Gene Expression Profiling of Adult t(4;11)(q21;q23)-associated Acute Lymphoblastic Leukemia Reveals a Different Signature from Pediatric Cases

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Abstract. *Chromosomal rearrangements involving the mixed-lineage leukemia (MLL) gene, located at chromosomal band 11q23, result in the generation of in-frame fusion transcripts with various partner genes from more than 60 distinct gene loci. Among them, the MLL/AFF1 (AF4/FMR2 family, member 1) fusion, associated with rearrangements between bands 4q21 and 11q23 is a recurrent event in pre-B acute lymphoblastic leukemia (ALL). Gene expression profiling (GEP) was performed for four adult patients with ALL. Their signatures were compared to those of ALL patients with a fusion gene involving c-abl oncogene 1, non-receptor tyrosine kinase (ABL1). The comparison of MLL–AFF1 cases with the ABL1 group identified 477 genes being differentially expressed at the statistically significant level of p<0.05, with 296 and 181 genes up- and down-regulated, respectively, in the MLL–AFF1 cases. Three GEP studies on t(4;11)(q21;q23) focusing on the age group of the patients have been reported in the literature. Different expression profiles based on the levels of the homeobox A (HOXA) signature were identified. Although comparison between studies is difficult because of differences in the microarrays and the control samples used, our results and those from the literature suggest that cells carrying t(4;11)(q21;q23) use different pathways to lead to leukemogenesis. Therefore, t(4;11)-associated ALL could represent different biological entities.*

Chromosomal rearrangements involving the mixed-lineage leukemia (*MLL*) gene (also known as *ALL-1*, *HRX*, and *HTRX1*), located at the chromosomal band 11q23, have been associated with many different types of hematological malignancies (1-3). They include reciprocal translocations, inversions and segmental insertions. These rearrangements result in the generation of in-frame fusion transcripts with various partner genes from more than 60 distinct gene loci (2, 4).

Microarray gene expression profiling (GEP) is well-suited to identify gene expression signatures associated with distinct clinical subtypes of leukemia and to reveal characteristic signatures of activation and/or silencing of multiple genes that reflect the underlying biology of the disease subtypes (5-7). Indeed, gene expression studies of leukemia have shown that recurrent chromosomal translocations found in leukemia cells indeed characterize unique diseases (8-11).

Although the function of the various MLL fusion proteins is still poorly understood, it appears that they disrupt the ability of wild-type MLL to regulate Homeobox (*HOX*) gene expression (12, 13). In the past 10 years, GEP has shown that acute leukemia associated with *MLL* rearrangements has a distinct signature, allowing it to be differentiated from those without *MLL* rearrangements (8, 13-16). However, whether specific fusion genes have distinct gene expression signatures remains controversial.

In the past 15 years, 27 patients with an *MLL* fusion gene were identified at the cytogenetic laboratory of the Brest University Hospital (17). High-quality RNA suitable for GEP was available from four of the seven adults with an *MLL–AFF1* fusion gene associated with a rearrangement involving bands 4q21 and 11q23; they are the subject of this report.

Patients and Methods

Patients. Seven patients with pre-B acute lymphoblastic leukemia (ALL) were found to have an *MLL–AFF1* fusion gene (Table I). The fusion resulted from a t(4;11)(q21;q23) in five patients and an insertion in two patients.

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Table I. *Cytogenetic and molecular data of 7 patients with the MLL–AFF1 fusion gene.*

