is an interesting observation that *ASXL* gene mutations [47] as well as cohesin gene mutations [48] are enriched in patients with *RUNX1* mutated-AML. Recently, several experiments have linked cohesin and *RUNX1* in hematopoiesis and leukemogenesis. Notably, *runx* genes expression has been showed to be dependent of *rad21* expression in a zebrafish model [49]. Kon et *al* showed that forced expression of wild-type *RAD21* in the Kasumi-1 cell line (carrying both t(8;21) and *RAD21* mutation p.K330PfsX6) induced significant growth suppression [33]. Moreover, haploinsufficiency of cohesin proteins appears to be associated with myeloid transformation and aberrant self-renewal linked with broad changes in chromatin occupancy [50,51]. Indeed, Mazumdar et *al* showed that cohesin mutations led to a state of elevated chromatin accessibility and higher binding at RUNX1 binding sites [50]. These findings suggest links between alterations in chromatin structure, mediated by cohesin or chromatin modifiers mutations, and cooperativity with the RUNX1-RUNXT1 fusion oncoprotein.

In our cohort, 18% of t(8;21) AML patients have mutations involving the cohesin member and 42% of t(8;21) AML patients have mutations involving chromatin modifiers. Overall, 52% of t(8;21) AML patients have at least one mutation in one of these two groups of genes. These findings suggest an important pathway that is specific to t(8;21) AML leukemogenesis and may have biological and clinical significance. Accordingly, transcriptome profiling supports the notion that t(8;21) and inv(16) AML are characterized by different genetic programs [18]. It is likely that future studies focused on the molecular basis of shared pathways as well as pathways specific to the two CBF AML subtypes may guide the development of new treatment approaches. Patients with t(8;21) AML who had at least one TK pathway mutation associated with at least one mutation in a chromatin modifier or cohesin gene had the worst prognosis which could indicate synergic cooperation between these events. Finally, evaluation of drugs targeting these pathways and translational research integrating these molecular findings with clinical trials will likely improve the treatment of patients with CBF AML.

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FIGURES AND TABLES

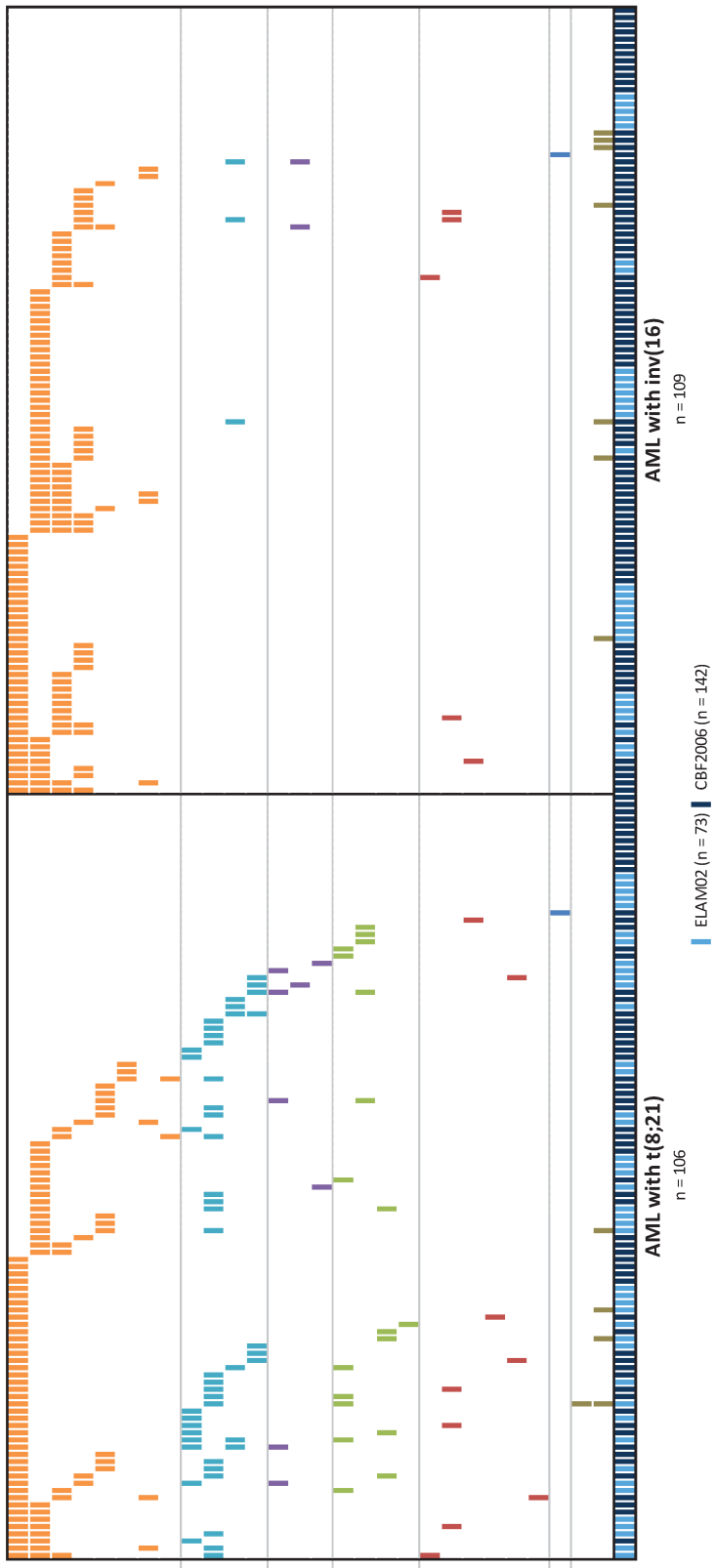


Figure 1: Molecular alterations in CBF AML. Each column represents the mutation pattern in one patient and each colored box represents a mutation of the gene. Genes have been grouped in seven categories according to reference [52]. Patients are grouped into AML with t(8;21) (on the left) and AML with inv(16) (on the right). Adult (CBF2006 trial, dark blue boxes) and pediatric patients (ELAM02, light blue boxes) are reported together.

Figure 2: Kaplan–Meier curves for cumulative incidence of relapse (CIR) in patients stratified according to their mutational profile. Patients are separated in 3 groups: (1) Tyrosine kinase (TK) pathway mutation associated with at least 1 mutation involving chromatin modifier or cohesin genes, (2) TK pathway mutation without mutations involving chromatin modifier or cohesin genes and (3) Other mutational profiles.

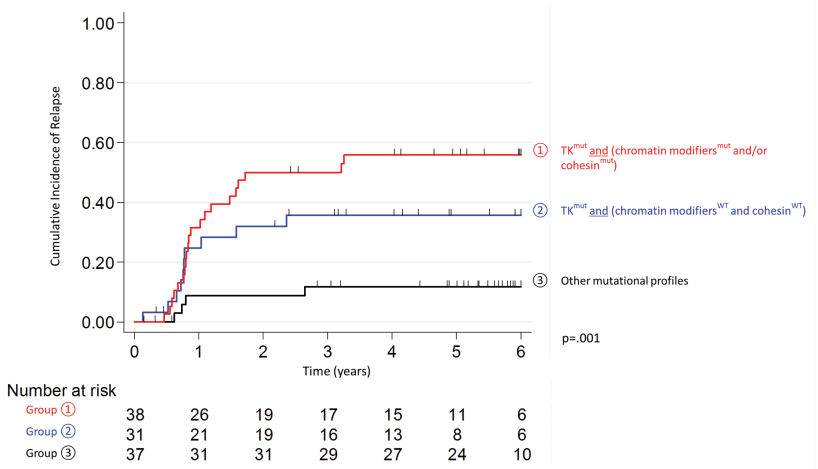


Table 1: Patient characteristics according to CBF subtype. Adult and pediatric patients are reported together.

	CBF-AML	AML with inv(16)	AML with t(8;21)	p-value
Patients, n	215	109	106	
Median age, y [range]	32 [1-60]	37 [1-60]	25 [2-59]	0.004 *
Median WBC, x10^9/L [range]	18,4 [1-232]	34,4 [1-232]	11,5 [1,3-163]	<0.001 *
Gender (male/female)	119/96	61/48	58/48	0.891
Trial (CBF2006/ELAM02)	142/73	79/30	63/43	0.046 *
Outcome				
Deaths, n (%)	35 (16)	15 (14)	20 (18)	0.358
Relapses, n (%)	70 (33)	34 (31)	36 (33)	0.771
Number of mutations per patient	2 [0-6]	1 [0-5]	2 [0-6]	0.277
Gene mutations				
Tyrosine kinase pathway, n (%)	156 (73)	87 (80)	69 (65)	0.021 *
KIT exon 8, n (%)	37 (17)	22 (20)	15 (14)	0.352
KIT exon 17, n(%)	42 (20)	16 (15)	26 (25)	0.080
- codon D416 (exon 17), n(%)	30 (14)	13 (12)	17 (16)	0.421
- codon N822 (exon 17), n (%)	12 (6)	2 (2)	10 (9)	0.032 *
KIT exon 11, n(%)	2 (1)	1 (1)	1 (1)	1.000
KIT all, n (%)	78 (36)	36 (33)	42 (40)	0.325
FLT3-TKD, n (%)	28 (13)	24 (22)	4 (4)	<0.001 *
FLT3-ITD, n (%)	14 (7)	3 (3)	11 (10)	0.028 *
FLT3 all, n (%)	41 (19)	26 (24)	15 (14)	0.083
RTK (*KIT* and/or *FLT3*), n (%)	105 (49)	53 (49)	52 (49)	1.000
NRAS, n (%)	66 (31)	42 (39)	24 (23)	0.012 *
- codon G12, n (%)	24 (11)	14 (13)	10 (9)	0.518
- codon G13, n (%)	19 (9)	9 (8)	10 (9)	0.813
- codon Q61, n (%)	34 (16)	26 (24)	8 (8)	0.001 *
KRAS, n (%)	36 (17)	29 (27)	7 (7)	<0.001 *
- codon G12, n (%)	15 (7)	14 (13)	1 (1)	<0.001 *
- codon G13, n (%)	13 (6)	8 (7)	5 (5)	0.569
- codon Q61, n (%)	6 (3)	5 (5)	1 (1)	0.212

RAS (*NRAS* and/or *KRAS*), n (%)	87 (40)	59 (54)	28 (26)	<0.001 *
JAK2, n (%)	3 (1)	0 (0)	3 (3)	0.118
PTPN11, n (%)	8 (4)	5 (5)	3 (3)	0.722
CBL, n (%)	2 (1)	0 (0)	2 (2)	0.242
Chromatin Modifiers, n (%)	51 (24)	6 (6)	45 (42)	< 0.001 *
ASXL1, n (%)	11 (5)	0 (0)	11 (10)	< 0.001 *
ASXL2, n (%)	23 (11)	0 (0)	23 (22)	< 0.001 *
ASXL (*ASXL1* or *ASXL2*), n (%)	34 (16)	0 (0)	34 (32)	< 0.001 *
KDM6A, n (%)	9 (4)	3 (3)	6 (6)	0.328
EZH2, n (%)	7 (3)	0 (0)	7 (7)	0.006 *
BCOR, n (%)	2 (1)	1 (1)	1 (1)	1.000
BCORL1, n (%)	6 (3)	3 (3)	3 (3)	1.000
DNA Methylation, n (%)	10 (5)	2 (2)	8 (8)	0.057
TET2, n (%)	5 (2)	0 (0)	5 (5)	0.028 *
IDH1, n (%)	3 (1)	2 (2)	1 (1)	1.000
IDH2, n (%)	2 (1)	0 (0)	2 (2)	0.242
Cohesin, n (%)	19 (9)	0 (0)	19 (18)	< 0.001 *
RAD21, n (%)	8 (4)	0 (0)	8 (8)	0.003 *
SMC1A, n (%)	5 (2)	0 (0)	5 (5)	0.028 *
SMC3, n (%)	5 (2)	0 (0)	5 (5)	0.028 *
STAG2, n (%)	1 (0)	0 (0)	1 (1)	0.493
Transcription Factors, n (%)	6 (3)	1 (1)	5 (5)	0.116
ETV6, n (%)	2 (1)	1 (1)	1 (1)	1.000
RUNX1, n (%)	1 (0)	0 (0)	1 (1)	0.493
GATA2, n (%)	2 (1)	0 (0)	2 (2)	0.242
IKZF1, n (%)	1 (0)	0 (0)	1 (1)	0.493
Spliceosome, n (%)	2 (1)	1 (1)	1 (1)	1.000
SRSF2, n (%)	2 (1)	1 (1)	1 (1)	1.000
Tumor Suppressors, n (%)	11 (5)	7 (6)	4 (4)	0.538
PHF6, n (%)	1 (0)	0 (0)	1 (1)	0.493
WT1, n (%)	11 (5)	7 (6)	4 (4)	0.538
Additional cytogenetic abnormalities				
Loss X or Y, n (%)	54 (25)	0 (0)	54 (51)	<0.001 *
Del(9q), n (%)	16 (7)	0 (0)	16 (15)	<0.001 *
Del(7q), n (%)	21 (10)	11 (10)	10 (9)	1.000
Trisomy 8, n (%)	18 (8)	12 (11)	6 (6)	0.218
Trisomy 22, n (%)	12 (6)	12 (11)	0 (0)	<0.001 *

* p-value < 0.05

Table 2: Univariate and multivariate analyses for specific hazard of relapse in patients with t(8;21) AML.

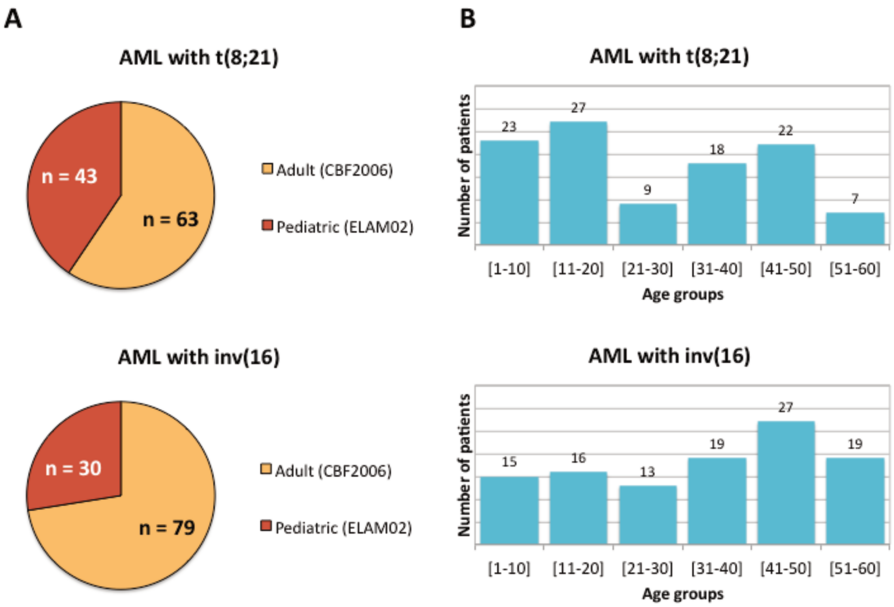
		Univariate analysis			Multivariate analysis		
	Patients	**SHR**	**95% CI**	**p-value**	**SHR**	**95% CI**	**p-value**
Age†	106	1.00	0.97-1.03	0.938	-	-	-
LogWBC†	106	2.47	1.18-5.19	0.017 *	1.94	0.89-4.27	0.097
TK^{mut} and ($chromatin\ modifiers^{WT}$ and $cohesin^{WT}$)	31/106	3.88	1.20-12.54	0.023 *	3.96	1.22-12.91	0.022 *
TK^{mut} and ($chromatin\ modifiers^{mut}$ and/or $cohesin^{mut}$)	31/106	6.21	2.11-18.26	0.001 *	5.44	1.82-16.27	0.002 *

* p-value < 0.05

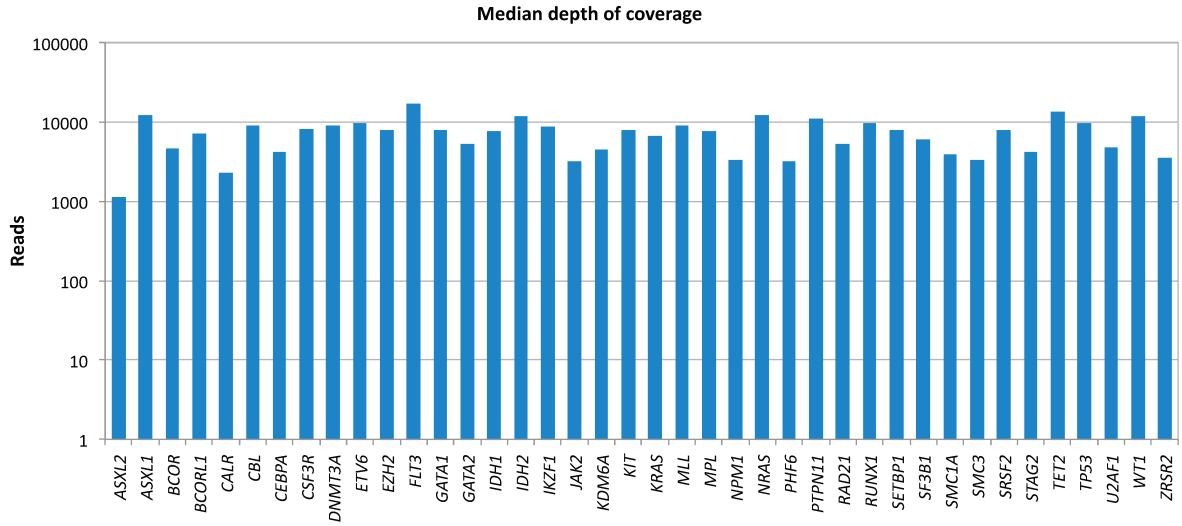
† Tested as continuous variable

SUPPLEMENTAL DATA

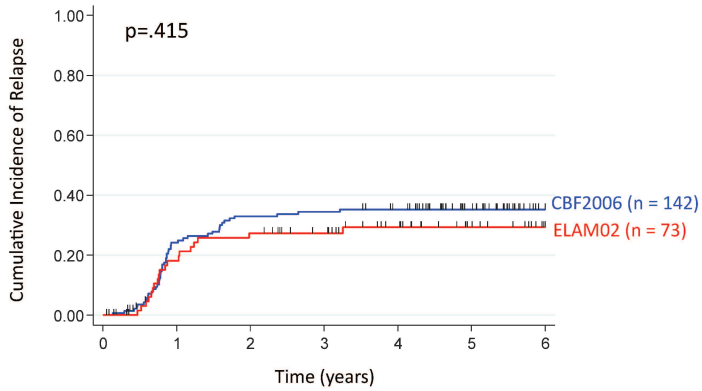
Supplementary Figure 1: (A) Pie chart representation of adult versus pediatric t(8;21) AML patients (n=106) and inv(16) AML patients (n=109) in the studied cohort. **(B)** Age groups by decade of t(8;21) AML patients and inv(16) AML patients in the studied cohort.



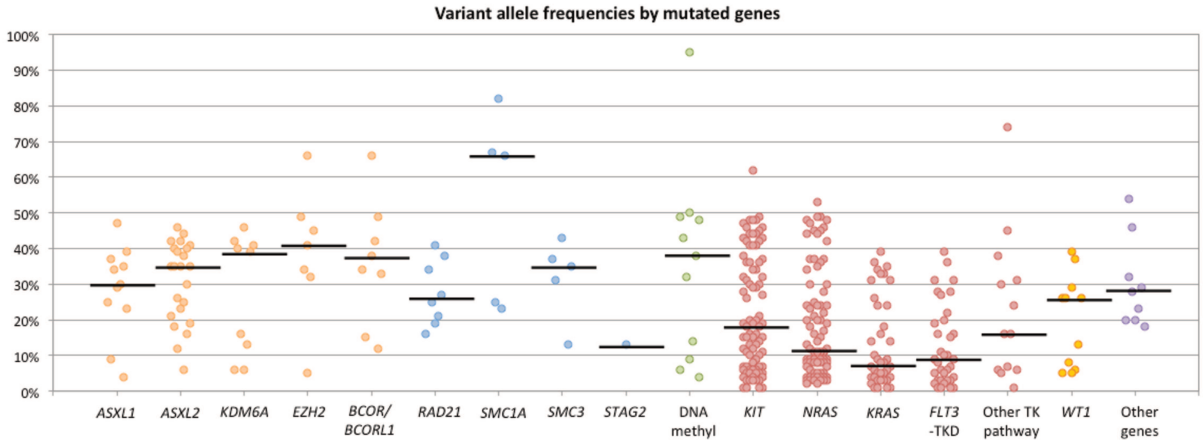
Supplementary Figure 2: Depth of coverage for all genes studied (n=40) by high-throughput sequencing.



