Gene Expression Profiling of Acute Myeloid Leukemia with Translocation t(8;16)(p11;p13) and *MYST3-CREBBP* Rearrangement Reveals a Distinctive Signature with a Specific Pattern of *HOX* Gene Expression

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Abstract

Acute myeloid leukemia (AML) with translocation t(8;16)(p11;p13) is an infrequent leukemia subtype with characteristic clinicobiological features. This translocation leads to fusion of *MYST3* (*MOZ*) and *CREBBP* (*CBP*) genes, probably resulting in a disturbed transcriptional program of a myelomonocytic precursor. Nonetheless, its gene expression profile is unknown. We have analyzed the gene expression profile of 23 AML patients, including three with molecularly confirmed *MYST3-CREBBP* fusion gene, using oligonucleotide U133A arrays (Affymetrix). *MYST3-CREBBP* cases clustered together and clearly differentiated from samples with *PML-RARα*, *RUNX1-RUNX1T1*, and *CBFβ-MYH11* rearrangements. The relative expression of 46 genes, selected according to their differential expression in the high-density array study, was analyzed by low-density arrays in an additional series of 40 patients, which included 7 *MYST3-CREBBP* AML cases. Thus, genes such as prolactin (*PRL*) and proto-oncogene *RET* were confirmed to be specifically overexpressed in *MYST3-CREBBP* samples whereas genes such as *CCND2*, *STAT5A*, and *STAT5B* were differentially underexpressed in this AML category. Interestingly, *MYST3-CREBBP* AML exhibited a characteristic pattern of *HOX* expression, with up-regulation of *HOXA9*, *HOXA10*, and cofactor *MEIS1* and marked down-regulation of other homeobox genes. This profile, with overexpression of *FLT3*, *HOXA9*, *MEIS1*, *AKR7A2*, *CHD3*, and *APBA2*, partially resembles that of AML with *MLL* rearrangement. In summary, this study shows the distinctive gene expression profile of *MYST3-CREBBP* AML, with overexpression of *RET* and *PRL* and a specific pattern of *HOX* gene expression. (Cancer Res 2006; 66(14): 6947-54)

Introduction

Chromosomal translocations resulting in fusion proteins are a common finding in acute myeloid leukemia (AML). The most frequent fusion products, *PML-RARα*, *RUNX1-RUNX1T1* (*AML1-ETO*), and *CBFβ-MYH11*, found in ~25% of *de novo* AML cases, constitute abnormal transcriptional factors causing a disturbed program of myeloid differentiation. Moreover, each of these translocations defines a specific leukemia subtype associated with a favorable prognosis (1, 2). In this setting, translocation t(8;16)(p11;p13) is an infrequent recurrent chromosomal abnormality found in both *de novo* and therapy-related AML cases after treatment with topoisomerase II inhibitors (3–6). These patients present with specific clinical and biological features, such as a blast population with a myelomonocytic stage of differentiation, frequent extramedullary involvement, severe coagulation disorder, and a poor outcome (3, 5, 6). At the molecular level, translocation t(8;16) fuses MYST histone acetyltransferase (monocytic leukemia)-3 (*MYST3*; formerly named *MOZ*) and CREB binding protein (Rubinstein-Taybi syndrome; *CREBBP*, or *CBP*) genes, both encoding proteins with histone acetyltransferase activity (4, 7–9). *MYST3* has been shown to modulate gene transcription through activation of the transcription factor complex *RUNX1* (8, 10). Moreover, the protein complex MYST3-RUNX1 has been found to increase during normal monocytic differentiation. In its turn, CREBBP protein also regulates transcription by means of histone acetyltransferase activity and by binding to several proteins with key cell cycle functions, such as p53 and nuclear factor κB (8, 9). Therefore, an inhibition of *RUNX1*-mediated transcription by MYST3-CREBBP fusion protein has been hypothesized to be the main mechanism of leukemogenesis in this AML variety (10). However, the precise pathways disrupted by this chimerical protein are mostly unknown.

Analysis of gene expression profile might contribute to refine the classification of AML based on biological grounds and to assign the prognostic risk of a given subtype more accurately (11–14). Furthermore, genomic analysis of AML might provide a deeper insight into the underlying disease mechanisms.