Patient	Gender	Age (years)	Banding cytogenetics	Revised cytogenetics (FISH)	Fusion gene	GEP
P13	F	68	46, XX, t(4;11)(q21;q23)[23]	t(4;11)(q21;q23)	*MLL/AFF1*	
P14	M	17	46, XY, t(4;11)(q21;q23)[14]/46, XY[1]	t(4;11)(q21;q23)	*MLL/AFF1*	X
P15	F	69	46, XX, ins(4;11)(q21;q13q23)[3]/46, idem, der(22)[18] ATG16L2/MLL	ins(4;11)(q21;q13q23)	*MLL/AFF1*	
P17	F	41	46, XX, t(4;11)(q21;q23)[20]/46, XX[1]	t(4;11)(q21;q23)	*MLL/AFF1*	
P18	M	45	46, XY, t(4;11)(q21;q23)[3]/46, idem, i(7)(q10)[19]	t(4;11)(q21;q23)	*MLL/AFF1*	X
P23	M	70	47, XY, +X, t(4;11)(q21;q23)[23]/46,XY[1]	t(4;11)(q21;q23)	*MLL/AFF1*	X
P28	F	65	46, XX, del(11)(q23)[3]/46, XX[30]	ins(4;11)(q21;q23),del(11)(q23->qter)	*MLL/AFF1*	
					Not determined	X

Patient numbers refer to the paper by De Braekeleer *et al*. (2011) (17). FISH: Fluorescent *in situ* hybridization; GEP: gene expression profile.

Four patients with B-cell ALL associated with a breakpoint cluster region–c-abl oncogene 1, non-receptor tyrosine kinase (*BCR–ABL1*) fusion gene resulting from a t(9;22)(q34;q11) and two with B-cell ALL (studied in duplicate) with t(1;9)(q24;q34) leading to a RCSD domain containing 1–c-abl oncogene 1, non-receptor tyrosine kinase (*RCSD1–ABL1*) gene, served as controls. They are referred to as the *ABL1* group.

Methods. Total RNA was extracted from bone marrow or peripheral blood mononuclear cells using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's guidelines and quantified on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA).

The integrity of the extracted RNA was assessed on a Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the RNA integrity number (RIN) was determined. By Illumina (San Diego, CA, USA) criteria, samples were required to have a RIN >7. Out of the five patients for whom cells were available, only four *MLL–AFF1* samples had a RIN higher than seven and were analyzed.

High-quality RNA was reverse-transcribed using T7-linked oligo-dT primers, and the obtained cDNA was used as a template to synthesize biotinylated cRNA. Labeled cRNA was then fragmented and hybridized to Human HT-12 v3 Expression BeadChips (Illumina), according to the manufacturer's instructions. Each array contains 48,803 probes, of which 27,455 are coding transcripts, 7,870 transcripts from genomic contigs (RefSeq Content, Build 36.2, Release 22) and 12,837 Expressed Sequence Tags (EST) (UniGene, Build 199). Following washing and staining, the BeadChips are imaged using an Illumina BeadArray Reader to measure the fluorescence intensity at each probe.

BeadChip data files were analyzed with GenomeStudio (Illumina) gene expression module and Bioconductor package to determine gene expression signal levels. Briefly, the raw intensity of Illumina Human HT-12 v3.0 gene expression array was scanned and extracted using BeadScan, with the data corrected by background subtraction in GenomeStudio module. The intensity data were then subjected to quantile normalization.

The ArrayMiner 5.3 software (Optimal Design, Brussels, Belgium http://www.optimaldesign.com/ArrayMiner/ArrayMiner.htm) was used to compare the gene expression profiles between *MLL–AFF1* and *ABL1* groups. The ClassMarker module (Optimal Design, Brussels, Belgium) was used to identify genes that can differentiate the two groups and to determine whether the two groups shared common molecular phenomena. A gene was considered to be overexpressed if the signal/noise (S/N) ratio was greater than 2. Analysis was also performed using 3-dimensional hierarchical clustering, principal component analysis (PCA) to assess the major grouping of the cases based solely on their gene expression profiles.