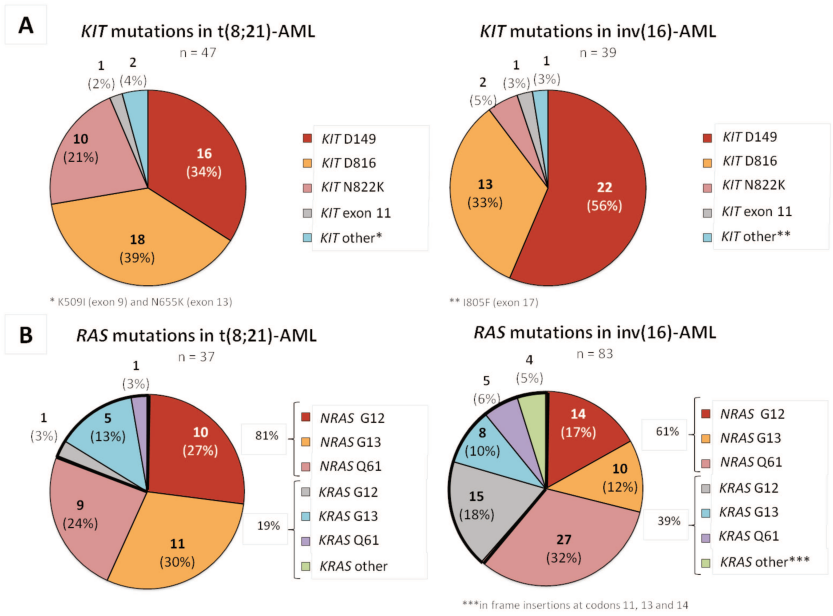
Supplementary Figure 3: Cumulative incidence of relapse (CIR) according to enrollment in ELAM02 trial (age 0-18) or CBF2006 trial (age: 18-60).



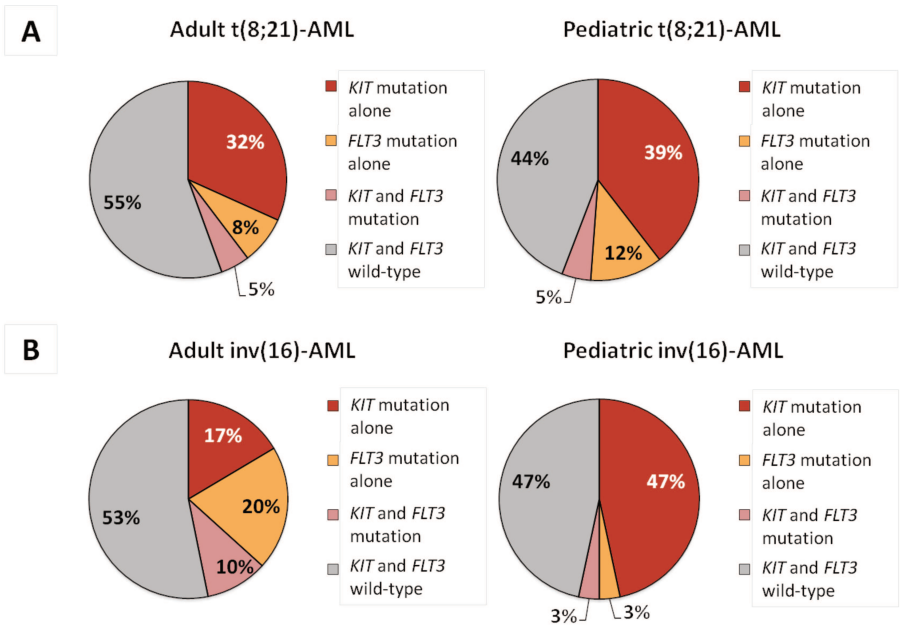
Supplementary Figure 4: Variant allele frequencies (VAFs) in selected genes. Quantitative measure of variant-containing reads by HTS estimates the abundance of these mutations (uncorrected for copy number, uniparental disomies or patient's sex for X-linked genes). Mutations of tyrosine kinase (TK) pathway genes are often present in small clones. ("DNA methyl" includes *TET2, IDH1* and *IDH2* mutations; "Other TK pathway" includes *JAK2, CBL* and *PTPN11*; "Other genes" includes *ETV6, GATA2, IKZF1, PHF6, RUNX1* and *SRSF2*).



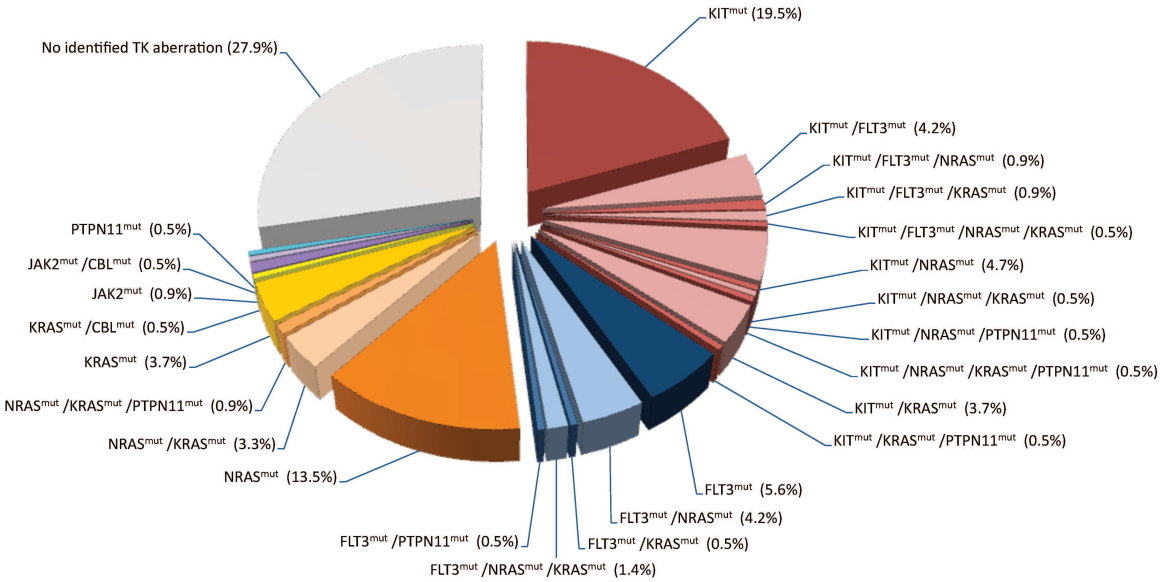
Supplementary Figure 5: Distribution of mutational hotspots identified in (A) *KIT* and (B) *RAS* genes amongst patient with t(8;21) AML and inv (16)-AML. *NRAS* and *KRAS* mutations are reported in the same pie charts. *KRAS* mutations have been surrounded by a thick line for more visibility.



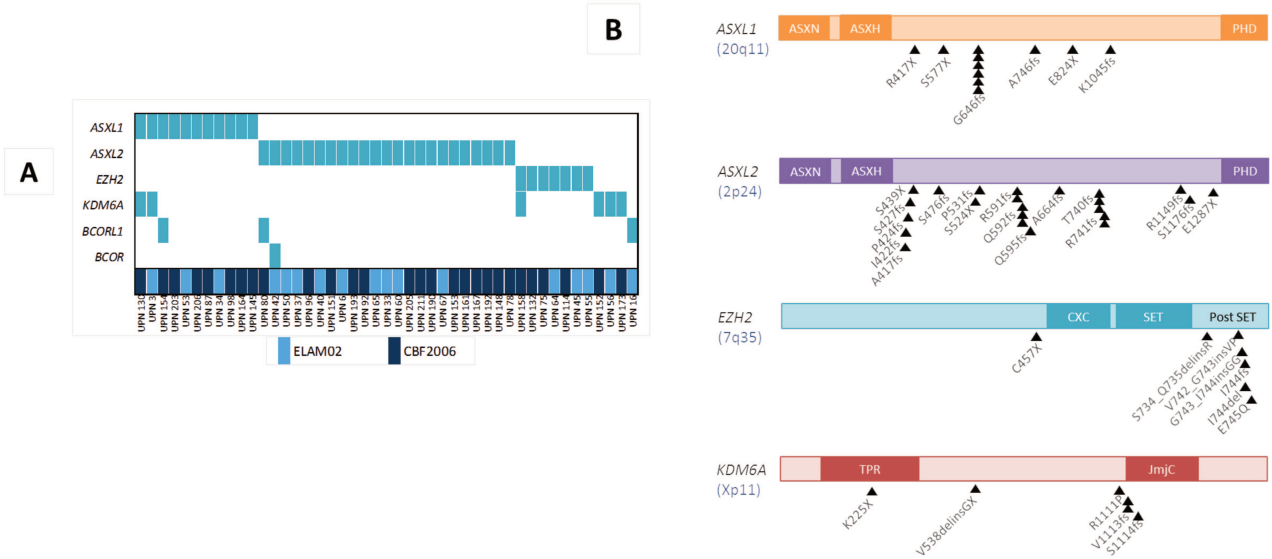
Supplementary Figure 6: Classification of pediatric and adult patients according to status for RTK mutations (*FLT3*-TKD or -ITD and *KIT* mutations) in (A) t(8;21) AML and (B) inv(16) AML.



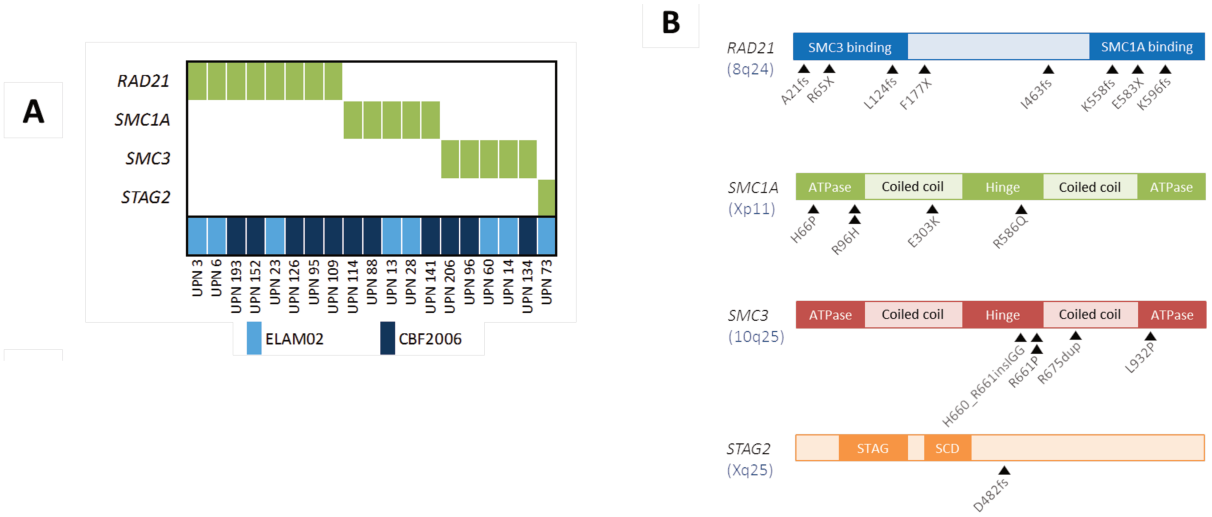
Supplementary Figure 7: Pie chart illustrating the molecular heterogeneity and coexistence of TK pathway mutations in the 215 CBF AML. The chart is based on mutational status for *KIT*, *FLT3* (TKD or ITD), *NRAS*, *KRAS*, *JAK2*, *CBL* and *PTPN11*.



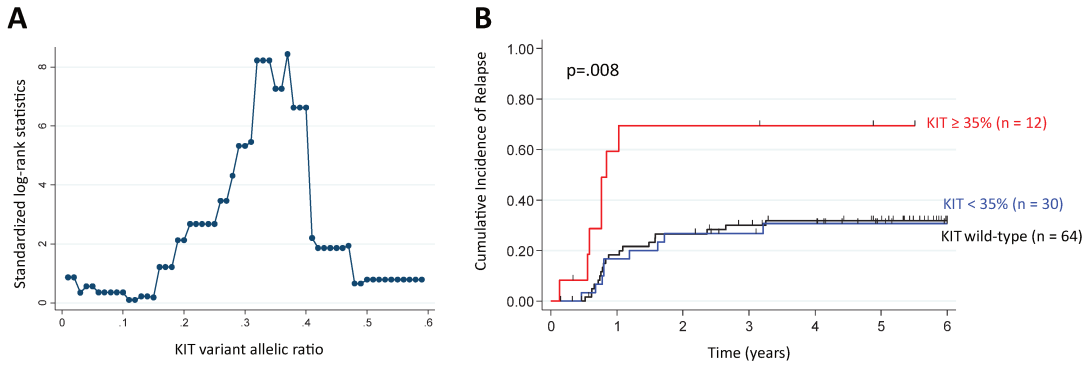
Supplementary Figure 8: (A) Distribution of chromatin modifiers mutations showing a nearly mutually exclusive pattern. Overall, 45 patients out of 106 (42%) with t(8;21) AML have at least one mutation in this set of genes. Co-occurring mutations are found in only 6 patients. (B) Identified mutations in *ASXL1*, *ASXL2*, *EZH2* and *KDM6A*.



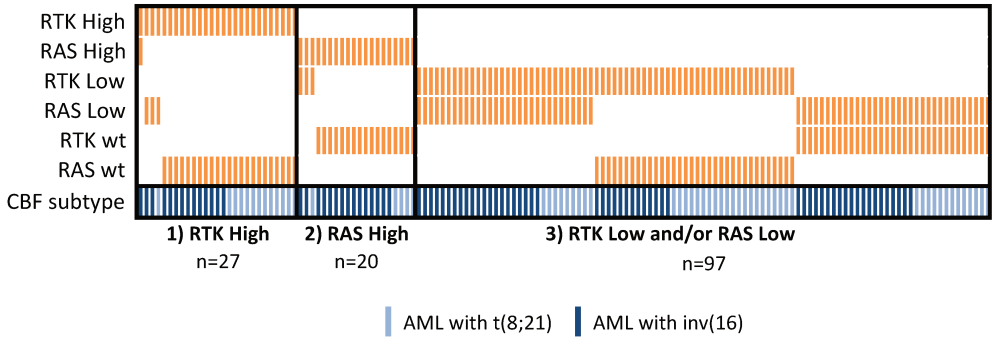
Supplementary Figure 9: (A) Distribution of cohesin complex mutations showing a mutually exclusive pattern. Overall, 19 out of 106 patients (18%) with t(8;21) AML have one mutation in this set of genes. (B) Identified mutations in the core components of the cohesin complex in t(8;21) AML.



Supplementary Figure 10: (A) Optimal cut-point for *KIT* VAF by maximally selected log-rank statistics. (B) Cumulative incidence of relapse in patients stratified according to *KIT* mutant allelic ratio in t(8;21) AML.



Supplementary Figure 11: Patient's classification according to their VAFs for RTK mutations (*KIT* and/or *FLT3*) and *RAS* mutations (*NRAS* and/or *KRAS*). A cut-off of 35% VAF was used for representation (high if ≥ 35%; low if < 35%). Here are shown only positive patients for one of these mutations.



Supplementary Table 1: Identified variants in the entire cohort of CBF AML. Variant allele frequencies (VAF) are given by HTS. The prediction of the effects of single nucleotide variants on protein function was performed by PredictSNP [22] with six established prediction tool.

Cf. version online.

Supplementary Table 2: Incidence of mutations in adult and pediatric AML with (A) inv(16) and (B) t(8;21).

Cf. version online.

Supplementary Table 3: Univariate analysis for CIR.

	CBF-AML		AML with inv(16)		AML with t(8;21)	
	SHR	**95% CI**	**SHR**	**95% CI**	**SHR**	**95% CI**
Age*	1.00	0.98-1.03	1.00	0.97-1.04	1.00	0.96-1.03
Sex	0.91	0.55-1.49	1.14	0.55-2.34	0.75	0.38-1.47
WBC*	1.00	0.99-1.01	1.00	0.99-1.01	1.00	0.99-1.01
Tyrosine kinase pathway	2.81	1.39-5.69	1.46	0.56-3.80	5.22	1.82-14.96
KIT	1.27	0.77-2.09	1.20	0.57-2.54	1.37	0.70-2.69
FLT3-TKD	1.51	0.80-2.84	1.77	0.84-3.72	NA	NA
FLT3-ITD	2.22	1.06-4.67	NA	NA	2.16	0.94-4.96
FLT3 all	1.78	1.04-3.05	1.63	0.77-3.45	1.86	0.84-4.11
RTK (*KIT* and/or *FLT3*)	1.72	1.05-2.81	1.70	0.84-3.46	1.72	0.86-3.41
NRAS	1.36	0.83-2.24	1.15	0.57-2.33	1.67	0.80-3.51
KRAS	1.44	0.80-2.61	1.19	0.55-2.58	NA	NA
Chromatin modifiers	1.25	0.73-2.15	NA	NA	1.44	0.74-2.80
ASXL1	1.87	0.80-4.34	NA	NA	1.86	0.77-4.52
ASXL2	1.41	0.72-2.77	NA	NA	1.34	0.64-2.79
ASXL (*ASXL1* or *ASXL2*)	1.64	0.93-2.88	NA	NA	1.71	0.88-3.33
DNA Methylation	0.62	0.15-2.57	NA	NA	NA	NA
Cohesin	1.09	0.50-2.39	NA	NA	1.01	0.44-2.33
WT1	1.58	0.57-4.37	NA	NA	NA	NA

Comparisons were based on cause-specific hazard Cox models, stratified on CBF subset, and the 14 patients who received allogeneic SCT in first hematologic CR were censored at SCT time. NA, not applicable because of too small size of the group.

*Tested as continuous variable.

ARTICLE 3 : PROFIL SNP-ARRAY DES LAM CBF

SNP-array lesions in core binding factor acute myeloid leukemia

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Résumé : Si la perturbation du CBF constitue l'anomalie initiatrice, elle reste insuffisante à elle seule pour entraîner la transformation leucémique. Le spectre des anomalies coopératrices est aujourd'hui mieux connu et intègre des anomalies chromosomiques additionnelles (trisomies, délétions) et des mutations géniques.

Nous avons étudié les prélèvements diagnostiques de 198 LAM CBF inclus dans les protocoles CBF2006 (adultes) et ELAM02 (enfants) par SNP-array permettant l'identification des anomalies du nombre de copies des gènes (CNAs) avec une haute résolution ainsi que des pertes d'hétérozygotie sans anomalie du nombre de copies (CN-LOH). Pour certains gènes d'intérêt, l'étude a été complétée par séquençage ciblé (haut-débit et Sanger) ou cytométrie en flux.

A l'exclusion des anomalies localisées aux points de cassure des réarrangements du CBF, les anomalies les plus fréquentes incluaient la perte d'un chromosome sexuel (53%), la délétion 9q (12%) et la délétion 7q (9%) dans les LAM avec t(8;21) ainsi que la trisomie 22 (13%), la trisomie 8 (10%) et la délétion 7q (12%) dans les LAM avec inv(16). Il n'existait aucune corrélation entre ces anomalies et le pronostic de la maladie. De nouvelles cibles potentiellement impliquées dans la leucémogénèse des LAM CBF ont pu être identifiées. Les mutations de *ZBTB7A* (20% des LAM avec t(8;21)) constituaient une cible de CN-LOH sur le bras court du chromosome 19. Les délétions ciblées et les mutations tronquantes de FOXP1 étaient identifiées dans 5% et 2% des LAM avec inv(16) mais pas dans les LAM avec t(8;21). Enfin, la désorganisation du locus de CCDC26 était identifiée dans les 2 sous-types de LAM CBF (4.5% de la cohorte totale). L'analyse en cytométrie en flux de la forme phosphorylée d'AKT dans les blastes de patients porteurs de cette anomalie ainsi que les données de la littérature suggéraient que la désorganisation de CCDC26 constituait un nouveau mécanisme conduisant à l'activation aberrante des voies tyrosine kinase.