In this study, we have examined the gene expression profile of AML with the *MYST3-CREBBP* fusion gene to determine the specific signature of this leukemia compared with other well-defined AML subtypes and to define possible molecular pathways involved in the pathogenesis of this leukemia.

Materials and Methods

Leukemia samples. Twenty-three AML patients were selected for a global gene expression profile analysis using the Affymetrix HU133A array (Affymetrix, Inc., Santa Clara, CA; subset A of patients, Supplementary Table A). These cases included three *MYST3-CREBBP* AML cases, together with other 20 samples of different leukemia subtypes classified according to WHO criteria (15) as acute promyelocytic leukemia with t(15;17)(q22;q12) (*PML-RARα*; $n = 3$); AML with t(8;21)(q22;q22) (*RUNX1-RUNX1T1*; $n = 3$); AML with inv(16)/t(16;16) (*CBFβ-MYH11*; $n = 3$); AML with t(9;11)(p22;q23) (*MLLT3-MLL*; $n = 1$); acute monocytic leukemias ($n = 8$); and two cases of AML with multilineage dysplasia. In 10 of these 23 cases, an internal tandem

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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duplication of the *FLT3* gene was detected whereas mutations of nucleophosmin (*NPM*) gene were found in 6 cases (Supplementary Table A). Two cases with *MYST3-CREBBP* rearrangement and one monocytic-differentiated AML (cases 1, 3, and 18) followed exposure to topoisomerase II inhibitors. Cases 1 and 18 also presented amplification of *MLL* gene.

To confirm the findings of the previous global gene expression profile study, a second subset of 40 AML patients (subset B, Supplementary Table B) was studied using TaqMan low-density arrays (Applied Biosytems, Foster City, CA; see below). This subset of patients included the three *MYST3-CREBBP* AML cases previously studied by high-density array and four additional *MYST3-CREBBP* samples with no appropriate material for the genome-wide assay. In addition, an independent set of 33 AML samples was included in this study. These samples corresponded to AML with well-characterized rearrangements (*PML-RARα, n* = 3; *RUNX1-RUNX1T1, n* = 3; *CBFβ-MYH11, n* = 3; *MLL*-rearranged AML, *n* = 9) and normal karyotype AML (*n* = 15). Mutations of *FLT3-ITD, NPM*, and *MLL* abnormalities are detailed in Supplementary Table B. None of the *MYST3-CREBBP* samples analyzed harbored either *FLT3-ITD* or *NPM* mutations. Four patients (cases 2, 3, 7, and 18) presented with a therapy-related AML. The clinical and laboratory data of the seven *MYST3-CREBBP* AML cases were previously reported (16). All peripheral blood and bone marrow samples were obtained with informed consent according to the guidelines of the Ethical Committee of the participating Institutions.

RNA extraction and cDNA synthesis. Total RNA was isolated from peripheral blood and bone marrow by standard methods (17). In one case, we obtained RNA from a paraffin-embedded tissue using a phenol-extraction method. In all cases, integrity of RNA was examined with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One microgram of RNA was reverse transcribed to cDNA using random primers with the High Capacity cDNA Archive Kit (Applied Biosystems).

Molecular analyses. The detection of transcript type I of *MYST3-CREBBP* rearrangement was analyzed by reverse transcription-PCR (RT-PCR) with primers MOZ3558F and CPB335R, as previously described (16). The presence of *PML-RARα, RUNX1-RUNX1T1*, and *CBFβ-MYH11* rearrangement was analyzed by RT-PCR following published conditions (18). The analysis of *FLT3-ITD* was done using primers and conditions previously published (19). The presence of mutations in exon 12 of *NPM* gene was studied by amplification of genomic DNA or cDNA with a fluorescently labeled forward primer and subsequent analysis of the PCR product in an Automatic sequencer (Abi Prism 310) using the Genescan software as described (20). *MLL* rearrangement was studied by Southern blot analysis using probe B859 after digestion with *Hind*III and *Bam*HI enzymes (21). Cases without available DNA, with a noninformative karyotype, or showing abnormalities at 11q23 region by conventional cytogenetics were studied by fluorescence *in situ* hybridization with the LSI *MLL* Dual Color Probe (Vysis) as described (22). Partial tandem duplication was specifically analyzed by RT-PCR as previously described (23).