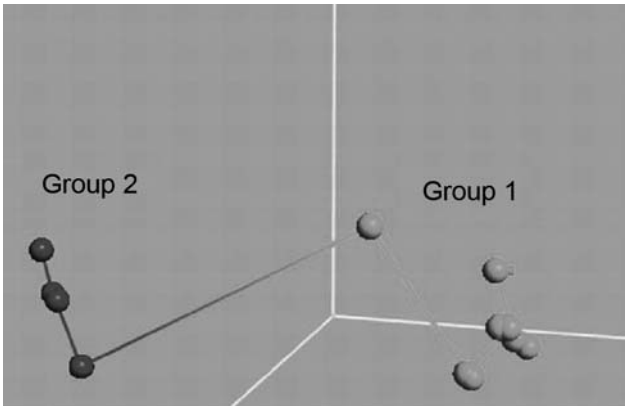

Figure 1. *Three-dimensional hierarchical clustering algorithm, principal component analysis (PCA) showing both clusters. Group 1 contains the ABL1 samples and group 2 the MLL–AFF1 samples.*

Results

The analysis of the data set using the 3-dimensional hierarchical clustering algorithm PCA differentiated the cases associated with an *MLL–AFF1* fusion from those with a rearrangement of the *ABL1* gene (Figure 1).

The comparison of the *MLL–AFF1* cases with those of the *ABL1* group identified 477 genes to be differentially expressed at the statistically significant level of $p<0.05$. Examination of the genes whose expression patterns distinguish ALL with a 4;11 rearrangement from those with

Table II. *Selection of genes differentially expressed in acute lymphoblastic leukemia (ALL) with a 4;11 rearrangement compared to those with an ABL1 fusion gene.*

Genes overexpressed in ALL with a 4;11 rearrangement	
*ADCY9**	Adenylate cyclase 9
*CCNA1**	Cyclin A1
*CD44**	CD44 molecule (Indian blood group)
*CD72**	CD72 molecule
CD320	CD320 molecule
*CSPG4**	Chondroitin sulfate proteoglycan 4
CYC1	Cytochrome c-1
DIABLO	IAP-binding mitochondrial protein
*FADD**	Fas (TNFRSF6)-associated via death domain
FANCA	Fanconi anemia, complementation group A
*GNA12**	Guanine nucleotide binding protein (G protein) alpha 12
HOXA3	Homeobox A3
*HOXA4**	Homeobox A4
*HOXA5**	Homeobox A5
HOXA6	Homeobox A6
*HOXA9**	Homeobox A9
*HOXA10**	Homeobox A10
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
*IGFBP7**	Insulin-like growth factor binding protein 7
IL28RA	Interleukin 28 receptor, alpha (interferon, lambda receptor)
*LGALS1**	Lectin, galactoside-binding, soluble, 1
*MAD2L2**	MAD2 mitotic arrest deficient-like 2 (yeast)
*MAP7**	Microtubule-associated protein 7
*MEIS1**	Meis homeobox 1
*MGST3**	Microsomal glutathione S-transferase 3
*MYC**	v-myc myelocytomatosis viral oncogene homolog (avian)
PTH1R	Parathyroid hormone 1 receptor
*RAB4A**	RAB4A, member RAS oncogene family
*SPIB**	Spi-B transcription factor (Spi-1/PU.1 related)
*VLDLR**	Very low density lipoprotein receptor

Genes underexpressed in ALL with a 4;11 rearrangement	
*ABLIM**	Actin binding LIM protein 1
*ALOX5**	Arachidonate 5-lipoxygenase
BCL6	B-cell CLL/lymphoma 6
*CCND2**	Cyclin D2
*CNN3**	Calponin 3, acidic
CD37	CD37 molecule
CD52	CD52 molecule
CD74	CD74 molecule
CD97	CD97 molecule
*DPEP1**	Dipeptidase 1
ELA2	Elastase, neutrophil expressed
FOXO1	Forkhead box O1
FYN	FYN oncogene related to SRC, FGR, YES
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HLA-A	Major histocompatibility complex, class I, A
HLA-B	Major histocompatibility complex, class I, B
HLA-DPA1	Major histocompatibility complex, class II, DP alpha 1
HLA-DPB1	Major histocompatibility complex, class II, DP beta 1
HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1
HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1
HLA-DRA	Major histocompatibility complex, class II, DR alpha
HLA-F	Major histocompatibility complex, class I, F
HLA-G	Major histocompatibility complex, class I, G
HLA-H	Major histocompatibility complex, class I, H
ICAM3	Intercellular adhesion molecule 3
IGJ	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides
*ITPR3**	Inositol 1,4,5-trisphosphate receptor, type 3
JAK1	Janus kinase 1
*LARGE**	Like-glycosyltransferase
LGMN	Legumain
*LTB**	Lymphotoxin beta (TNF superfamily, member 3)
*MME**	Membrane metallo-endopeptidase
MPO	Myeloperoxidase
*PDE4B**	Phosphodiesterase 4B, cAMP-specific
PDE4D	Phosphodiesterase 4D, cAMP-specific
PIM1	Pim-1 oncogene
PIM2	Pim-2 oncogene
PRTN3	Proteinase 3
*PTP4A3**	Protein tyrosine phosphatase type IVA, member 3
RAI14	Retinoic acid-induced 14
RUNX3	Runt-related transcription factor 3
SMAD7	SMAD family member 7
TCF4	Transcription factor 4
TP53INP1	Tumor protein p53 inducible nuclear protein 1