SNP-array lesions in core binding factor acute myeloid leukemia

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Abstract: Acute myeloid leukemia (AML) with t(8;21) and inv(16), together referred as core binding factor (CBF)-AML, are recognized as unique entities. Both rearrangements share a common pathophysiology, the disruption of the CBF, and a relatively good prognosis. Experiments have demonstrated that CBF rearrangements were insufficient to induce leukemia, implying the existence of cooperating events. To explore these aberrations, we performed single nucleotide polymorphism (SNP)-array in a well-annotated cohort of 198 patients with CBF-AML. Excluding breakpoint-associated lesions, the most frequent events included loss of a sex chromosome (53%), deletions at 9q21 (12%) and 7q36 (9%) in patients with t(8;21) compared with trisomy 22 (13%), trisomy 8 (10%) and 7q36 deletions (12%) in patients with inv(16). SNP-array revealed novel recurrent genetic alterations likely to be involved in CBF-AML leukemogenesis. *ZBTB7A* mutations (20% of t(8;21)-AML) were shown to be a target of copy-neutral losses of heterozygosity at chromosome 19p. *FOXP1* focal deletions were identified in 5% of inv(16)-AML while sequence analysis revealed that 2% carried *FOXP1* truncating mutations. Finally, *CCDC26* disruption was found in both subtypes (4.5% of the whole cohort) and possibly highlighted a new lesion associated with aberrant tyrosine kinase signaling in this particular subtype of leukemia.

Keywords: acute myeloid leukemia; core binding factor; t(8;21); inv(16); *RUNX1-RUNX1T1*; *CBFB-MYH11*; SNP-array; tyrosine kinase; *CCDC26*; *FOXP1*; *ZBTB7A.*

INTRODUCTION

Core binding factor (CBF)-acute myeloid leukemia (AML), including AML with t(8;21) and AML with inv(16)/t(16;16), accounts for approximately 25% of pediatric and 15% of adult *de novo* AML patients. Compared to other AML subsets, CBF-AML is considered to have a good prognosis. Both alterations result in disruption of genes encoding subunits of the CBF (*i.e*. *RUNX1* and *CBFB*), a heterodimeric transcription factor complex required for normal hematopoiesis[1]. Importantly, experiences from murine models[2], as well as the existence of preleukemic cells harboring a CBF rearrangement in healthy individuals[3,4], have demonstrated that CBF disruption is insufficient to induce leukemia. CBF-AML is therefore considered as a model of multistep pathogenesis. Evidences supporting this model have been generated by the high frequency of cooperative events at time of diagnosis. Notably, mutations in genes encoding tyrosine kinase pathways effectors (especially *KIT*, *FLT3* and *RAS* mutations) are found in up to 80% of CBF-AML patients[5–7]. Additional chromosomal aberrations are detected in approximately 70% of patients with t(8;21)-AML and 40% of patients with inv(16)-AML by conventional karyotype[5,8–10]. These aberrations are nonrandom events and some of them are extremely rare in non-CBF-AML. In this context, the identification of recurrent events involved in CBF-AML pathophysiology and heterogeneity remains of great interest. We report here the single nucleotide polymorphism (SNP)-array profiling of a large and well-annotated cohort of pediatric and adult patients with CBF-AML and the identification of new recurrent lesions in this particular subtype of leukemia.

METHODS

Patients and samples

This study focused on diagnostic bone marrow (BM) samples from 198 CBF-AML patients including 116 AML with t(8;21) and 82 AML with inv(16). CBF-AML was identified by conventional cytogenetics and/or reverse-transcriptase PCR as previously described[5]. The cohort included 125 adults (aged from 18 to 60 years) and 73 children (aged from 1 to 17 years) enrolled in the French trials CBF2006 (a phase 3 trial of systematic versus response-adapted timed-sequential induction in patients with CBF-AML; ClinicalTrials.gov NCT00428558) and ELAM02 (a randomized study of maintenance treatment with interleukin-2 in patients with childhood AML; ClinicalTrials.gov NCT00149162) respectively. Median age was 30 years (range: 1-60). Samples were screened for known AML-associated mutations by high-throughput sequencing (HTS) as previously reported for 176 of the 198 CBF-AML patients included in the present study[6]. Patient's characteristics are summarized in Supplementary Ta-

ble 1. Studies were approved by the Ethics Committee of Nîmes University Hospital and by the Institutional Review Board of the French Regulatory Agency and were conducted in accordance with the Declaration of Helsinki.

SNP-array karyotyping

DNA was extracted from diagnostic cell pellets using the QIAamp Tissue Kit (Qiagen) according to the manufacturer's instructions. Patient's genomic DNA was processed and hybridized to Cytoscan HD array (Affymetrix) according to the manufacturer's protocol. Data were analyzed using the Chromosome Analysis Suite (ChAS) software (Affymetrix). In a first step, only copy number variants with a size over 20 kb including at least 20 consecutive markers as well as copy-neutral losses of heterozygosity (CN-LOH) over 3 Mb were considered for this analysis. In a second step, we adopted a stringent and conservative algorithm in order to distinguish somatic from constitutional SNP-array lesions. Variations were excluded as known copy number variants (CNVs) if there was more than 50% overlap with variants from the public Database of Genomic Variants (DGV). Based on previous studies[11–13], only interstitial CN-LOH over 10 Mb and CN-LOH extending to telomeres were considered to be acquired abnormalities. Remaining CN-LOH were considered as possibly constitutional and then rejected for subsequent analyses. Finally, all copy-number abnormalities (CNAs) and CN-LOH fulfilling the above criteria were validated by visual inspection and annotated for size, position and location of genes based on the human genome version 19 (hg19) of the UCSC Genome Browser.

Mutational analysis

Considering data from SNP-array karyotyping, target sequencing was performed for all coding exons of *FOXP1* (NM_001244810) and *ZBTB7A* (NM_015898). Libraries were prepared using the Ampliseq system according to the manufacturer's instruction and run on Personal Genome Machine (PGM, Life Technologies). Raw HTS data from PGM sequencing were processed by Torrent Browser (Life Technologies) and SeqNext (JSI Medical System). The depth of coverage was more than 2000X for both genes. Variants were confirmed by direct Sanger Sequencing as previously described[14]. Frameshift and nonsense variants were always considered as relevant mutations. Single nucleotide variants were retained in the absence of description into public databases of human polymorphisms and effects on protein function were predicted with SIFT and Polyphen-2.

Multiparameter flow cytometry

Diagnostic blast cells were obtained from thawed cryopreserved BM samples after red blood cell lysis. Fixation, permeabilization and staining (with both intracellular and cell surface markers) were performed using the PerFix-no centrifuge assay kit (Beckman Coulter) according to the manufactur-

er's instructions. The antibody panel contained: anti-AKTpS473-Vio515 (clone: REA359, Miltenyi Biotec), anti-CD33-PC5.5 (clone D3HL60.251, Beckman Coulter), anti-CD34-PC7 (clone 581, Beckman Coulter), anti-CD117/KIT-APC (clone 104D2D1, Beckman Coulter), anti-CD3-AA750 (clone UCHT1, Beckman Coulter), anti-CD4-PB (clone 13B8.2, Beckman Coulter) and anti-CD45-KO (clone J33, Beckman Coulter). Blast cells were gated as $CD45^{dim}$, SSC^{low}, CD33+, excluding lymphocytes ($CD45^{bright}$, SSC^{low}, CD33−), monocytes (CD45int/bright, SSC^{int}, $CD33^{bright}$) and mature myelomonocytic cells ($CD45^{int}$, SSC^{high}, $CD33^{dim/neg}$). Isotype control (clone REA293, Miltenyi Biotec) was used to better define the threshold of AKTpS473-positive cells. AKTpS473 expression levels were calculated as [mean fluorescent intensity (MFI) of blast cells/MFI of isotype IgG control]. Measurements were performed on a Navios flow cytometer and analyzed with Kaluza software (Beckman-Coulter).

Statistical Methods

Details of statistical analysis are located in supplemental Methods.

RESULTS

CBF AML genomes are characterized by a limited number of SNP-array-lesions

SNP-array analysis of 116 t(8;21)-AML and 82 inv(16)-AML revealed a total of 319 lesions, including 277 CNAs (187 losses and 90 gains; median size: 26.1 Mb [range: 26 kb-155.1 Mb]) and 42 CN-LOH (Supplementary Table 2). Overall, 97 (84%) patients with t(8;21)-AML and 55 (64%) patients with inv(16)-AML had at least one genomic aberration (CNA and/or CN-LOH). There was no significant difference in the number of lesions between adult and pediatric patients (Supplementary Table 3) arguing for similar diseases as previously described[6]. Recurrent focal lesions associated with t(8;21) and inv(16) breakpoints were common events, occurring in 27 (14%) CBF-AML cases especially in the inv(16) subtype (22% *vs.* 7%, p=0.005). Considering them as part of the primary event, t(8;21) or inv(16), breakpoint-associated lesions (accounting for 41 of the 319 identified lesions) were excluded for subsequent descriptions. Finally, CBF-AML genomes exhibited a mean of 1.40 SNP-array aberrations per case (range: 0-7)(Table 1). CNAs were more numerous in t(8;21)-AML than in inv(16)-AML, mostly due to genomic deletions (0.98 *vs.* 0.44 losses/case respectively; p<0.001). Neither the presence of SNP-array lesions nor the number of lesions was a predictor of outcome (Supplementary Figure 1).

SNP-array karyotyping in CBF AML shows nonrandom copy number changes

Recurrent CNAs are listed in Table 2. Considering lesions that are non-associated with breakpoints, a large proportion of detected CNAs were broad aberrations or involved whole chromosomes (Figure 1). Most of them appeared to be nonrandom events and are usually seen by conventional karyotype[1]. Among t(8;21)-AML patients, loss of a sex chromosome (LOS) was by far the most common event, occurring in 62 (53%) patients, followed by interstitial deletion of the long arm of chromosome 9 [del(9q)] in 15 (13%) patients ($p<0.001$). Both aberrations were virtually absent among inv(16)-AML patients. All but one case with del(9q) shared a minimal deleted region (MRD) of 6.1Mb in size containing 19 genes (Supplementary Figure 2) in which *TLE1* and *TLE4* have been the most studied[15,16]. By contrast, trisomy 22 was restricted to inv(16)-AML and occurred in 11 (13%) patients. Trisomy 8 and interstitial deletion of the long arm of chromosome 7 [del(7q)] were found in both genetic subtypes. Trisomy 8 was observed in 8 (10%) cases with inv(16) and 6 (5%) cases with t(8;21). Gain of the long arm of chromosome 8 (+8q) was seen is 2 additional cases with inv(16) (2 other cases with t(8;21) had +8q related to the rarely described duplication of the derivate chromosome der(21)t(8;21)[17]; Supplementary Figure 3). Del(7q) was found in 20 (10%) patients, including 10 (9%) cases with t(8;21) and 10 (12%) cases with inv(16). All cases with del(7q), whatever their genetic subtype t(8;21) or inv(16), shared a MDR of 4.2 Mb in size containing 71 genes in which the 2 epigenetics-related genes *EZH2* and *KMT2C* (*MLL3*) were the more relevant and have already been studied by others[18–20] (Supplementary Figure 4). Overall, we did not find any association between these recurrent genetic aberrations and clinical outcome (Supplementary Figure 5). Other broad recurrent aberrations included a previously undescribed deletion 2q which appeared to be restricted to patients with t(8;21)-AML (n=5) as well as gains 1q (n=2), 4q (n=3) and 13q (n=3). All patients with gain(13q) had also del(7q) in SNP-array while conventional karyotype showed additional material of unknown origin on the long arm of chromosome 7 [add(7q)]. Whole chromosome 13 painting performed in one of them by fluorescent *in situ* hybridization confirmed transfer of material from chromosome 13 to chromosome 7 leading to both gain(13) and del(7q) (Supplementary Figure 6).

SNP-array identifies recurrent target genes involved in CBF AML pathogenesis

One of the most common alterations was copy number gains at locus 8q24, which concerned 27 (13.5%) CBF-AML patients through several mechanisms (Supplementary Figure 7): 14 (7%) patients had trisomy 8, 4 (2%) patients had broad gains of the long arm of the chromosome 8 and the 9 (4.5%) remaining patients harbored focal gains that contained a single putative gene referred to as *CCDC26* which has been recently linked to myeloid leukemia cell growth[21]. *FOXP1* focal deletions were identified in 4 patients, all with inv(16)-AML. Interestingly, subsequent sequencing of all coding exons of *FOXP1* by HTS in the whole cohort identified 2 other inv(16) patients with *FOXP1* truncating mutations (Supplementary Figure 8).

Other recurrent focal aberrations included deletions of *WT1* (n=4), *BCORL1* (n=2), the cohesin core component *RAD21* (n=2) and the RAS pathway modulator *NF1* (n=2) whose mutations are recurrent in CBF-AML[6,7]. Additionally, some focal unique CNAs involved highly relevant genes such as deletions of the transcription factors *IKZF1* (n=1) and *ETV6* (n=1), gain of *MYB* (n=1), deletions of the cohesin regulator *PDS5A* (n=1) or the potential tumor suppressor *MGA* (n=1). A gain of *CNOT4*, which amplification is expected to enhance JAK/STAT signaling[22], was seen in one patient as the sole secondary abnormality.

ZBTB7A is a target of copy-neutral loss of heterozygosity in t(8;21) AML

The short arm of chromosome 19 was recurrently affected by CN-LOH in 3 patients with restriction to the t(8;21) subtype. The minimal affected region was about 6 Mb in size and contained 209 genes. Notably, this region was previously reported by Kühn et *al.* in 2 t(8;21)-AML patients with paired samples[20]. This region contained the *ZBTB7A* gene recently described as highly mutated in patients with t(8;21)-AML but not in patients with inv(16)-AML[23,24]. In order to validate *ZBTB7A* as a target of CN-LOH in 19p, we performed HTS of all coding exons of *ZBTB7A* in the whole cohort. We identified 23 *ZBTB7A* mutations in 19 patients (4 patients had 2 mutations) restricted to the t(8;21) subgroup (16%; Supplementary Table 4). Three patients harbored the same frameshift mutation at alanine 175. All patients with 19p CN-LOH harbored concomitant *ZBTB7A* mutation validating *ZBTB7A* as the target of this aberration (Figure 2A). Missense mutations clustered in the N-terminal BTB domain while frameshift mutations occurred through the whole protein as previously described by others[23,24] (Figure 2B). We did not identify any association or exclusion with other known mutations (Figure 2C). There was no difference in age, sex or white blood cell count according the *ZBTB7A* mutational status. Finally, there was no impact of *ZBTB7A* mutations on OS and RFS in t(8;21)-AML patients (Supplementary Figure 9).

CCDC26 disruption is likely to be associated with aberrant tyrosine kinase signaling in CBF AML

Nine (4.5%) patients harbored focal gains confined to the *CCDC26* locus (Supplementary Figure 10). Interestingly, it has been recently suggested that *CCDC26* could control myeloid leukemia cell growth through regulation of *KIT* expression[21]. Considering that hyperactive *KIT* mutations are highly prevalent in CBF-AML (about 35% of cases)[6] and that *CCDC26* focal amplification (*CCDC26*^{amp}) is found more frequently in CBF-AML than in non CBF-AML[25,26], these findings could reveal a new lesion associated with aberrant tyrosine kinase pathway activation in CBF-AML patients. Importantly, all but one patient harboring *CCDC26*^{amp} were *KIT* wild-type. In order to explore this hypothesis, expressions of the *KIT* receptor and the phosphorylated downstream effector AKT (AKTp473) were estimated on diagnostic blast cells by flow cytometry in patients with *CCDC26*^{amp} (n=3). Results were compared

with blast cells isolated from patient with normal *CCDC26* copy number (*CCDC26*^{nor}) and *KIT* mutation (*KIT*^{mut}; n=4), *FLT3*-ITD (n=2) or *KIT*/*FLT3* wild-type (*KIT*^{wt}/*FLT3*^{wt}; n=3). All patients were *NRAS* and *KRAS* wild-type (*RAS*^{wt}). Overall, there was no correlation between *KIT* expression and *CCDC26* copy number or *KIT*/*FLT3* mutational status. Median expression of AKTp473 showed a trend of higher expression in *CCDC26*^{amp}-cells (+24%) compared with *CCDC26*^{nor}/KIT^{wt}/FLT3^{wt}/RAS^{wt}-cells (Figure 3). Although we could not directly linked AKTp473 and *CCDC26*^{amp}, these data suggest an underlying mechanism leading to the activation of tyrosine kinase pathway in cells harboring *CCDC26*^{amp}. On the other hand, median expression of AKTp473 clearly increased in *CCDC26*^{nor}/*KIT*^{wt}/*FLT3*-ITD/RAS^{wt}-cells (+107%) compared with *CCDC26*^{nor}/KIT^{wt}/FLT3^{wt}/RAS^{wt}-cells while it was not observed for *CCDC26*^{nor}/KIT^{mut}/FLT3^{wt}/RAS^{wt}-cells.

DISCUSSION

SNP-array karyotyping of 198 patients with CBF-AML highlight great differences between t(8;21)-AML and inv(16)-AML as described in most recent studies focused on cooperating mutations[6,7,23,24,27–29]. Notably, these studies identified frequent mutations in genes encoding epigenetic regulators and cohesin complex in t(8;21)-AML while they were absent in inv(16)-AML[6,7,23,24]. Overall, we found more CNAs in t(8;21)-AML than in inv(16)-AML, mostly due to genomic deletions. Excluding breakpoint-associated lesions, the most common CNAs included large chromosomal lesions usually seen by conventional cytogenetics including LOS, del(9q), del(7q) and trisomy 8 in t(8;21)-AML compared with trisomy 22, del(7q), trisomy 8, trisomy 21 and trisomy 9 in inv(16)-AML. While it is clear that these events are nonrandom and contribute to the pathogenesis of CBF-AML, there was no association between clinical outcome and the number of SNP-array lesions nor the presence of these specific aberrations. Remarkably, there was no significant difference in the number of lesions between adult and pediatric patients suggesting they reflect the same entity and could be studied together in further biological experiments.

Concerning recurrent broad deletions, SNP-array led us to identify MDRs on chromosome 9 (involving *TLE1* and *TLE4*) and chromosome 7 (containing *EZH2* and *KMT2C*). In previous experiments, Dayyani et *al* shown that haploinsufficiency of *TLE1* and *TLE4* could overcome the negative survival and anti-proliferative effects of *RUNX1-RUNXT1* on myeloid progenitors and promote leukemogenesis[16]. Using SNP-array profiling, Kühn et *al* previously identified a MDR on 7q containing only 4 genes including *KMT2C*[20]. By sequence analysis of 46 CBF-AML without *KMT2C* deletion, they identified a single somatic heterozygous frameshift mutation in this gene. More recently, it was shown that *KMT2C* act as a tumor suppressor gene in AML[19]. Together, these data suggest that *KMT2C* haploin-

sufficiency is likely to be a cooperating event in CBF-AML pathogenesis. While the MDR defined by Kühn et *al* did not contain *EZH2,* by contrast with the present study*,* the high frequency of polycomb mutations *(ASXL1, ASXL2* and *EZH2)* in t(8;21)-AML suggest that *EZH2* haploinsufficiency could be of interested, at least in t(8;21)-AML patients [6,14].

Although the number of CNAs was low, our analysis identified recurrent deletions and subsequent mutations in known and potentially new cancer genes. These included deletions in *WT1*, *BCORL1*, *RAD21*, *EZH2* or *NF1* whose mutations have been recurrently found in CBF-AML patients[6,7,23]. Interestingly, we identified *FOXP1* aberrations (deletions or truncated mutations) in 7% of patients with inv(16)-AML, arguing for a pathogenic role in this particular subtype. *FOXP1* (*forkhead box P1*) encodes one of the 4 members of the FOXP subfamily of forkhead transcription factors, known to be involved in human malignancies, cell survival and differentiation[30]. *FOXP1* has been described as a target of chromosomal translocations and amplifications in B-cell lymphomas and prostate cancer[30]. By contrast, *FOXP1* losses have been described in clear cell-type kidney cancer but also rarely in myeloproliferative neoplasms[31] and AML with normal[32] or complex karyotype[33]. *FOXP1* has been shown to function as a transcriptional repressor in monocytic differentiation[34]. Thus, it is likely that *FOXP1* loss-of-function could contribute to leukemogenesis especially in inv(16)-AML which is most often diagnosed as AML with a monocytic compartment[1].

Three patients with t(8;21)-AML had CN-LOH of 19p leading to homozygous *ZBTB7A* mutation. *ZBTB7A* (also known as *LRF* or *Pokemon*) encodes a transcription factor of the POK (*poxvirus and zinc finger and Krüppel*)/ZBTB (*zinc finger and broad complex, tramtrack, and bric-a-brac*) family involved in the hematopoietic development and the negative regulation of glycolysis[24]. Sequence analysis of *ZBTB7A* in the whole cohort identified mutations in 16% of t(8;21)-AML while no mutation was found in inv(16)-AML. Our results are in line with other studies previously reporting *ZBTB7A* mutations in 10% to 23% of t(8;21)-AML[7,23,24]. Somatic *ZBTB7A* mutations are also reported at low frequencies in various solid malignancies[35]. Missense mutations identified in our analysis clustered in the BTB domain which mediates the homodimerization and/or heterodimerization with other proteins[35]. Truncated mutations were distributed through the whole gene leading to the loss of the zinc-finger domain involved in DNA binding and/or nuclear localization signal. Previous experiments from Hartmann et *al* suggest that *ZBTB7A* act as a tumor suppressor in t(8;21)-AML[23]. Overexpression of *ZBTB7A* in Kasumi-1 cells leads to reduced proliferation while its haploinsufficiency should result in the induction of glycolysis promoting tumor progression[23,35].