*Identifies genes common in this study and in that by Rozovskaia et al. (53).

a *ABL1* fusion gene identified a large number of genes involved in cell growth, apoptosis and differentiation, as well as in cell transformation.

A total of 296 genes were up-regulated in *MLL–AFF1* cases (Table II). Among them, several genes could be of particular importance as they are expressed in hematopoietic lineages and/or involved in cell growth, apoptosis and differentiation.

CCNA1 encodes cyclin A1 that binds both cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase 1 (CDK1), whose activity is essential for G_1/S and G_2/M cell cycle phase transitions, thus regulating separate functions in cell cycle. This cyclin was found to bind to important cell cycle regulators, such as Rb family proteins, transcription factor E2F-1, and the p21 family proteins (18).

The protein encoded by the *CD44* gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration (19). High CD44 expression, by opposing p53 function of inhibition of cell proliferation and induction of apoptosis and senescence, can serve as an important growth-promoting and survival factor in early stages of tumor progression (20). The CD44 molecule has been shown to be an important factor for the progression of acute myeloid leukemia (21, 22).

The *CD72* gene, whose expression is restricted to B-lineage cells, encodes a protein that may be involved in signals for B-cell proliferation (23, 24).

CSPG4 encodes chondroitin sulfate proteoglycan 4, which is not expressed by normal hematopoietic cells, but was found to be expressed by leukemia blast cells having abnormalities in chromosome band 11q23. Smith *et al*. hypothesized that this gene was controlled by a transcription factor encoded by the *MLL* gene and that certain types of alterations in MLL resulted in the aberrant expression of *CSPG4* (25). Furthermore, Behm *et al*. showed that the cell surface expression of CSPG4 was useful for identifying patients with t(4;11)(q21;q23) or t(11;19)(q23;p13) translocations (26).

The homeobox (*HOX*) gene family, among which *HOXA3, HOXA4, HOXA5, HOXA6, HOXA9* and *HOXA10* are overexpressed in patients with *MLL–AFF1*, and they encode DNA-binding transcription factors that may regulate gene expression, morphogenesis, and differentiation. Some may function in regulation of hematopoietic lineage commitment or myeloid cell proliferation and differentiation (27-29).

Meis homeobox 1 (MEIS1) is a homeobox protein belonging to the Three Amino Acid Loop Extension (TALE) family of homeodomain-containing proteins. *MEIS1* is a common collaborator with two divergent *HOX* genes, *HOXA9* and *HOXB3*, in leukemic transformation. Using overexpression studies in bone marrow cells, Thorsteinsdottir *et al*. demonstrated that each *HOX* gene studied predisposed to phenotypically distinct types of leukemia and that MEIS1 acted to accelerate the occurrence of these leukemias (30). Wong *et al*. showed that MEIS1 was essential for induction and maintenance of MLL leukemogenesis, regulating differentiation arrest, cycling activity, *in vivo* progression, and self-renewal of MLL leukemia cells (31).