Finally, we identified *CCDC26* (*coiled-coil domain containing* 26) focal amplifications in 4.5% of the total cohort, consistent with previous SNP-array investigations showing such lesions in 4.7% of CBF-AML genomes[20,25]. The nature of *CCDC26* remains ambiguous but it is more plausible that the

CCDC26 locus encodes a long non-coding-RNA[36] involved in tumors, including low-grade gliomas[37] and pancreatic cancer[38]. This locus, also known as *RAM* (*retinoic acid modulator*), was initially reported as required for retinoic acid (RA)-induced myeloid differentiation. Retroviral DNA integration into this locus has been shown to generated RA-resistant cells[39]. Interestingly, Hirano et *al* showed that *CCDC26*-knockdown resulted in *KIT* up-regulation and enhanced survival in myeloid leukemia cell lines. First of all, these results appeared conflicting with our data showing *CCDC26* amplification in CBF-AML. However, this paradox could be explicable by the fact that *CCDC26* amplification does not extend to the whole gene. Partial amplification restricted to exons 1 and 1a could result in *CCDC26* disruption leading to an abnormal mRNA structure without any activity or able to interfere with the remaining intact gene[40]. Thus, considering the high frequency class I mutations (especially in *KIT*, *FLT3* and *RAS* genes) in CBF-AML[6], it is likely that *CCDC26* disruption could highlight a new class I aberration leading to increased cell survival and proliferation in leukemia. In order to explore this hypothesis, we studied phosphoAKT expression by flow cytometry in CBF-AML cells harboring *CCDC26* focal amplification. Although we were able to study only 3 patients with this lesion, blast cells from patients with *CCDC26* disruption showed a subtle increased expression of phosphoAKT compared with blast cells from patients with normal *CCDC26* copy number and no class I mutation (*KIT*, *FLT3* and *RAS* wild-type). However, this was not observed blast cells from 4 *KIT*-mutated patients suggesting other activated pathways associated with *KIT* mutations. By contrast phosphoAKT expression were clearly increased in blast cells from 2 patients with *FLT3-ITD*. Unfortunately, we were not able to study other cytoplasmic effectors such as ERK, SRC or STAT proteins that could be deregulated in leukemia. Also, because we studied a very small number of patients, we were not able to give strong conclusions but our data suggest a mechanism leading to tyrosine kinase signaling in cells with *CCDC26* disruption. Of course, further studies are needed to directly link *CCDC26* disruption and aberrant tyrosine kinase signaling in CBF-AML.

In conclusion, we defined the landscape of SNP-array lesions in a cohort of 198 adult and pediatric CBF-AML at time of diagnosis. As no cell culture is required, we described the frequency of known cytogenetic abnormalities with an unbiased approach and found no association with clinical outcome. Although, the number of SNP-array lesions appeared very low in CBF-AML, when combining with sequence analyses, we were able to identify recurrent involvement of known and potentially new cancer genes including *FOXP1* loss-of-function in inv(16)-AML, *ZBTB7A* homozygous mutations through CN-LOH in t(8;21)-AML and *CCDC26* disruption in both genetic subgroups of CBF-AML. Because of the low frequency of recurrent events, further studies focused on specific genetic subgroups of AML are needed to specify the incidence and the role of these aberrations in leukemogenesis.

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FIGURES AND TABLES

Table 1: Mean number of SNP-array lesions per CBF AML case according to genetic subtype.

	CBF AML	t(8;21) AML	inv(16) AML	p-value
Patients, n	198	116	82	
Number of CNAs†, mean (range)	1.19 (0-6)	1.35 (0-5)	0.96 (0-6)	0.004 *
Gains†, mean (range)	0.43 (0-4)	0.37 (0-4)	0.52 (0-3)	0.084
Losses†, mean (range)	0.76 (0-4)	0.98 (0-3)	0.44 (0-4)	<0.001 *
Number of CN-LOH†, mean (range)	0.21 (0-6)	0.17 (0-6)	0.27 (0-4)	0.372
Breakpoint lesions, mean (range)	0.21 (0-2)	0.09 (0-2)	0.38 (0-2)	0.002 *
Total CNAs/CN-LOH†, mean (range)	1.40 (0-7)	1.53 (0-7)	1.23 (0-7)	0.020 *

CNA: copy number abnormality; CN-LOH: copy neutral-loss of heterozygosity.
† excluding breakpoint-associated lesions.

Table 2: Recurrent copy number abnormalities in CBF AML patients.

Recurrent SNP-array lesions	Start ¥	End ¥	Size	Gene count	CBF AML	inv(16)	t(8;21)	p-value
gain(1)(q42.13q44)	227 833 996	249 224 684	21391 kb	197 genes	2 (1%)	0 (0%)	2 (2%)	0.512
del(2)(q33.2q35)	204 563 014	220 260 561	15698 kb	127 genes	5 (3%)	0 (0%)	5 (4%)	0.078
del(3)(p13)	71 194 153	71 523 438	329 kb	***FOXP1***	4 (2%)	4 (5%)	0 (0%)	0.028 *
gain(4)(q32.1q35.2)	158 379 102	190 957 473	32578 kb	124 genes	3 (2%)	1 (1%)	2 (2%)	1.000
del(7)(q35q36.1)	147 660 930	151 908 681	4248 kb	71 genes including ***EZH2*** and ***KMT2C***	20 (10%)	10 (12%)	10 (9%)	0.476
del(8)(q24.11)	117 823 216	117 914 100	91 kb	***RAD21***, *RAD21-AS1, MIR3610*	2 (1%)	0 (0%)	2 (2%)	0.512
focal gain(8)(q24.21)†	130 586 319	130 697 500	111 kb	***CCDC26***	9 (5%)	4 (5%)	5 (4%)	1.000
gain(8)(q24.11q24.3)†	118 660 515	140 821 810	22161 kb	92 genes including ***CCDC26*** and ***MYC***	2 (1%)	2 (2%)	0 (0%)	0.170
trisomy 8†	whole chromosome		-	-	14 (7%)	8 (10%)	6 (5%)	0.264
del(9)(q21.2q21.33)	80 806 493	86 951 615	6145 kb	19 genes including ***TLE1*** and ***TLE4***	14 (7%)	0 (0%)	14 (12%)	<0.001 *
trisomy 9	whole chromosome		-	-	2 (1%)	2 (2%)	0 (0%)	0.170
del(11)(p13)	31 972 741	32 633 735	661 kb	*RCN1,* ***WT1****, WT1-AS, EIF3M, CCDC73*	4 (2%)	2 (2%)	2 (2%)	1.000
gain(13)(q31.1q34)	85 412 329	115 107 733	29695 kb	135 genes	3 (2%)	1 (1%)	2 (2%)	1.000
del(17)(q11.2)	29 357 586	29 520 056	162 kb	*MIR4733,* ***NF1***	2 (1%)	2 (2%)	0 (0%)	0.170
trisomy 21	whole chromosome		-	-	3 (2%)	3 (4%)	0 (0%)	0.070
trisomy 22	whole chromosome		-	-	11 (6%)	11 (13%)	0 (0%)	<0.001 *
del(X)(q26.1)	129 129 272	129 211 954	83 kb	***BCORL1****, ELF4*	2 (1%)	2 (2%)	0 (0%)	0.170
loss X or Y					63 (32%)	1 (1%)	62 (53%)	<0.001 *
loss X§	whole chromosome		-	-	22 (24%)	0 (0%)	22 (42%)	<0.001 *
loss Y§	whole chromosome		-	-	41 (39%)	1 (2%)	40 (63%)	<0.001 *
Breakpoint-associated lesions								
del(8)(q21.3)	93 096 598	93 128 271	32 kb	***RUNX1T1***	5 (3%)	0 (0%)	5 (4%)	0.078
del(16)(p13.11)	15 828 494	16 056 322	228 kb	***MYH11****, FOPNL, ABCC1*	16 (8%)	16 (20%)	0 (0%)	<0.001 *
gain(16)(p13.11)	15 725 039	15 814 747	90 kb	*KIAA0430, NDE1, MIR484,* ***MYH11***	1 (1%)	1 (1%)	0 (0%)	0.414
del(16)(q22.1)	67 132 654	67 176 123	43 kb	***CBFB****, C16orf70*	13 (7%)	13 (16%)	0 (0%)	<0.001 *
gain(16)(q21q22.1)	65 352 347	67 131 638	1779 kb	23 genes including ***CBFB***	1 (1%)	1 (1%)	0 (0%)	0.414
del(21)(q22.12)	36 183 871	36 210 100	26 kb	***RUNX1***	3 (2%)	0 (0%)	3 (3%)	0.268
gain(21)(q22.12)	36 355 481	36 423 085	68 kb	***RUNX1****, RUNX1-IT1*	2 (1%)	0 (0%)	2 (2%)	0.512
+der(21)t(8;21)(q22;q22)	whole chromosome		-	-	2 (1%)	0 (0%)	2 (2%)	0.512

* p-value < 0.05.
† Partial chromosomal lesions were considered separately from abnormalities involving the whole chromosome. Gains 8q related to der(21)t(8;21) duplication (n=2) were considered apart from other lesions.
¥ Chromosomal locations were obtained from the Human Feb. 2009 (GRCh37/hg19) assembly of the UCSC Genome Browser (http://genome.ucsc.edu/).
§ Proportions are given within the female population for loss of X and the male population for loss of Y.

Figure 1: Karyograms of detected SNP-array lesions by genetic subtype. Each vertical line represents 1 event in 1 patient. Gains are in red, losses in green and CN-LOH in blue. Part (A) shows cases with t(8;21) AML and part (B) shows cases with inv(16) AML. Schematic representations were obtained using the Genomic Recurrent Event ViEwer (GREVE) web tool (http://www.well.ox.ac.uk/GREVE)[41].

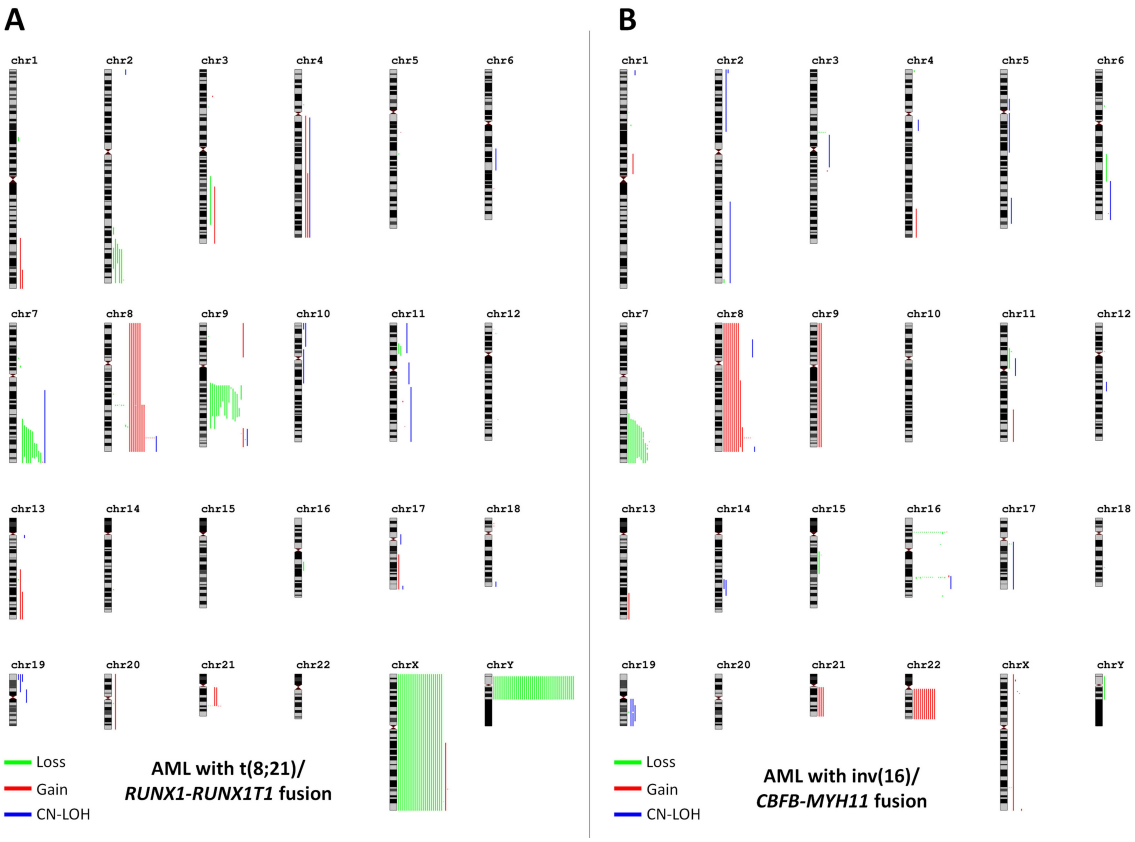


Figure 2: *ZBTB7A* aberrations in AML patients with t(8;21). (A) Concomitant mutation and copy-neutral loss of heterozygosity in 3 patients with t(8;21) AML. (B) ZBTB7A protein (NP_056982.1) and identified mutations (red = truncating; green = missense). BTB: BR-C ttk and bab; NLS: nuclear localization sequence; Zf: zinc finger. (C) Genomic landscape of t(8;21) AML including *ZBTB7A* mutations.

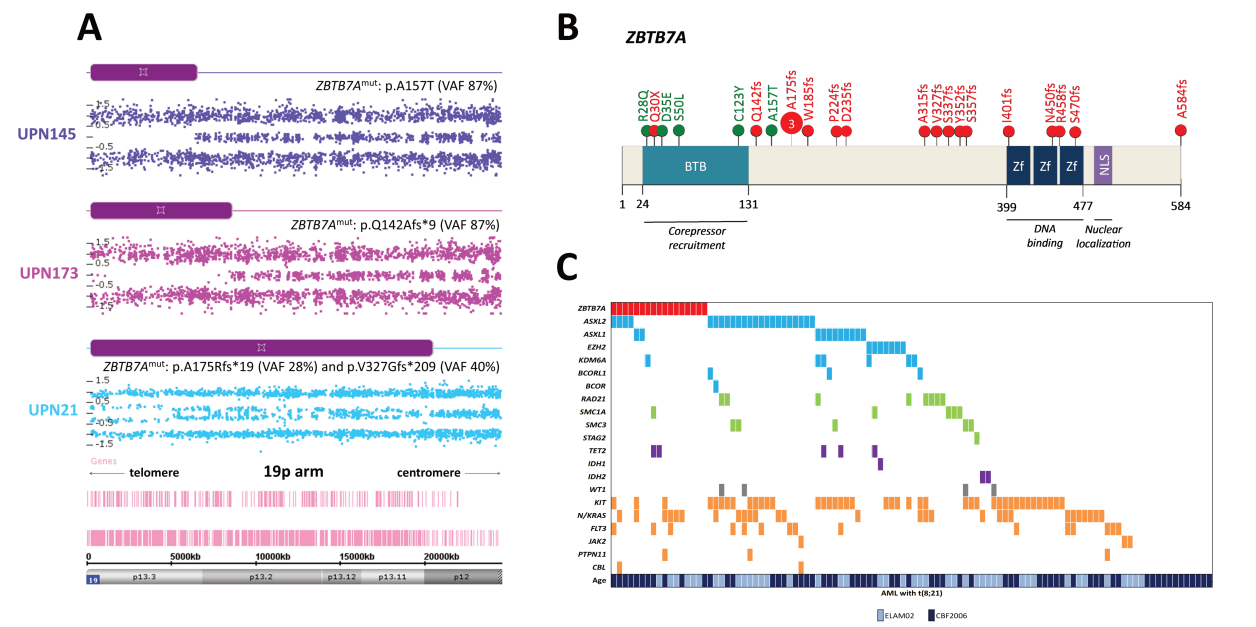
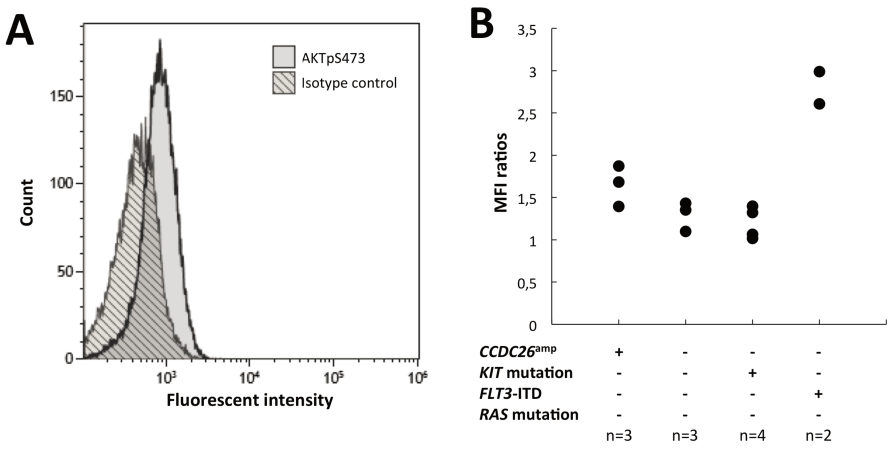


Figure 3: AKTp473 expression in blasts from CBF AML patients. (A) AKTpS473 expression level in a patient with *CCDC26* focal amplification (*CCDC26*^{amp}). (B) AKTpS473 mean fluorescent intensity ratios according to *CCDC26* focal amplification and *KIT/FTL3/RAS* mutational status. Ratios were calculated as (MFI of blast cells)/(MFI of isotype IgG control).



SUPPLEMENTAL DATA

Supplemental methods

Statistical analysis: Failure time data were analyzed and compared after censoring at transplant for patients who received allogeneic stem cell transplantation (SCT) in first complete remission (CR). Relapse-free survival (RFS) and overall survival (OS) were estimated by the Kaplan-Meier method. RFS was estimated taking into account death in first CR for competing risk. Comparisons between patient subgroups were performed by the Mann-Whitney test for continuous variables and by Fisher's exact test for categorical variables. All statistical tests were performed with the SPSS Statistics software (IBM).

Supplementary Table 1: Patient characteristics according to CBF AML subtype.

	CBF-AML	AML with inv(16)	AML with t(8;21)	p-value	
Patients, n	198	82	116		
Median age, y [range]	30 [1-60]	33 [1-60]	28 [2-60]	0.554	
Median WBC, x10^9/L [range]	16,8 [1,3-215]	38,7 [1,9-215]	12,8 [1,3-163]	<0.001	*
Gender (male/female)	105/93	42/40	63/53	0.773	
Trial (CBF2006/ELAM02)	125/73	52/30	73/43	1.000	
Outcome					
Deaths, n (%)	27 (14)	10 (12)	17 (15)	0.679	
Relapses, n (%)	59 (30)	20 (24)	39 (34)	0.207	
Gene mutations					
KIT, n (%)	68/176 (39)	28/75 (37)	40/101 (40)	0.876	
FLT3-TKD, n (%)	21/176 (12)	17/75 (23)	4/101 (4)	<0.001	*
FLT3-ITD, n (%)	12/176 (7)	2/75 (3)	10/101 (10)	0.073	
NRAS, n (%)	50/176 (28)	26/75 (35)	24/101 (24)	0.130	
KRAS, n (%)	24/176 (14)	17/75 (23)	7/101 (7)	0.004	*
ASXL1, n (%)	10/176 (6)	0/75 (0)	10/101 (10)	0.005	*
ASXL2, n (%)	22/176 (13)	0/75 (0)	22/101 (22)	<0.001	*

* p-value < 0.05

Supplementary Table 2: CBF AML cases studied by SNP-array. Identified copy number alterations (CNAs) and copy-neutral losses of heterozygosity (CN-LOH) are listed for each CBF AML case (n=198) with chromosome location, start and end positions according to human genome version 19 (hg19) and gene count (gene symbols are given if < 10). Mutational profiling from Duployez et al, Blood, 2016 is also reported. ND: not determined.

Cf. version online.

Supplementary Table 3: Mean number of SNP-array lesions per CBF AML case according to trial.

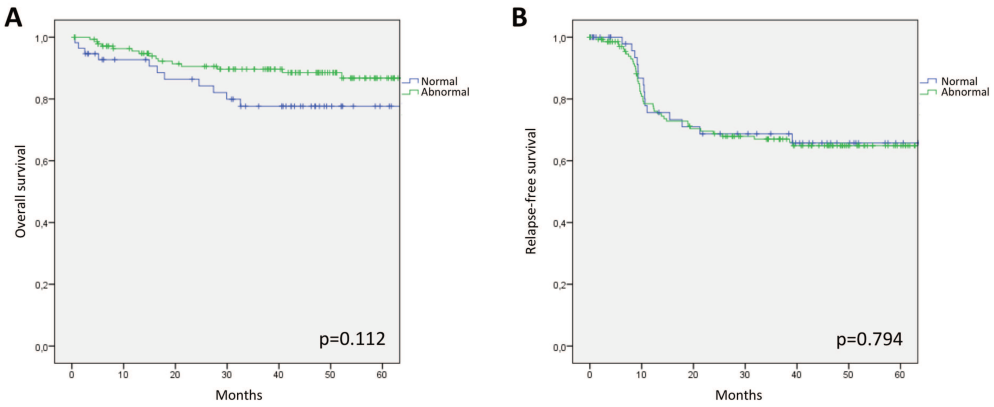
	CBF AML	Adults (CBF2006)	Children (ELAM02)	p-value
Patients, n	198	125	73	
Number of CNAs†, mean (range)	1.19 (0-6)	1.18 (0-6)	1.22 (0-5)	0.804
Gains†, mean (range)	0.43 (0-4)	0.42 (0-4)	0.45 (0-3)	0.567
Losses†, mean (range)	0.76 (0-4)	0.75 (0-4)	0.77 (0-3)	0.582
Number of CN-LOH†, mean (range)	0.21 (0-6)	0.19 (0-3)	0.25 (0-6)	0.798
Breakpoint lesions, mean (range)	0.21 (0-2)	0.16 (0-2)	0.29 (0-2)	0.087
Total CNAs/CN-LOH†, mean (range)	1.40 (0-7)	1.37 (0-7)	1.47 (0-7)	0.778

CNA: copy number abnormality; CN-LOH: copy neutral-loss of heterozygosity.
† excluding breakpoint-associated lesions

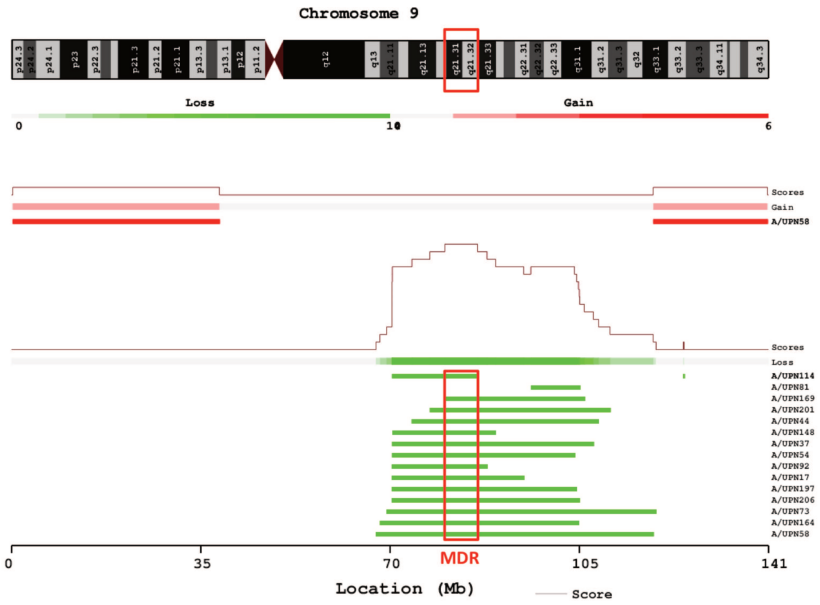
Supplementary Table 5: *ZBTB7A* variants (NM_015898) in CBF AML. AA: amino-acid; CN-LOH: copy neutral-loss of heterozygosity; NA: not applicable; UPN: unit patient number; VAF: variant allele frequency.

UPN	CBF	Type	Exon	Nuc. change	AA change	VAF	Polyphen-2	SIFT	CN-LOH
UPN151	t(8;21)	Frameshift	2	c.1067_1068insTGTTC	p.S357Vfs*71	31%	NA	NA	
UPN145	t(8;21)	Single AA change	2	c.469G>A	p.A157T	87%	probably damaging	tolerated	Yes
UPN39	t(8;21)	Frameshift	2	c.551dupC	p.W185Vfs*9	32%	NA	NA	
UPN203	t(8;21)	Frameshift	2	c.522dupC	p.A175Rfs*19	30%	NA	NA	
UPN78	t(8;21)	Frameshift	2	c.1054dupT	p.Y352Lfs*188	55%	NA	NA	
UPN149	t(8;21)	Frameshift	2	c.522dupC	p.A175Rfs*19	33%	NA	NA	
UPN173	t(8;21)	Frameshift	2	c.423dupG	p.Q142Afs*9	87%	NA	NA	Yes
UPN36	t(8;21)	Nonsense	2	c.88C>T	p.Q30X	36%	NA	NA	
UPN171	t(8;21)	Single AA change	2	c.368G>A	p.C123Y	12%	probably damaging	damaging	
UPN301	t(8;21)	Frameshift	2	c.703delG	p.D235Tfs*89	20%	NA	NA	
UPN63	t(8;21)	Single AA change	2	c.149C>T	p.S50L	9%	probably damaging	damaging	
UPN307	t(8;21)	Frameshift	2	c.1200_1201delCA	p.I401Pfs*138	26%	NA	NA	
UPN190	t(8;21)	Frameshift	3	c.1349dupA	p.N450Kfs*90	35%	NA	NA	
UPN192	t(8;21)	Frameshift	2	c.1008_1009insG	p.S337Efs*203	48%	NA	NA	
		Frameshift	3	c.1407dupC	p.S470Qfs*70	24%	NA	NA	
UPN30	t(8;21)	Single AA change	2	c.105C>A	p.D35E	20%	probably damaging	damaging	
		Single AA change	2	c.83G>A	p.R28Q	23%	probably damaging	damaging	
UPN79	t(8;21)	Frameshift	3	c.1372delC	p.R458Afs*99	20%	NA	NA	
		Frameshift	2	c.944_966del	p.A315Dfs*217	26%	NA	NA	
UPN21	t(8;21)	Frameshift	2	c.522dupC	p.A175Rfs*19	28%	NA	NA	Yes
		Frameshift	2	c.980_990del	p.V327Gfs*209	40%	NA	NA	
UPN308	t(8;21)	Frameshift	3	c.1749_1750insTGGGG	p.A584Wfs*62	26%	NA	NA	
UPN1	t(8;21)	Frameshift	2	c.671_676delinsTCGGTGA	p.P224Lfs*26	12%	NA	NA	

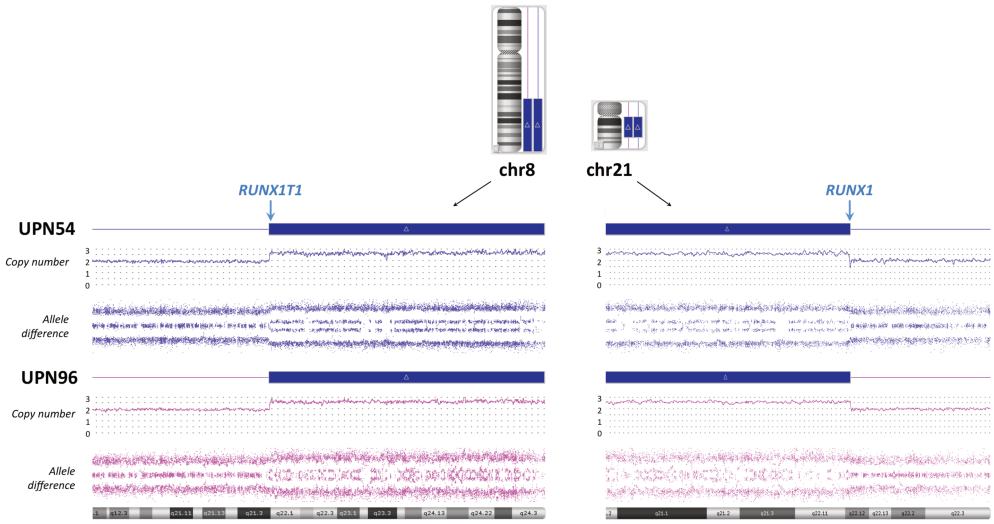
Supplementary Figure 1: Clinical outcome according to SNP-a profile. Overall survival (A) and relapse-free survival (B) in CBF AML according to the presence (abnormal) or absence (normal) of SNP-array lesions. Patients who received allogeneic stem cell transplantation were censored at time of transplant.



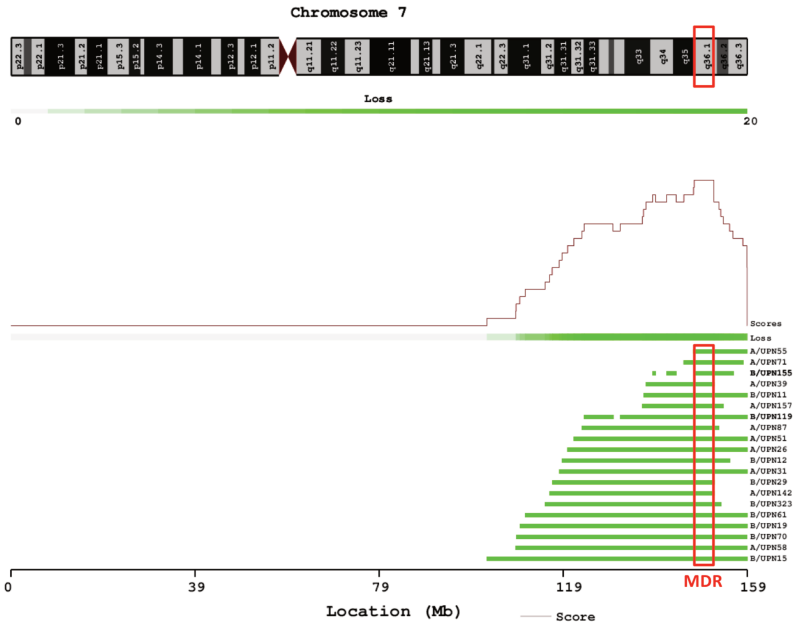
Supplementary Figure 2: Deletion 9q. Interstitial deletion of the long arm of chromosome 9 occurred in 15 cases, all of which were t(8;21) AML (referred as "A" next to UPNs). The minimal deleted region (MDR, showing the highest score), shared by 14 of 15 patients, was of 6.1 Mb in size (location: chr9:80 806 493-86 951 615) and contained 19 genes (including *TLE1* and *TLE4*). A last patient (UPN81) had a more distal del(9)(q22.32q31.1) of 9.1 Mb in size containing 84 genes. UPN58 had probable duplication of the der(9)del(9)(q13q33.1) showing gains of the non-deleted regions. (Figure restricted to patients with del(9q)).



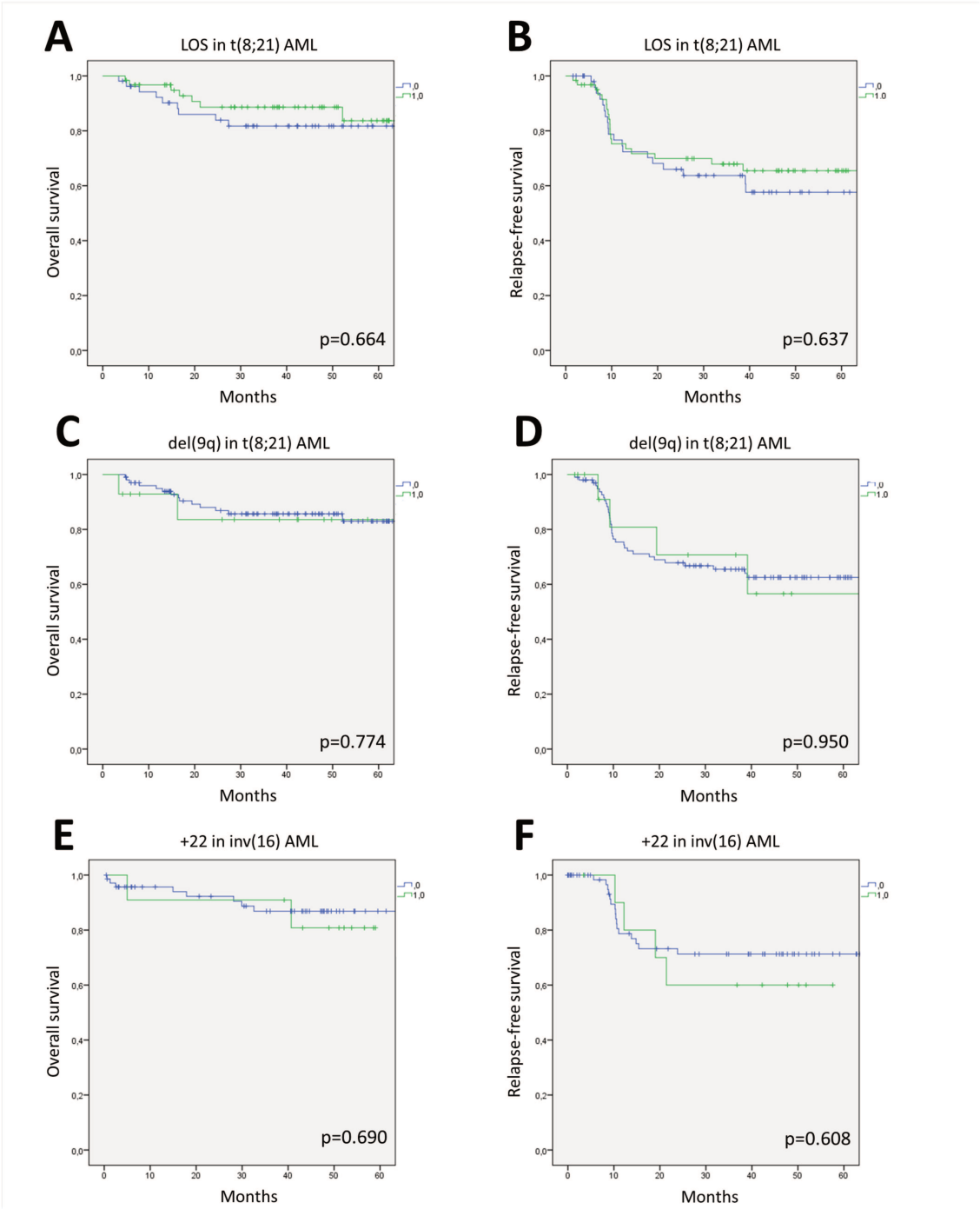
Supplementary Figure 3: Duplication of the der(21)t(8;21). One pediatric patient (UPN54) and one adult patient (UPN96) showed trisomy of the regions upstream of the RUNX1 gene and downstream of the RUNX1T1 gene. Conventional karyotype confirmed the duplication of the der(21)t(8;21) in both patients as the sole additional abnormality in UPN96 and associated with del(9q) and loss of Y in UPN54.

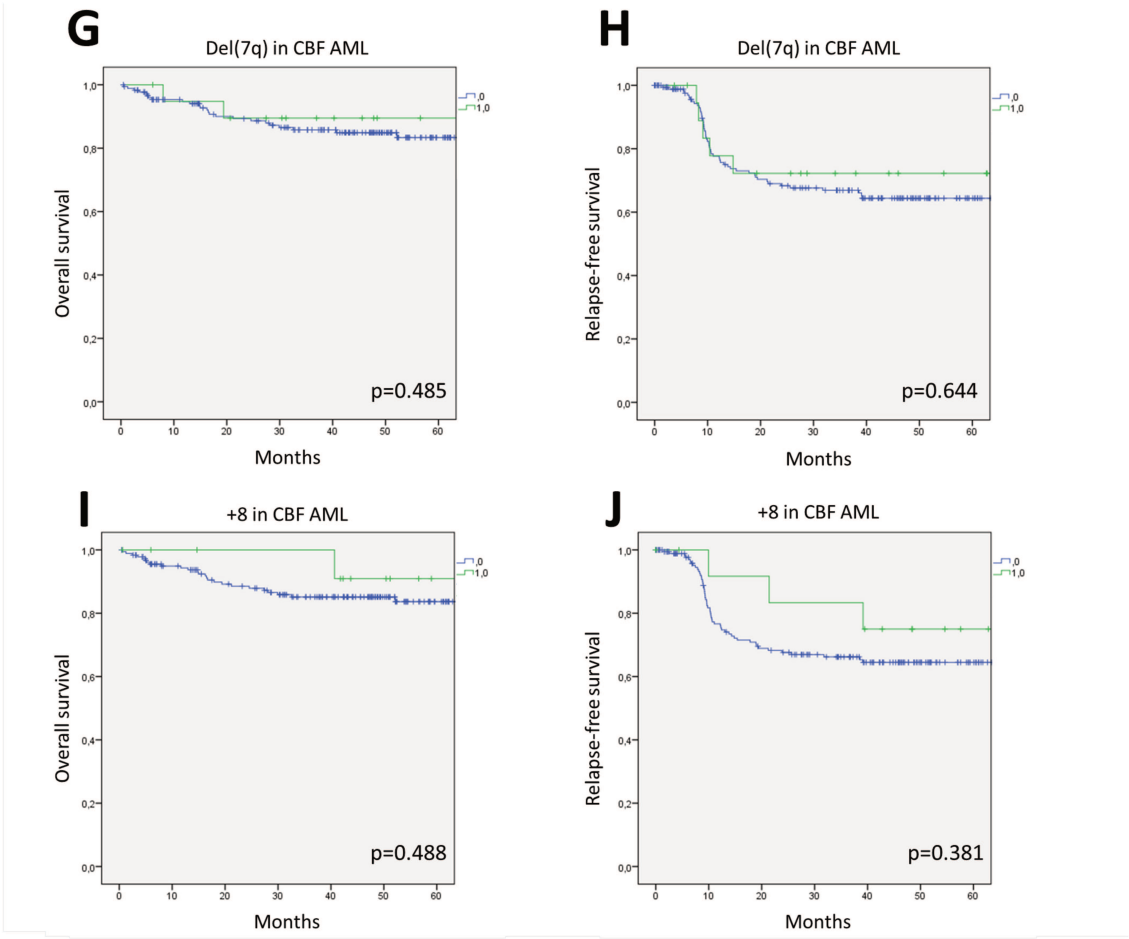


Supplementary Figure 4: Deletion 7q. Interstitial deletion of the long arm of chromosome 7 occurred in 20 cases, including 10 cases with t(8;21) and 10 cases with inv(16) (referred as "A" and "B" next to UPNs). The MDR (showing the highest score), shared by all patients, was of 4.2 Mb in size (location: chr7:147 660 930-151 908 681) and contained 71 genes (including *EZH2* and *KMT2C*). (Figure restricted to patients with del(7q)).

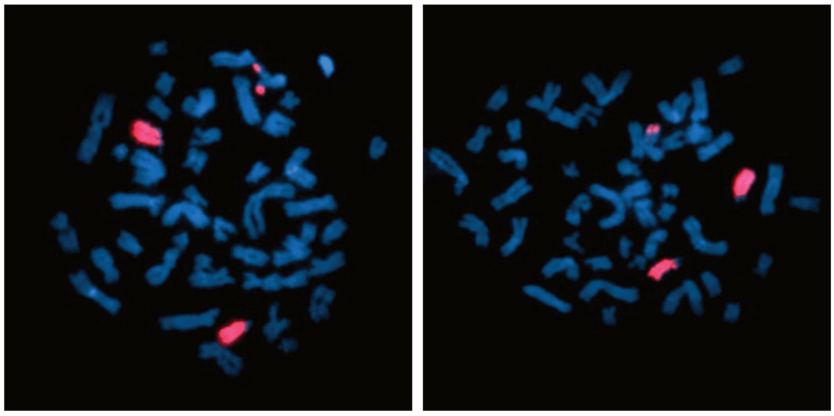


Supplementary Figure 5: Clinical outcome according to recurrent genetic aberrations. Recurrent genetic aberrations observed in at least 10 patients were studied for their impact on outcome: loss of sex chromosome (A,B), del(9q) (C,D), trisomy 22 (E, F), del(7q) (G,H) and trisomy 8 (I,J). Patients who received allogeneic stem cell transplantation were censored at time of transplant.