LGALS1 encodes soluble galactoside-binding lectin, 1, a member of an ubiquitously-expressed beta-galactoside-binding family that has been shown to be involved in many physiological and pathological processes, such as tumor progression, by promoting cancer cell invasion and metastasis, apoptosis, embryogenesis and immunobiology (32). It was shown that LGALS1 expression was a highly sensitive and specific marker of MLL-rearranged B-ALL in infant/pediatric and adult patients (33). Although the mechanisms of LGALS1 overexpression differ in specific hematological malignancies, this protein plays a general role in limiting host antitumor immune responses (34).

The MAD2 mitotic arrest deficient-like 2 (yeast) gene (*MAD2L2*) encodes a protein that is a component of the mitotic spindle assembly checkpoint, preventing the onset of anaphase until all chromosomes are properly aligned at the metaphase plate (35). Colonic tumors with up-regulated MAD2L2 expression have significantly higher numbers of aberrant mitotic figures, indicating chromosomal instability (36).

The protein encoded by the *MYC* gene [v-myc myelocytomatosis viral oncogene homolog (avian)] is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Induction of the transcription factor MYC promotes cell proliferation and transformation by activating growth-promoting genes (37). Mutations, overexpression, rearrangement and translocation of this gene have been associated with a variety of hematopoietic tumors, leukemias and lymphomas (38).

The *SPIB* gene encodes a transcriptional activator, Spi-B transcription factor (Spi-1/PU.1 related) that binds to the 5-prime-GAGGAA-3-prime (PU)-box and acts as a lymphoid-specific enhancer (39). It plays a role in the control of differentiation of human B-cells by repressing the induction of the plasma cell gene expression program; its overexpression in $CD19^+$ B-cells inhibits B-cell differentiation (40).

A total of 181 genes were down-regulated in *MLL–AFF1* patients (Table II). Among them, B-cell CLL/lymphoma 6 gene (*BCL6*), which encodes a zinc finger transcription factor that acts as a sequence-specific repressor of transcription (41). BCL6 was found to repress a number of genes involved notably in B-cell differentiation and the cell cycle; its inhibition altered their expression (42).

The *TP53INP1* gene, encoding the tumor protein p53 inducible nuclear protein 1 was also under-expressed in patients with *MLL–AFF1*. *TP53INP1* is a p53 target gene that induces cell growth arrest and apoptosis by modulating p53 transcriptional activity (43). It is interesting to note that the oncoprotein MYC, the gene for which is overexpressed in patients with *MLL–AFF1*, binds to the core promoter of *TP53INP1* and recruits DNA methyltransferase 3A to methylate the local promoter region, thus leading to the inhibition of *TP53INP1* expression (44).

CCND2 encodes cyclin D2, a protein that forms a complex with and functions as a regulatory subunit of cyclin-dependent kinase 4 (CDK4) or cyclin-dependent kinase 6 (CDK6), whose activity is required for G_1/S cell cycle transition (45).

Discussion

Gene expression analyses of *MLL*-rearranged acute leukemia showed a specific signature that could differentiate them from other leukemias (13-16). *MLL*-rearranged leukemia exhibits elevated *HOX* gene expression, with *HOXA9* and *HOXA10* being highly-expressed regardless of disease phenotype, and up-regulation of the HOX co-factor *MEIS1* and the homeotic regulator polycomb ring finger oncogene (*BMI-1*) (10, 14, 16). Comparison of the gene expression signatures between *BCR–ABL1* and *MLL*-rearranged ALL cases showed major differences between both groups, including *HOX* gene up-regulation in the latter group (8, 9,

46). Our results, based on a comparison with patients carrying a t(9;22)(q34;q11) or a t(1;9)(q24;q34) associated with an *ABL1* fusion gene, confirmed the *HOX* gene signature. Thus, it is likely that the presence of an *MLL* translocation in leukemia cells sets a program of homeotic expression as part of its leukemogenic mechanism (16).

Using unsupervised PCA on 130 AML, 132 B-cell ALL and 5 T-cell ALL associated with an *MLL* fusion gene, Ross *et al*. found that patients did not cluster as a unique subgroup, but instead segregated according to their lineage (11). The same conclusions were reached by others (47, 48).