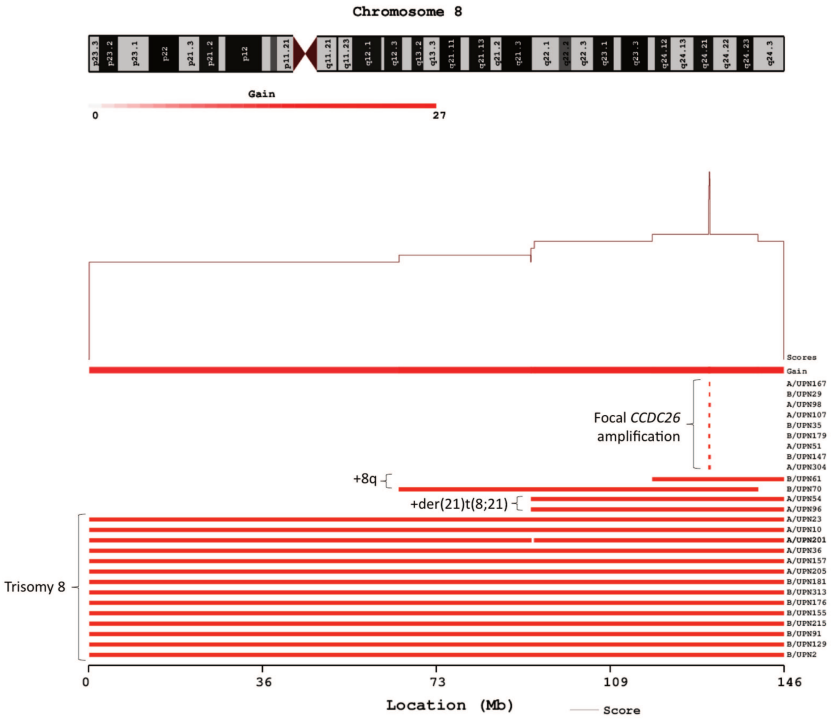




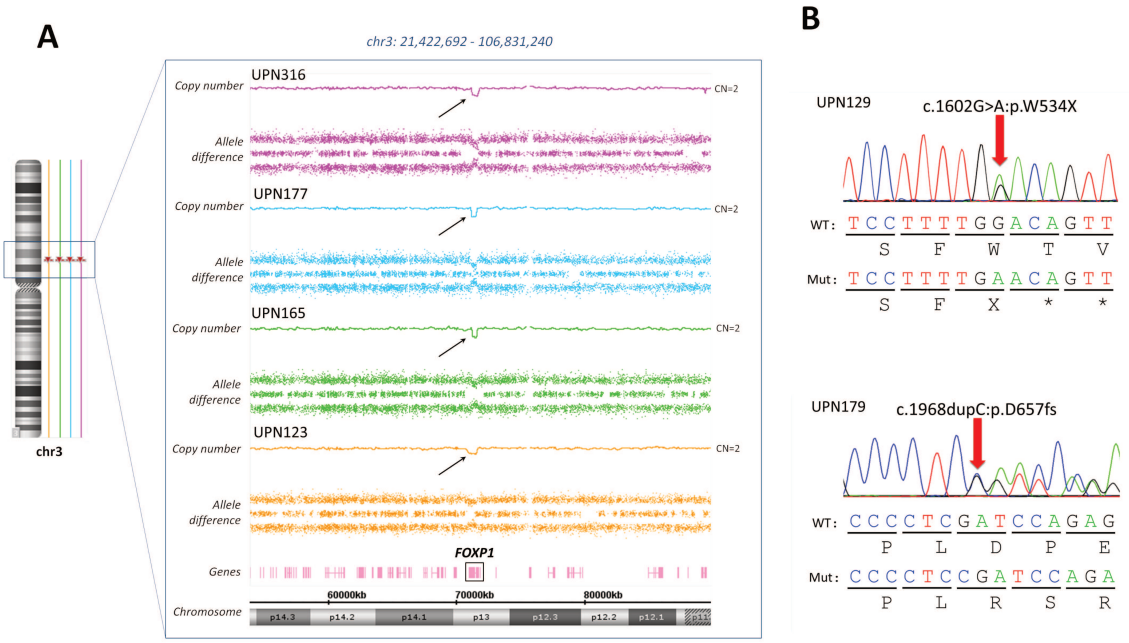
Supplementary Figure 6: Whole chromosome 13 painting in a patient with gain(13q). Whole chromosome 13 painting (Metasystems) by fluorescent in situ hybridization was applied to fixated metaphases cells according to standard procedures. The result showed transfer of material from chromosome 13 to chromosome 7.



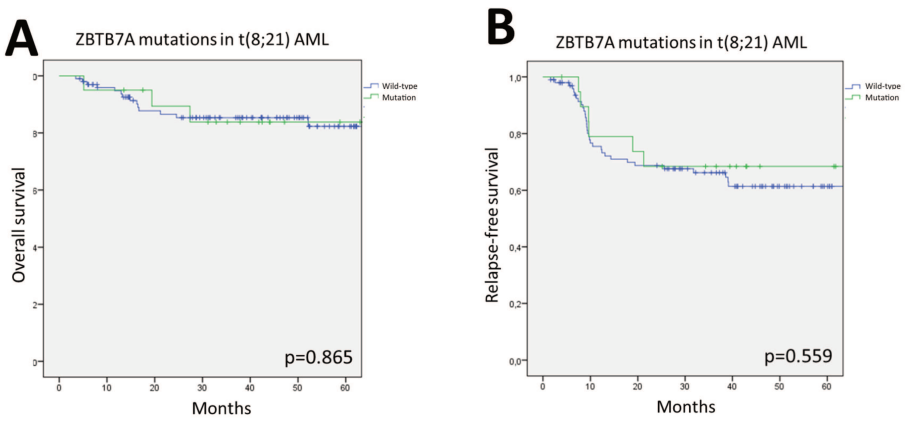
Supplementary Figure 7: Gain 8q. Amplification of the 8q24 was found in 27 cases, including 13 cases with t(8;21) and 14 cases with inv(16) (referred as "A" and "B" next to UPNs). Fourteen cases had trisomy 8. Two inv(16) AML patients had broad gains of the long arm of chromosome 8 (8q). Two t(8;21) AML patients had gains 8q from the RUNX1T1 gene to the telomere region, related to the duplication of the der(21)t(8;21). Finally, focal gains focused on *CCDC26* concerned 9 cases. (Figure restricted to patients with gains 8q).



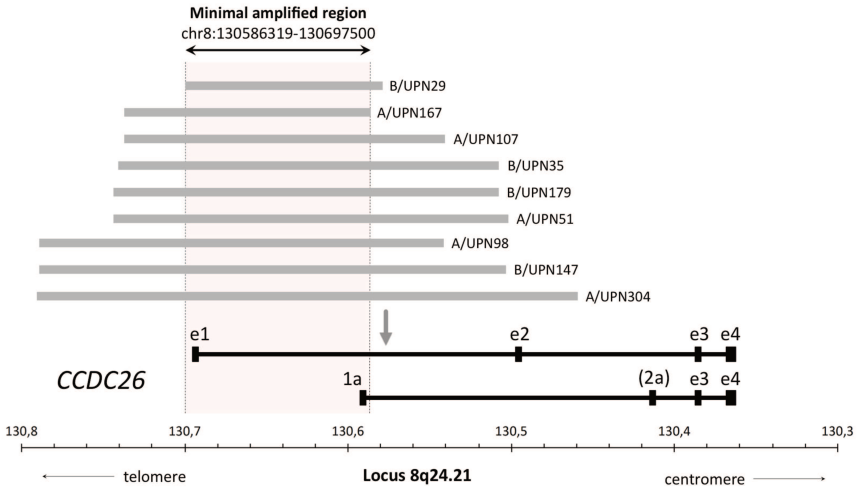
Supplementary Figure 8: *FOXP1* aberrations in inv(16) AML patients. (A) Focal deletions targeting the FOXP1 gene were detected in 4 patients harboring an inv(16). (B) Targeted sequencing of *FOXP1* revealed to other inv(16) patients with *FOXP1* truncating mutations.



Supplementary Figure 9: Clinical outcome according to *ZBTB7A* mutations in patients with t(8;21). (A) Overall survival. (B) Relpase-free survival.



Supplementary Figure 10: Focal *CCDC26* amplifications. Horizontal grey lines illustrate focal recurrent amplifications detected in 9 CBF AML genomes across the *CCDC26* locus. The area inside the dotted lines represents the minimal amplified region shared by all patients. Major variants of *CCDC26* mRNA are shown in black below the figure. Exons are indicated by boxes. The long transcript consists of four exons (from e1 to e4) and the short transcripts comprise 3 or 4 exons (from 1a to e4, more or less exon 2a). The arrow shows the retrovirus insertion site defined by Yin et al.



TRAVAUX NON PUBLIÉS : AUTRES ANOMALIES DES LAM CBF

1. Séquençage du gène *DHX15*

Le gène *DHX15* (DEAH-box helicase 15), localisé en 4p15, code pour une ARN-hélicase ATP-dépendante de la famille DEAH/RHA (Asp-Glu-Ala-His/RNA-Helicase A) dont l'homologue Prp43 identifié chez la levure est impliqué dans la biogénèse des 2 sous-unités ribosomiques et l'épissage des pré-ARN messagers [146]. Le rôle de *DHX15* a également été montré dans l'immunité antivirale [147]. Chez la levure, Prp43 intervient après excision de l'intron dans la dissociation du complexe intron-spliceosome permettant le recyclage des ribonucléoprotéines [148]. Cette étape fait intervenir son partenaire Ntr1 dont l'homologue TP1F11 est identifié chez l'Homme. Ainsi, la disparition de *Prp43/DHX15* ou *Ntr1/TPIF11* s'associe à l'accumulation de complexes intron-spliceosome et d'intermédiaires d'épissage.

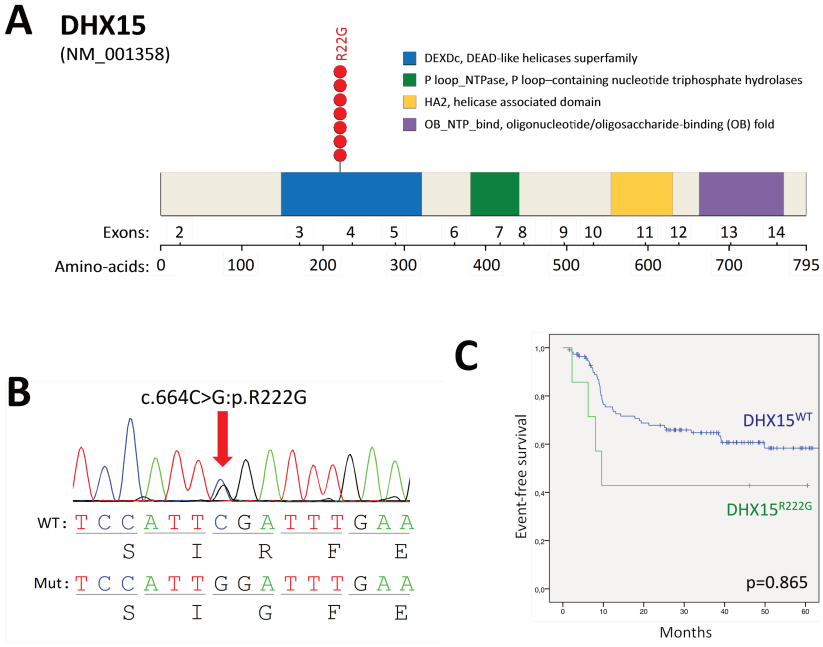


Figure 18 : Mutations de *DHX15* dans les LAM avec t(8;21) (n=121). (A) Structure schématique de la protéine DHX15 et localisation des mutations. (B) Séquence Sanger de la mutation R222G de *DHX15*. (C) Survie sans rechute des patients atteints de LAM avec t(8;21) selon le statut muté ou non muté de *DHX15*.

Par séquençage de l'exome entier d'une cohorte de 165 LAM CBF, les mutations de *DHX15* ont récemment été identifiées comme une anomalie récurrente dans ce sous-groupe de LAM. Toutes les mutations identifiées impliquaient le remplacement de l'arginine 222 par une glycine et concernaient exclusivement le groupe des LAM avec t(8;21) [149]. Les auteurs rapportaient une fréquence d'environ 7% (6/85) dans ce sous-groupe. L'expression *in vitro* du mutant DHX15^{R222G} montrait des

effets similaires à l'abolition de l'expression de *DHX15* sur l'épissage des pré-ARNm. Via des études de co-immunoprécipitation, les auteurs mettaient en évidence une perte de l'affinité de TFIP11 pour le mutant $DHX15^{R222G}$ [149]. Afin d'établir la fréquence de la mutation R222G dans notre cohorte, nous avons séquencé l'exon 3 de *DHX15* par méthode Sanger. Ce séquençage a été réalisé sur 237 LAM CBF incluant 121 LAM avec t(8;21) et 115 LAM avec inv(16). La mutation R222G a été identifiée chez 7 patients, tous atteints de LAM avec t(8;21), soit 6% dans ce sous-groupe (Figure 18A et B). Il n'était pas observé d'impact pronostique significatif des mutations de *DHX15 (*pouvant refléter un manque d'effectifs ; Figure 18C). Une rechute était observée pour 3 patients sur 7 (à respectivement 6, 8 et 10 mois). L'implication des mutations de *DHX15* dans la leucémogénèse et leur association dans les LAM avec t(8;21) restent à définir. De manière intéressante, nous avons montré dans le chapitre précédent (page 77) que les mutations des gènes du spliceosome (*SF3B1*, *SRSF2*, *U2AF1* et *ZRSR2*) étaient exceptionnelles dans ce sous-groupe de LAM suggérant une coopération spécifique des mutants $DHX15^{R222G}$ avec la protéine de fusion RUNX1-RUNX1T1.

2. Séquençage du gène *CCDN2*

Le gène *CCND2*, situé en 12p13 code pour la cycline D2. Les cyclines sont des régulateurs des sérine-thréonine kinases CDK (*cyclin-dependent kinases*) contrôlant le cycle cellulaire. La cycline D2 agit plus particulièrement au point de contrôle G1/S par activation de CDK4 et CDK6, lesquelles vont phosphoryler la protéine de susceptibilité au rétinoblastome pRb (produit du gène *RB1*). En phases G0 et G1 précoce, pRb sous forme non phosphorylée exerce ses fonctions de blocage de la prolifération en séquestrant le facteur de transcription E2F. Sa phosphorylation provoque la libération de E2F actif qui va ainsi pouvoir activer la transcription de nombreux gènes intervenant dans la progression du cycle cellulaire et la synthèse d'ADN [150]. Les mutations de *CCND2* ont été récemment identifiées dans 4.2% des LAM CBF par séquençage d'exome d'une cohorte de 165 patients [149]. Par séquençage ciblé d'une seconde cohorte de 177 patients, une autre étude du groupe américain confirmait la présence des mutations de *CCND2* dans 5.1% des LAM CBF [151]. En revanche, le séquençage d'une cohorte de 1426 adultes atteints de LAM non-CBF ne retrouvait des mutations de *CCND2* que chez 6 patients (0.4%), suggérant une association significative des mutations de *CCND2* avec les LAM CBF [151]. Toutes les mutations identifiées dans les 2 séries concernaient directement la thréonine 280 ou sa région proche. La thréonine 280 est un site de phosphorylation hautement conservé intervenant dans la régulation de l'ubiquitination de la lysine 270 précédant la dégradation de la cycline D2 par le protéasome et l'arrêt du cycle cellulaire en phase G0 [152,153]. D'un point de vue fonctionnel, Eisfeld et *al* ont montré que la mutation T280A était associée à une augmentation de la forme phos-

phorylée de pRb et une augmentation de la prolifération cellulaire [151]. Faber et *al* ont démontré que les mutations P281R et T282fs de *CCND2* augmentaient la stabilité de la cycline D2 et favorisaient ainsi la poursuite du cycle cellulaire [149].

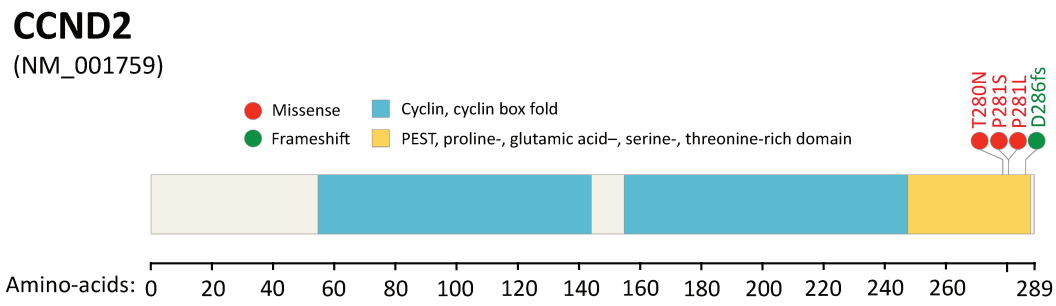


Figure 19 : Mutations de *CCN2* dans les LAM CBF (n = 237).

Le séquençage ciblé de *CCND2* par méthode Sanger dans notre cohorte de 237 LAM CBF identifiait 4 mutations (1.7% ; Figure 19) qui impliquaient les codons 280 (T280N), 281 (P281S et P281L) et 286 (D286fs) et concernaient 2 LAM avec t(8;21) et 2 LAM avec inv(16). A noter que la plus faible fréquence observée dans notre cohorte (1.7% *vs.* 4.2 et 5.1% dans la littérature) est susceptible d'être liée à la méthode de séquençage utilisée (Sanger *vs.* séquençage à haut débit).

3. Synthèse des différents travaux dans les LAM CBF (Figure 20)

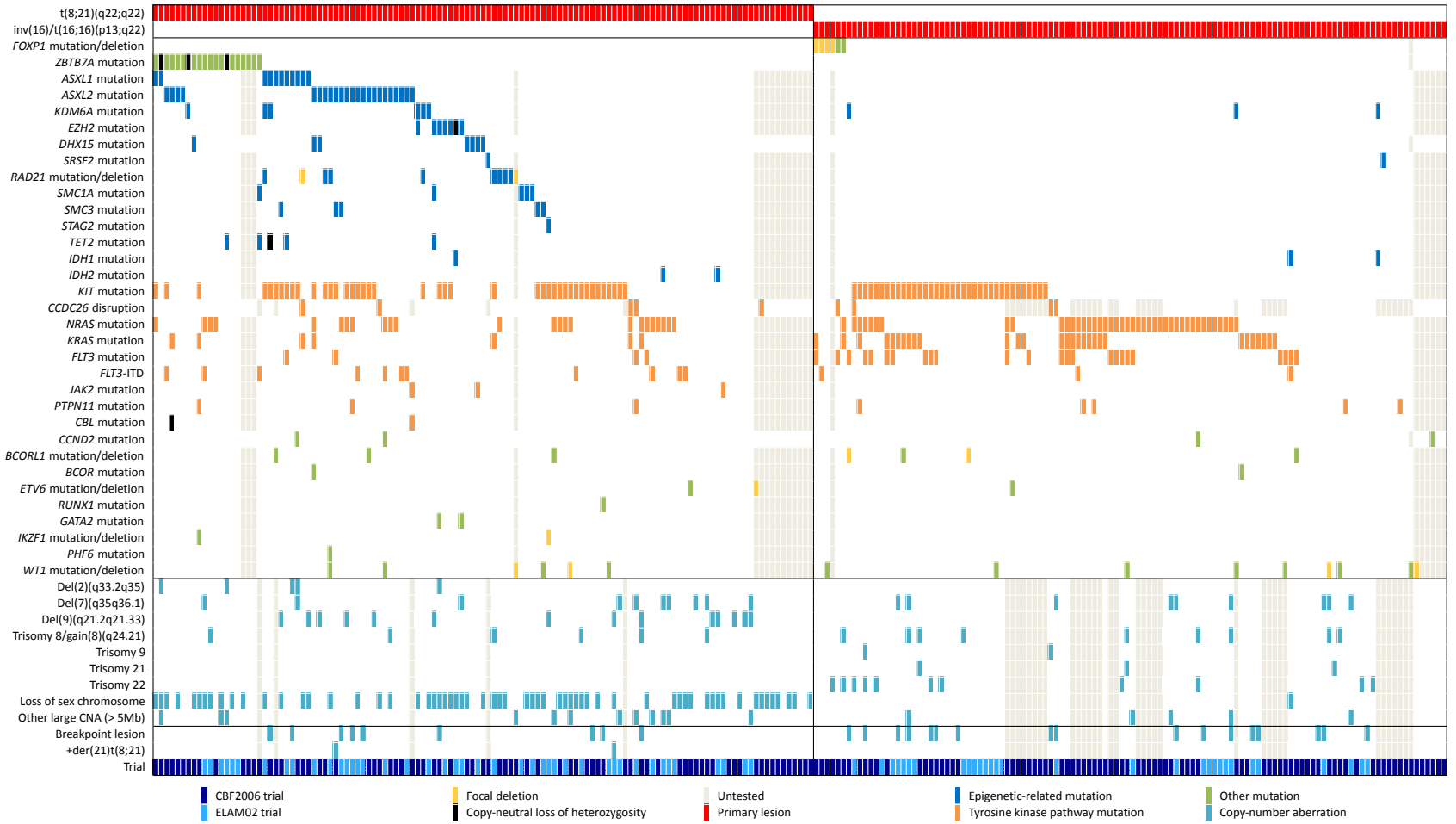


Figure 20 : Diagramme en barcode synthétisant l'ensemble des anomalies retrouvées par séquençage (haut-débit, Sanger) et SNP-array dans les LAM à core binding factor incluses dans les protocoles CBF2006 et ELAM02.

ARTICLE 4 : ANOMALIES ACQUISES CHEZ LES PATIENTS FPD/AML

Somatic mutations associated with leukemic progression of familial platelet disorder with predisposition to acute myeloid leukemia

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Résumé : La thrombopénie familiale avec prédisposition aux hémopathies malignes (FPD/AML) est un syndrome rare de transmission autosomique dominante lié à des mutations germinales du gène *RUNX1* (21q22). Environ un tiers des individus atteints développeront une leucémie aiguë, généralement après plusieurs années. L'acquisition d'événements secondaires paraît ainsi nécessaire au processus de transformation leucémique et constitue un modèle *in vivo* de leucémogénèse multi-étapes.

Nous avons étudié par séquençage haut-débit un total de 25 individus atteints de FPD/AML issus de 15 familles indépendantes identifiées entre 2005 et 2014. Dix d'entre eux avaient développé une LAM (de 6 à 60 ans) et 3 avaient développé une LAL-T (de 14 à 28 ans). L'acquisition d'un second événement était identifiée chez la totalité des individus ayant développé une leucémie aiguë. Ces événements impliquaient notamment les voies des récepteurs à tyrosine kinase, certains gènes de l'épigénétique et de la cohésine, du spliceosome ou des facteurs de transcription tel que *RUNX1* dont l'acquisition d'une seconde anomalie apparaît comme un événement majeur de la transformation en LAM mais pas en LAL-T. Parmi les 9 patients étudiés ayant développé une LAM, 6 avaient une mutation acquise du second allèle de *RUNX1* (dont 2 par perte d'hétérozygotie sans anomalie du nombre de copies) et 3 avaient une trisomie 21 acquise dupliquant le chromosome muté.

A l'inverse des LAM CBF, aucune mutation des gènes *ASXL1* et *ASXL2* n'était identifiée dans les LAM secondaires aux mutations germinales de *RUNX1*. Il n'était pas non plus retrouvé de mutations de *CDC25C,* lesquelles ont été rapportées avec une haute fréquence dans une cohorte japonaise de patients FPD/AML.

Somatic mutations associated with leukemic progression of familial platelet disorder with predisposition to acute myeloid leukemia

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LETTER TO THE EDITOR:

The familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML) is an autosomal dominant disorder characterized by germline *RUNX1* alterations. Missense, nonsense and frameshift mutations as well as intragenic duplications and large deletions of *RUNX1* (21q22) have been reported in less than 45 pedigrees. Transformation to acute leukemia (AL) occurs with an incomplete penetrance and at a variable age in FPD/AML patients, suggesting that germline *RUNX1* mutations alone are insufficient to induce AL. Thus this disorder represents a unique model to study leukemic progression. *RUNX1* mutation could be considered like an inherited 'first hit', leading to a preleukemic state, but additional genetic alterations are required for the formation of fully transformed leukemic cells. To date, the most frequent additional event implicates an acquired mutation in the second allele of *RUNX1*.[1] Despite this, recurrent mutations coinciding with transformation to AL are not yet well defined. Recently, somatic mutations in the cell cycle regulator *CDC25C* were identified in 53% of FPD/AML patients. *CDC25C* mutations appeared to occur in the preleukemic clone, and subsequent mutations in other genes, such as *GATA2*, coincided with leukemia progression.[2] Moreover, a number of somatic mutations have been recently described to significantly co-occur with somatic *RUNX1* aberrations in AML, including mutations in the Polycomb-associated genes *ASXL1/2* in t(8;21) AML, as well as in *RUNX1*-mutated AML.[3] Intriguingly, an acquired *ASXL1*

mutation has been also described in a FPD/AML patient who developed T-ALL.[4] Given these recent discoveries and the lack of recurrent mutations known to coincide with leukemic progression of FPD/AML patients, we explored the status of 44 AML-associated genes in 25 individuals from 15 FPD/AML pedigrees. Interestingly, we identified a second alteration of *RUNX1* in all patients who developed AML, in contrast to patients who developed T-ALL.

The FPD/AML patients were identified from 2005 to 2014 (**Supplementary Material**, **Supplementary Figure S1A**), and biological samples were collected after informed consent, in accordance with the Declaration of Helsinki. Genomic DNA was extracted from peripheral blood or bone marrow mononuclear cells, using standard procedures. Next-generation sequencing (NGS) was performed at thrombocytopenia stage if the patients were thrombocytopenic or at leukemia stage if they developed AL. For one patient, samples for both stages have been collected (patient 9). For patients who progressed to AL, samples were also collected at the time of complete remission to confirm acquired status of mutations. NGS was performed using a custom-designed 44 gene panel (MiSeq, Illumina, San Diego, CA, USA), including the entire coding region of *ASXL1, ASXL2, CDC25C, BCOR, BCORL1, BRAF, CSF3R, CALR, CBL, CEBPA, DNMT3A, ETV6, EZH2, FBXW7, FLT3, GATA1, GATA2, IDH1, IDH2, JAK2, JAK3, KIT, KRAS, MPL, NIPBL, NPM1, NOTCH1, NRAS, PHF6, PTEN, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, STAG2, TET2, TP53, U2AF1, WT1* and *ZRSR2*, with a median depth of 2692 ×. For *CDC25C*, the entire coding region was covered with a mean depth of 2633 × (range: 1837–3656). Hotspot locations at codons 233–234 described by Yoshimi et al.[2] were visually checked without any filters. Depth at these codons was 43000 for all patients. *FLT3* internal tandem duplication (*FLT3*-ITD) was detected as previously described.[5]

RUNX1 loss of heterozygosity was deducted from variant allele frequencies (VAF) found by NGS and standard cytogenetic analysis. For the patient 25, quality of DNA did not allow a good quantification of the VAF. For the patients 5, 7 and 24, NGS was not performed at AL stage owing to a lack of DNA. We report here the mutations already described for these patients.[1] Cytogenetic G-banding analysis was performed according to standard methods in each center. Comparative genomic hybridization array analysis was performed for patients 8 and 21 as previously described.[1,6]

Characteristics of the 25 FPD/AML patients studied here are reported in Table 1.[1,6–8] Of the 15 pedigrees, 8 were described for the first time. Germinal *RUNX1* mutations were heterogeneous with six missense mutations, one non-sense mutation, six frameshift mutations and two large deletions of *RUNX1*. Most mutations (8/13) were located in the Runt homology domain (DNA-binding domain), where the majority of *RUNX1* mutations in FPD/AML are reported (**Supplementary Figure S1B**).[9] Of the 25 patients, 13 patients developed AL, 10 AML and 3 T-ALL, attesting to the importance of

RUNX1 mutations to the myeloid as well as T-lymphoid lineage. The age of AML onset was heterogeneous (6–60 years), whereas the patients who developed T-ALL were younger (14–28 years).

Mutational analysis identified a second leukemogenic event in all patients who developed AL. Consistent with our previous report,[1] we identified a second aberration of *RUNX1* in all AML patients, for whom material was available (9/10). Of the nine patients, six have a mutation in the second allele, including two by copy-neutral loss of heterozygosity, and three have a duplication of the *RUNX1*-mutated or -deleted chromosome (**Figures 1a and b**). These data highlight the importance of *RUNX1* dosage in AML development. Analysis of the variant allelic frequency (**Supplementary Table S1**) was critical in identifying copy-neutral loss of heterozygosity, as well as duplication of the *RUNX1*-mutated allele. In contrast to AML transformation of FPD/AML, most *RUNX1* mutations in sporadic AML are monoallelic.[10] These results suggest that FPD/AML should be suspected in the diagnosis of AML in any patient with a *RUNX1* biallelic mutation or with a single *RUNX1* mutation with a VAF>50%, which could indicate trisomy 21 with a duplication of the mutated chromosome or copy-neutral loss of heterozygosity. Routine analysis of *RUNX1* mutant allele ratios may greatly facilitate identification of FPD/AML patients in the clinical setting. These findings in FPD/AML are analogous to what has been observed in familial MDS/AML owing to *CEBPA* mutations or in recently described familial MDS/AML with mutations in the RNA helicase *DDX41*, where biallelic mutations have been reported at AML transformation.[11,12] Subsequent germline *CEBPA* mutations have been found in 10% of a cohort of AML patients harboring biallelic *CEBPA* mutations. Interestingly, no additional *RUNX1* mutations were found in patients who developed T-ALL.

Additional mutations identified at the AL stage were heterogeneous and affected genes recurrently implicated in leukemogenesis. This included mutations affecting signaling intermediates (*FLT3, KRAS, KIT, MPL, CBL, NOTCH1*), tumor suppressors (*TP53, WT1, PHF6, BCORL1*), cohesins (*RAD21*), splicing proteins (*SRSF2, SF3B1*) or DNA methylation (*TET2, DNMT3A*) (**Table 1, Figure 1a**).[13] A possibility for this heterogeneity is that, as low levels of wild-type *RUNX1* cause genetic instability in FPD/AML,[14] the cells are more prone to acquire mutations. Analysis of the allelic frequency for each somatic mutation reveals that, in most of the cases, the second *RUNX1* alteration is one of the initial events for leukemia progression (**Supplementary Table S1**, **Figure 1c**). None of these additional mutations were found in samples at complete remission (available for 10/13 patients), attesting to their role in leukemogenesis.

In contrast to the AL stage, no additional mutations were found in patients at the thrombocytopenic stage, except for one patient with a *DNMT3A* mutation and another with a *KRAS* mutation. Interestingly, this last patient (patient 9) developed AML and analysis of samples from both stages revealed

that the minor clone with the *KRAS* mutation (4%) expanded at the leukemic stage with a VAF at 45% (**Supplementary Table S1**).

Surprisingly, no association between germline *RUNX1* mutation and *ASXL1/2* mutations were found. We also did not identify *CDC25C* or *GATA2* mutations in these pedigrees. This is intriguing given that *CDC25C* mutations were recently identified in 7/13 FPD/AML patients, including 4 at AML stage and 3 at thrombocytopenia stage.[2] One possible explanation could be the geographic origin of the patients, as the patients described with *CDC25C* and *GATA2* mutations were from Japan and the majority of the patients described here are from France. Additional studies will be needed to corroborate this hypothesis and evaluate more precisely the frequency of *CDC25C* and *GATA2* mutations in FPD/AML.

In this study, we analyzed a large cohort of FPD/AML patients for a panel of genes recurrently mutated in AML, including many genes never studied previously in the context of FPD/AML, and identified genetic events co-existing with germline *RUNX1* mutations in all patients at AL stage. These events implicate signaling, RNA splicing or epigenetic regulation genes, reflecting the heterogeneity of additional mutations with AL progression in FPD/AML. Moreover, a second acquired aberration of *RUNX1* was associated with AML progression in all patients, suggesting that biallelic *RUNX1* alterations are crucial for AML development.

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AUTHORSHIP: N Duployez, MB, SG, AR and CP performed genetic analysis and analyzed mutational data. IA-D, N Duployez, NB and CP conceptualized the idea, designed the research and analyzed data. N Dhédin, DR, BN, CB, TL, M-JM, RF, P-GH, HR, VL-C and CP provided samples and data. IA-D, N Duployez, J-BM, OA-W and CP wrote the manuscript, which was approved by all the authors.

CONFLICT-OF-INTEREST DISCLOSURE: The authors declare no conflict of interest.

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FIGURES AND TABLES

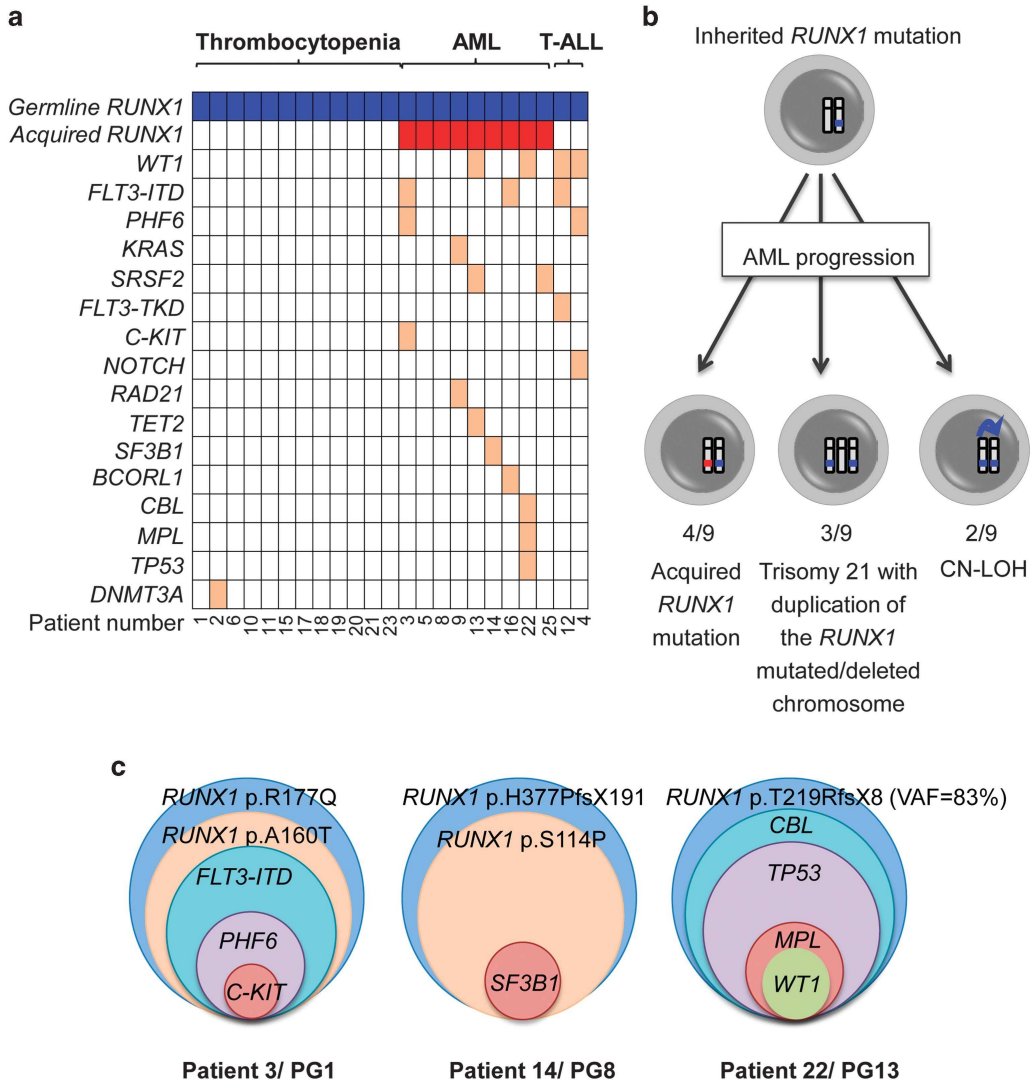


Figure 1: Associated mutations in FPD/AML patients. **(a)** Aberrations of *RUNX1* and associated mutations in FPD/AML patients at all stages. Germline *RUNX1* aberrations (mutations and deletions) are represented in blue and acquired *RUNX1* aberrations (second mutation, mutation by copy-neutral loss of heterozygosity (CN-LOH) and duplication of *RUNX1*-mutated or -deleted chromosome) in red. Other acquired mutations are represented in orange. Twenty-three patients are represented; the patients 7 and 24 have been excluded because of a lack of material at T-ALL and AML diagnosis, respectively. The patient 9 is represented at AML stage. **(b)** Acquired *RUNX1* secondary events in FPD/ AML patients who developed AML. Of the 10 patients who developed AML, DNA was available for 9. Of these 9, all had a second somatic event affecting the other allele of *RUNX1*: 4 had a novel acquired *RUNX1* mutation, 3 had trisomy 21 with duplication of the *RUNX1*-mutated or -deleted chromosome and 2 harbor a second mutation by CN-LOH. *RUNX1* germline mutation is represented in blue, *RUNX1* acquired mutation in red. **(c)** Schematic representation of mutations found in FPD/AML patients who developed AML. The circles are proportional to the variant allelic frequency found for each mutation (values in Supplementary Table S1). Representative examples of three patients are shown (patient 3 from pedigree 1, patient 14 from pedigree 8, patient 22 from pedigree 13) and highlight that the second aberration of RUNX1 is an early event in leukemia transformation. PG, pedigree; VAF, variant allelic frequency.

Table1: Characteristics of FPD/AML patients

Patient	*Pedigree*	*Sex*	*Germline RUNX1 alteration*	*Age at AL*	*AL*	*Acquired RUNX1 anomalies*	*Other acquired anomalies identified*
1	1/brother	M	p.R177Q	NA	No	No	No
2	1/brother	M	p.R177Q	NA	No	No	DNMT3A[a]
3	1/proband	M	p.R177Q	60	AML	p.A160T	FLT3-ITD, PHF6, KIT
4	1/son	M	p.R177Q	28	T-ALL	No	PHF6, WT1, NOTCH1
5	2/proband	F	p.Q308RfsX259	55	AML	p.G138PfsX12	ND
6	2/daughter	F	p.Q308RfsX259	NA	No	No	No
7	2/daughter	F	p.Q308RfsX259	24	T-ALL t-AML 5y later	ND No	ND KRAS, t(1;3)(p36;q26)
8	3	F	Complete deletion of *RUNX1*	12	AML	Duplication of *RUNX1*-deleted chromosome	No
9	4	F	p.R139X	48	AML	p.R139X (CN-LOH)	KRAS[a], RAD21
10	5/proband	M	p.P218S	NA	No	No	No
11	5/identical twin	M	p.P218S	NA	No	No	No
12	6	M	p.G108V	14	T-ALL	No	WT1, FLT3-ITD, FLT3-TKD
13	7	F	p.D305TfsX262	37	AML	Duplication of the *RUNX1*-mutated chromosome	SRSF2, WT1, TET2
14	8	F	p.H377PfsX191	12	AML	p.S114P	SF3B1
15	9	F	p.G108V	NA	No	No	No
16	10	M	p.G143RfsX43	36	AML	p.K83Q	BCORL1, FLT3-ITD
17	11/proband	M	p.T169R	NA	No	No	No
18	11/daughter	F	p.T169R	NA	No	No	No
19	11/daughter	F	p.T169R	NA	No	No	No
20	11/son	M	p.T169R	NA	No	No	No
21	12	F	Complete deletion of *RUNX1*	NA	No	No	No
22	13	F	p.T219RfsX8	43	AML	p.T219RfsX8 (CN-LOH)	CBL, MPL, TP53, WT1, del(11)(q21)
23	14/proband	F	p.T121HfsX9	NA	No	No	No
24	14/son	M	p.T121HfsX9	6	AML	ND	ND
25	15	F	p.A129E	42	AML	Duplication of the *RUNX1*-mutated chromosome	SRSF2

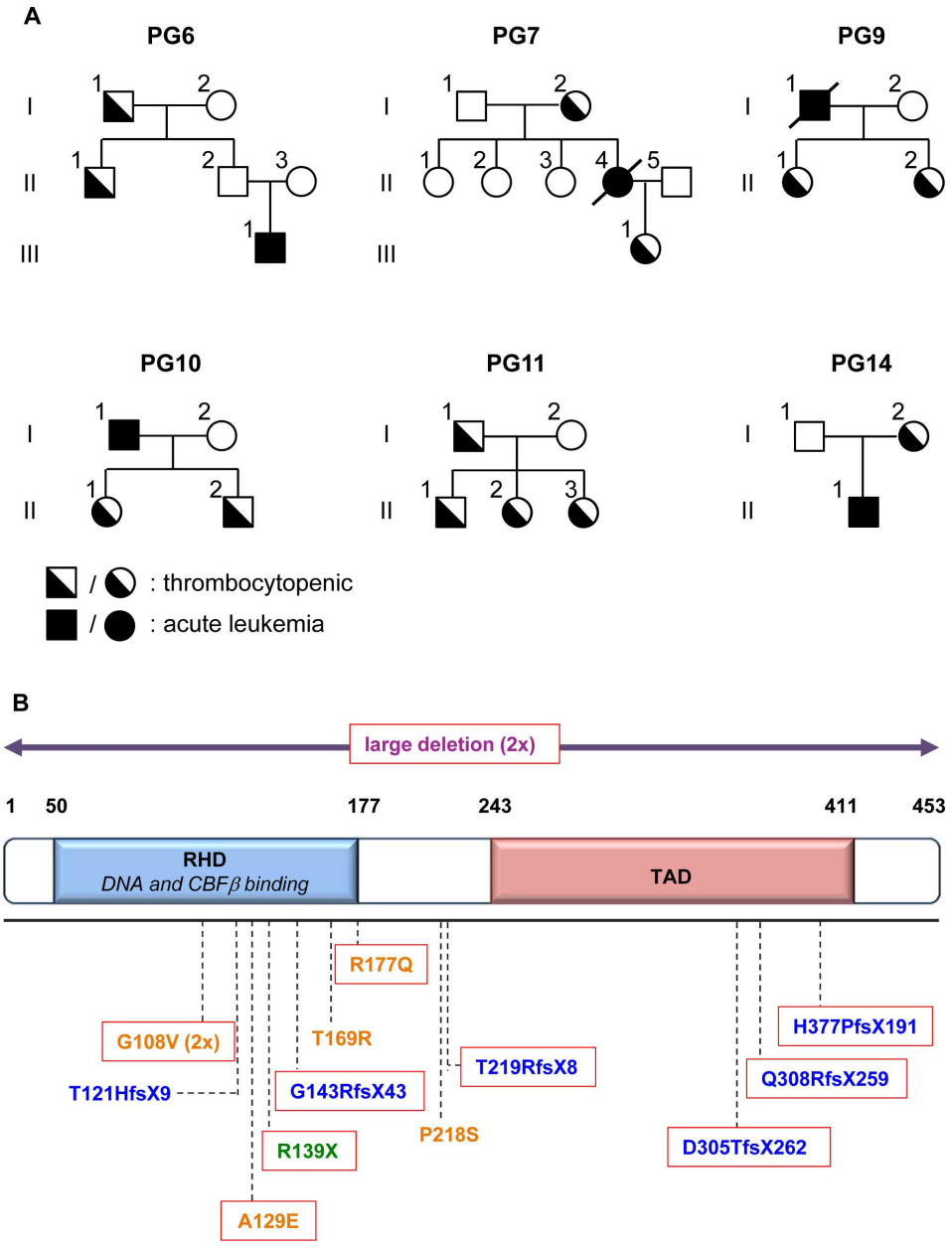
Abbreviations: AML, acute myeloid leukemia; AL, acute leukemia; CN-LOH, copy-neutral loss of heterozygosity; F, female; *FLT3-ITD, FLT3* internal tandem duplication; *FLT3-TKD, FLT3* tyrosine kinase domain mutation ; M, male; NA, not applicable; ND, not determined; T-ALL, T-acute lymphoblastic leukemia; t-AML: therapy-related AML. [a]Acquired mutation at thrombocytopenic stage.