Kohlmann *et al*. tried to determine whether specific *MLL* fusion genes had distinct gene expression signatures. The gene expression profile did not support the hypothesis of a clear distinct signature associated with the specific *MLL* fusion genes analyzed in their cohort of 65 MLL-rearranged cases (47). However, controversy remains as to whether specific fusion genes have distinct gene expression signatures (49-51). One cannot exclude that in addition to the common pathways in *MLL*-rearranged leukemia, there are other pathways that could be more dependent on the *MLL* fusion partner, age, previous anticancer treatment, *etc*.

Three GEP studies on t(4;11)(q21;q23) focusing on the age group of the patients have been published (50, 52, 53). Rozovskaia *et al*. analyzed the expression profiles of 12 patients with t(4;11)-associated ALL, including 10 adults (age range=19-58 years), one infant (<1 year) and one child, using human U95 oligonucleotide probe arrays (Affymetrix, Santa Clara, CA, USA) (53). Profiles were compared with those of a set of patients with ALL lacking the t(4;11), some of them having T-cell ALL or Philadelphia-positive B-cell ALL. Supervised analysis revealed 130 overexpressed and 107 underexpressed genes in t(4;11)-ALL, in comparison to ALL without t(4;11).

Trentin *et al*. analyzed the expression profiles of 20 patients with t(4;11)-associated ALL, including 11 infants (<1 year) and 9 children (non-infants, between 1 and 15 years), using HU133 Plus 2.0 oligonucleotide arrays (Affymetrix) (52). Comparison with GEP of three bone marrow samples of healthy individuals identified two distinct t(4;11) subgroups sharing a common set of 36 overexpressed and 150 underexpressed genes. The two subgroups could be distinguished by the high or low expression of *HOXA* genes (*HOXA5*, *HOXA9* and *HOXA10*). The low HOXA signature was found in infants and the high HOXA signature in children.

Using HU133 Plus 2.0 oligonucleotide arrays (Affymetrix), Stam *et al*. compared the expression profiles of 59 infants (<1 year) with B-cell ALL with an *MLL* rearrangement with those of 14 infants with *MLL* germline B-cell ALL (50). They showed that GEP of *MLL*-rearranged ALL was different from that of *MLL* germline ALL. Individualizing the expression profiles by *MLL* fusion partner showed that patients carrying a t(4;11), t(11;19) or t(9;11) clustered completely separately from one another. Furthermore, among the 29 t(4;11)-positive ALL infants, two separate clusters could be identified based on the presence or absence of *HOXA9, HOXA7, HOXA10, HOXA5* and *HOXA3* expression.

Our study, based on Illumina Human HT-12 v3 Expression BeadChips, identified a set of 477 differentially expressed genes among the four patients with a 4;11 rearrangement. Comparison of our results with those from the three previously reported studies is difficult because of differences in the microarrays and the control samples used. With these restrictions in mind, we tried to identify up- and down-regulated genes common to our study and the other studies.

Gene expression profiling of pediatric ALL cases (less than 15 years) revealed at least two clusters. Comparing the profile observed in the patients reported here showed that *HOX* genes (*HOXA9, HOXA7, HOXA10, HOXA5* and *HOXA3*) were highly expressed, as observed in a subset of pediatric patients. However, the genes differentially expressed in our patients are different from those found in pediatric cases with *HOX* gene overexpression. In fact, our results are in agreement with the results of Rozovskaia *et al*. who analyzed 12 patients with t(4;11), 10 of them being adults (54). Indeed, 21 overexpressed and 11 underexpressed genes are shared by both studies (Table II).

Our results and those from the literature suggest that cells carrying a t(4;11)(q21;q23) use different pathways to lead to leukemogenesis. Therefore, t(4;11)-associated ALL could represent different biological entities. It would also be interesting to compare the GEP of patients having undergone or not chemotherapy with topoisomerase II inhibitors. Studies on clinically and biologically well-characterized patients are needed to decipher the heterogeneity of ALL associated with t(4;11).

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