SUPPLEMENTAL DATA

Supplementary Figure 1: Characteristic of FPD/AML pedigrees studied.

(A) Family trees of the newly described pedigrees (PGs). PGs 6, 7, 9, 10, 11, 14 are represented.

(B) Gene diagram depicting RUNX1 anomalies in the FPD/AML pedigrees. RUNX1 isoform b is represented. Abbreviations: RHD: Runt homology domain, TAD: transactivation domain. In blue: frameshift mutation, in orange: missense mutation, in green: nonsense mutation, red square: mutations found to predispose to leukemia development in FPD/AML patients.



Supplementary Table 1: Variant allelic frequency for mutated genes in FPD/AML patients who developed acute leukemia.

Cf. version online.

CONCLUSION

Ce travail de thèse a mis en lumière le large spectre d'anomalies coopératrices intervenant dans les LAM impliquant le CBF. Indispensable au développement de l'hématopoïèse définitive, le *core binding factor* est l'un des éléments les plus fréquemment dérégulés en hématologie maligne via divers mécanismes incluant mutations, délétions, translocations ou amplifications. Dans la plupart des situations, l'altération du CBF constitue l'événement primitif initiateur de la leucémie. Néanmoins, qu'elle soit acquise (LAM CBF) ou constitutionnelle (FPD/AML), la seule altération du CBF est insuffisante au développement de la leucémie. Un temps de latence de plusieurs mois à plusieurs dizaines d'années est nécessaire pour permettre l'acquisition d'événements coopérateurs et l'expansion du clone leucémique (leucémogénèse multi-étapes) [143,144,154]. D'autre part, il existe une grande hétérogénéité phénotypique et pronostique entre ses différentes entités. En particulier, bien que les LAM CBF soient associées à un pronostic relativement favorable, l'incidence des rechutes atteint 30-40% dans ces pathologies. L'identification des patients à risque de rechute est primordiale afin d'adapter ou d'intensifier les traitements. Chez les patients FPD/AML, l'enjeu est de prédire au mieux la transformation leucémique.

Dans les LAM avec réarrangement acquis du CBF (LAM CBF), nous avons démontré la grande hétérogénéité moléculaire, déjà entrevue par la découverte des mutations d'*ASXL2* [133], distinguant les LAM avec inv(16) des LAM avec t(8;21). Si les deux sous-types ont en commun la grande fréquence des anomalies de type tyrosine kinase (*KIT*, *RAS*, *FLT3*, etc.), les LAM avec t(8;21) sont caractérisées par des altérations moléculaires qui leurs sont hautement spécifiques. Les mutations des gènes de la cohésine (*SMC1A, SMC3, RAD21, STAG2*) ou du remodelage chromatinien (*ASXL2, ASXL1, EZH2, KDM6A*) concernent 18% et 41% des LAM avec t(8;21) de notre cohorte tandis qu'elles sont exceptionnelles dans les autres catégories de LAM, y compris les LAM avec inv(16). Depuis la publication de ce travail, ces données ont été confirmées par les groupes allemand [155], américains [149,151] et canadien [46]. Ces travaux mettent en avant l'intérêt d'étudier des sous-groupes homogènes de patients et identifient une voie pathologique unique aux LAM avec t(8;21).

Récemment, Jean-Baptiste Micol et *al* ont exploré l'association spécifique des mutations d'*ASXL2* et de la fusion *RUNX1-RUNX1T1* (résumé présenté en annexe de cette thèse). La protéine ASXL2 apparaît comme un élément indispensable de l'auto-renouvellement des CSH. La perte d'*ASXL2* est associée à une augmentation de l'accessibilité de la chromatine sur certains loci impliqués en leucémogénèse tels que les gènes *HOXA*. De manière intéressante, les gènes dérégulés par l'inactivation d'*ASXL2* recoupent les cibles transcriptionnelles de *RUNX1* et *RUNX1-RUNX1T1*. Ces données suggèrent un rôle d'ASXL2 comme promoteur de la leucémogenèse en présence de la protéine de fusion RUNX1-RUNX1T1. Ceci explique, au moins en partie, l'absence de mutation d'*ASXL2* dans les LAM avec mutations de *RUNX1* (germinale ou somatique) où la capacité transcriptionnelle de RUNX1 est

abolie ou diminuée. Concernant les mutations de la cohésine, Claire Mazumdar et *al* ont montré qu'elles étaient associées à une augmentation de l'accessibilité de la chromatine au niveau des gènes cibles de RUNX1 mais également de GATA2 et ERG [23]. La cohésine est un complexe multi-protéique hautement conservé à travers les espèces, initialement décrit comme intervenant dans la ségrégation des chromatides sœurs au cours de la division cellulaire mais l'effet leucémogène des mutations est plus probablement lié à la régulation de la transcription de gènes clés de l'hématopoïèse. Les mutations des gènes de la cohésine sont décrites dans l'ensemble des hémopathies malignes dont 12% de l'ensemble des LAM [156]. De manière intéressante, l'expression de *RUNX1* chez le poisson-zèbre est dépendante de l'expression de la cohésine [157]. Dans la lignée Kasumi-1 (lignée t(8;21) mutée *RAD21*), l'expression forcée de *RAD21* sauvage est associée à une baisse significative de la prolifération cellulaire [156]. Enfin, des interactions étroites entre système de la cohésine et protéines polycomb (dont les protéines ASXL) ont été décrites, renforçant l'idée d'un rôle de ce complexe dans le remodelage chromatinien [158]. De manière intéressante, nous avons montré que la coexistence des mutations des gènes de la cohésine et/ou du remodelage chromatinien avec les mutations de type tyrosine kinase était associée à un pronostic défavorable dans les LAM avec t(8;21).

Par ailleurs, ce travail a également mis en évidence l'intervention d'autres classes d'altérations ciblant notamment la glycolyse (*ZBTB7A*) et l'épissage des pré-ARN messagers (*DHX15*) dans les LAM avec t(8;21) mais aussi les délétions et mutations perte-de-fonction du gène *FOXP1* dans les LAM avec inv(16), lequel est notamment impliqué dans la différenciation monocytaire [159]. Enfin, l'étude en SNP-array a également révélé l'existence d'amplifications ciblées du gène *CCDC26* ayant pour conséquence sa désorganisation. Bien que la fonction et la nature de *CCDC26* restent ambiguës, nos données préliminaires, couplées aux données de la littérature [160], suggèrent que son altération pourrait venir compléter le spectre des anomalies de type tyrosine kinase impliquées dans les LAM CBF.

Chez les patients atteints de FPD/AML, la transformation en LAM s'accompagne systématiquement (dans notre cohorte) de l'apparition d'une seconde anomalie impliquant *RUNX1*, soit par mutation acquise du second allèle, soit par duplication de la mutation germinale (via trisomie 21 acquise ou perte d'hétérozygotie sans anomalie du nombre de copies). En se basant sur l'étude des ratios alléliques, il apparaît que cet événement est le plus souvent à l'origine du clone fondateur, les autres anomalies acquises étant classiquement sous-clonales. En pratique clinique, ces résultats ont des implications directes dans l'aide à l'identification des patients atteints de FPD/AML au décours d'un diagnostic de LAM. Ainsi, l'identification chez un patient souffrant de LAM, de deux mutations de *RUNX1* ou d'une mutation unique à une fréquence allélique supérieure à 50% (reflétant la duplication de l'allèle muté) doit faire discuter la possibilité d'un FPD/AML. Ceci est d'autant plus important

si une greffe intrafamiliale de CSH est envisagée. Bien que plus rare, la transformation en LAL-T n'était en revanche pas associée à l'acquisition d'une deuxième anomalie de *RUNX1* dans notre cohorte.

Récemment, des mutations acquises du gène *CDC25C* (codant une phosphatase impliquée dans la régulation de la division cellulaire) ont été rapportées par le groupe japonais chez environ la moitié des patients atteints de FPD/AML au moment de la transformation en LAM [161]. Les mutations de *CDC25C* étaient associées à une augmentation de l'entrée en cycle des cellules et correspondaient à un événement précoce de la transformation, rapidement suivi par l'apparition d'autres mutations, en particulier celles de *GATA2*. Néanmoins, aucune mutation de *CDC25C* ou de *GATA2* n'a été identifiée dans notre travail, de même que dans l'étude du groupe américain [154] pouvant refléter les différences ethniques entre les séries.

Concernant la prise en charge des patients atteints de FPD/AML en l'absence de leucémie aiguë et dans l'état actuel des connaissances, il n'existe pas de marqueur suffisamment robuste permettant d'identifier les patients à risque de transformation. Aussi, en l'absence de données cinétiques et de consensus sur les modalités d'intervention thérapeutique, une recherche plus systématique et régulière d'anomalies génétiques acquises ne semble pas justifiée. Actuellement, il paraît licite de proposer une simple surveillance par numération-formule sanguine et de réserver le myélogramme et l'étude génétique (caryotype, biologie moléculaire) aux patients présentant des cytopénies. Dans tous les cas, cette surveillance doit être réalisée dans le cadre du conseil génétique tout en considérant la complexité des antécédents personnels et familiaux des individus.

En conclusion, les LAM impliquant le CBF sont caractérisées par la haute fréquence et la récurrence d'anomalies additionnelles acquises de manière non aléatoire. Le développement des LAM au niveau cellulaire peut être considéré comme un processus darwinien impliquant la sélection de cellules variant génétiquement dans le cadre d'une écologie microenvironnementale complexe [162]. La dérégulation du CBF constitue par ailleurs un facteur d'instabilité génétique important et s'associe à des défauts de réparation de l'ADN favorisant ainsi l'acquisition d'anomalies génétiques [163]. Les techniques actuelles d'étude du génome des LAM, en particulier le séquençage à haut débit, permettent d'apprécier la diversité génétique intraclonale (sous-clones et clones minoritaires), substrat essentiel de l'évolution/sélection clonale, de la progression de la maladie et le cas échéant des rechutes. Ainsi, si dans certains cas l'architecture présumée semble obéir à un modèle linéaire d'acquisition d'événements additionnels, une hétérogénéité sous-clonale plus marquée est retrouvée chez une grande partie des patients de notre cohorte avec l'existence de plusieurs anomalies d'une même classe (essentiellement de type tyrosine kinase) supposées appartenir à des clones différents et déri-

ver d'un clone ancestral commun. Parmi les perspectives de ce travail figure l'étude à l'échelle unicellulaire de la hiérarchie clonale des LAM CBF dont les premiers essais tendent à montrer que les mutations de type tyrosine kinase présentes chez un même patient sont portées par des clones différents. Enfin, des études fonctionnelles seront nécessaires afin de comprendre comment les anomalies identifiées dans la présente étude peuvent influencer la physiopathologie de ces LAM et éventuellement permettre le développement de nouvelles pistes thérapeutiques.

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ANNEXES

ASXL2 is essential for hematopoiesis and acts as a haploinsufficient tumor suppressor in leukemia

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ABSTRACT: Additional Sex Combs-Like (ASXL) proteins are mammalian homologs of Additional sex combs (Asx), a regulator of Trithorax and Polycomb function in *Drosophila.* While there has been great interest in *ASXL1* due to its frequent mutation in leukemia, little is known about its paralog *ASXL2*, which is frequently mutated in acute myeloid leukemia patients bearing the *RUNX1-RUNX1T1* (*AML1-ETO*) fusion. Here we report that ASXL2 is required for normal hematopoiesis with distinct, non-overlapping effects from ASXL1 and acts as a haploinsufficient tumor suppressor. While *Asxl2* was required for normal hematopoietic stem cell self-renewal, Asxl2 loss promoted AML1-ETO leukemogenesis. Moreover, ASXL2 target genes strongly overlapped with those of RUNX1 and AML1-ETO and ASXL2 loss was associated with increased chromatin accessibility at putative enhancers of key leukemogenic loci. These data reveal that *Asxl2* is a critical regulator of hematopoiesis and mediates transcriptional effects that promote leukemogenesis driven by AML1-ETO.

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RÉSUMÉ

ÉTUDE DES ALTÉRATIONS GÉNOMIQUES ACQUISES DANS LES LEUCÉMIES AIGUËS MYÉLOÏDES IMPLIQUANT LE CORE BINDING FACTOR

Les gènes *RUNX1* et *CBFB* codent pour les sous-unités du core binding factor (CBF), facteur de transcription hétérodimérique essentiel de l'hématopoïèse définitive. La dérégulation du CBF est l'une des anomalies les plus fréquemment rencontrées dans les hémopathies malignes. Puisque la perturbation seule du CBF est insuffisante au développement d'une leucémie aiguë myéloïde (LAM), les LAM impliquant le CBF sont considérées comme des modèles de leucémogénèse multi-étapes, nécessitant la coopération d'anomalies génétiques additionnelles.

Dans ce travail, nous nous sommes intéressés aux LAM de type CBF, caractérisées soit par une t(8;21)/fusion *RUNX1-RUNX1T1* soit par une inv(16)/fusion *CBFB-MYH11*, ainsi qu'aux LAM avec mutations germinales de *RUNX1* (définissant la thrombopénie familiale avec prédisposition aux leucémies aiguës ou FPD/AML). Afin d'identifier des anomalies additionnelles, nous avons étudié les prélèvements de patients atteints de LAM CBF inclus dans les essais français ELAM02 (0-18 ans) et CBF2006 (18-60 ans) par séquençage à haut débit (n=215) et single nucleotide polymorphism-array (n=198). Les échantillons de 25 individus atteints de FPD/AML (issus de 15 familles), diagnostiqués entre 2005 et 2014, ont également été séquencés au stade thrombopénique et au moment de la transformation en leucémie aiguë.

Dans les LAM CBF, les mutations activatrices des voies tyrosines kinases (TK) sont les événements les plus fréquents quel que soit le sous-type de LAM CBF, comme cela a déjà été rapporté dans d'autres études. En revanche, les mutations affectant les gènes du remodelage chromatinien ou du complexe de la cohésine sont identifiées à des fréquences élevées (41% et 18% respectivement) dans les LAM avec t(8;21) tandis qu'elles sont pratiquement absentes dans les LAM avec inv(16). Dans les LAM avec t(8;21), la coexistence de ces mutations avec les mutations de type TK est associée à un pronostic défavorable suggérant une synergie entre ces événements. D'autres événements fréquemment retrouvés incluent les mutations de *ZBTB7A* et *DHX15* dans les LAM avec t(8;21) (20% et 6% respectivement) et les délétions/mutations de *FOXP1* dans les LAM avec inv(16) (7%). Enfin, nous avons décrit la perturbation de *CCDC26* comme une possible lésion associée à une signalisation aberrante des TK dans les LAM CBF (4,5% des cas).

Dans les FPD/AML, l'analyse mutationnelle a révélé l'acquisition d'un deuxième événement impliquant *RUNX1* chez tous les patients ayant développé une LAM. Ce deuxième événement correspondait soit à une mutation somatique du second allèle de *RUNX1* soit à la duplication de la mutation germinale de *RUNX1* (par perte d'hétérozygotie sans anomalie du nombre de copies ou trisomie 21 acquise). En pratique clinique, cela suggère que la présence de deux mutations différentes de *RUNX1* ou d'une seule mutation avec un ratio allélique supérieur à 50% chez un patient atteinte de LAM doit alerter sur la possibilité d'un syndrome FPD/AML sous-jacent.

ABSTRACT

ACQUIRED GENOMIC ABERRATIONS IN ACUTE MYELOID LEUKEMIA WITH CORE BINDING FACTOR INVOLVEMENT

RUNX1 and *CBFB* encode subunits of the core binding factor (CBF), a heterodimeric transcription factor required for the establishment of definitive hematopoiesis. Deregulation of the CBF is one of the most frequent aberrations in hematological malignancies. Since CBF disruption alone is insufficient to induce acute myeloid leukemia (AML) on its own, AML with CBF involvement is considered as a model of multistep leukemogenesis requiring additional genetic aberrations.

Here, we focused on acute myeloid leukemia (AML) with t(8;21)/*RUNX1-RUNX1T1* fusion and AML with inv(16)/*CBFB-MYH11* fusion, reported together as CBF AML, as well as AML with germline *RUNX1* mutation (defining the familial platelet disorder with propensity to develop leukemia or FPD/AML).

In order to explore additional genomic aberrations, we performed comprehensive genetic profiling in CBF AML patients enrolled in the French trials ELAM02 (0-18 years) and CBF2006 (18-60 years) using both high-throughput sequencing (n=215) and single nucleotide polymorphism-array (n=198). In addition, we sequenced samples from 25 individuals with FPD/AML (15 pedigrees) diagnosed between 2005 and 2014 at thrombocytopenic stage and during leukemic progression.

In CBF AML, mutations in genes activating tyrosine kinase (TK) signaling were frequent in both subtypes as previously described by others. By contrast, we found mutations in genes encoding chromatin modifiers or members of the cohesin complex with high frequencies in t(8;21) AML (41% and 18% respectively) while they were nearly absent in inv(16) AML. Interestingly, such mutations were associated with a poor prognosis in patients with TK mutations suggesting synergic cooperation between these events. Other events included *ZBTB7A* and *DHX15* mutations in t(8;21) AML (20% and 6% respectively) and *FOXP1* deletions or truncating mutations in inv(16) AML (7%). Finally, we described *CCDC26* disruption as a possible new lesion associated with aberrant TK signaling in this particular subtype of leukemia (4.5% of CBF AML).

In FPD/AML, mutational analysis revealed the acquisition of a second event involving *RUNX1* in all patients with AML including somatic mutation of the second allele or duplication of the germline *RUNX1* mutation through copy-neutral loss of heterozygosity and trisomy 21. In clinical practice, we suggest that the occurrence of two different *RUNX1* mutations or a *single* RUNX1 mutation with a variant allele frequency higher than 50% in a patient with AML should alert about the possibility of FPD/AML.