High-density array study: RNA purification, labeling, and hybridization. In this part of the study, 23 AML samples with confirmed high quality RNA by Agilent 2100 Bioanalyzer analysis and sufficient amount of RNA (5 μg) were included. Amplified biotinylated complementary RNA was produced with an *in vitro* transcription labeling reaction and was subsequently hybridized to Affymetrix HU133A oligonucleotide arrays following the Affymetrix protocol for high-density arrays (details are provided in supplementary material). Scans were carried out on Agilent G2500A GeneArray scanner (Agilent Technologies, Waldbronn, Germany) and the fluorescence intensities of scanned arrays were analyzed with the Affymetrix GeneChip software.

Statistical analysis. Affymetrix Microarray Suite Software version 5.0 (MAS5.0) was used for the quantification of the expression level of target genes. HU133A microarray raw expression intensities were scaled to a target intensity of 200 units. To exclude genes with minimal variation across samples, only genes with a mean (SD) of normalized values between 0.7 and 10 were filtered. Thereafter, we selected those genes with an expression level of ≥20 in ≥25% of samples. The 2,683 resulting genes were studied by means of unsupervised two-dimensional cluster analysis with dChip v1.3 software using the default clustering algorithm defined as $1 - r$, where r is the Pearson correlation coefficient between standardized expression values (make 0 and SD 1) and the centroid linkage method. To identify those genes with significant differences in their expression level among different AML categories, we used a random-variance F test as described in BRB ArrayTools software (BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam)[5] with all probe sets, but assigning an arbitrary value of 10 to genes with an expression level below 10 units. A significance level of 0.001 was chosen to reduce the number of false positive results. For each one of the differentially expressed genes, a ratio between the mean expression value in *MYST3-CREBBP* samples and the mean expression in every AML category was calculated. Additionally, a t test comparing the gene expression in *MYST3-CREBBP* and that in the remaining samples as a whole, with a significance level of 0.001, was used as another method of detection of differentially expressed genes in *MYST3-CREBBP* samples.

Quantitative real-time RT-PCR. A selection of 46 genes was subsequently studied by real-time PCR using TaqMan Low Density Arrays (Applied Biosystems) in an additional series of 40 AML patients (subset B, Supplementary Table B). These genes were selected on the basis of their differential expression in *MYST3-CREBBP* according to high-density array analysis or their oncogenic potential in leukemia. The list of 46 genes is provided in Supplementary Table C. Briefly, cDNA was obtained from these cases, loaded onto the low-density arrays, and amplified using standard conditions in an Abi Prism 7900HT Sequence Detection System (Applied Biosystems). All samples were tested in duplicate and the average value between replicates was taken as the specific level of expression of a given gene. To quantify the relative expression of each gene, the Ct values were normalized for endogenous reference ($\Delta Ct = Ct_{target} - Ct_{\beta 2\text{-microglobulin}}$) and compared with a calibrator using the ΔΔCt method. As calibrator, the average Ct value of each gene in all samples grouped together was taken. Comparison of the relative expression of the 46 genes in *MYST3-CREBBP* AML with that in the remaining AML samples was done using a t test comparing two groups, with a significance level of 0.05. In addition, an ANOVA test was also used to compare the relative expression of these genes after defining different AML categories (significance level, 0.05).

Results

High-density Array Analysis

Unsupervised analysis. After applying a variation filter, the resulting 2,683 genes were visualized by hierarchical clustering method (Fig. 1*A*). Two main branches were seen in the dendrogram, corresponding mainly to cases with myeloid and monocytic differentiation, respectively. Samples clearly grouped in five clusters, which were constituted by cases of AML with well-defined gene rearrangements [i.e., *MYST3-CREBBP* (cluster 1), *RUNX1-RUNX1T1* (cluster 2), *CBFβ-MYH11* (cluster 3), and *PML-RARα* (cluster 4), and, on the other hand, AML with multilineage dysplasia (cluster 5)]. In contrast, the remaining samples (no. 13-21), defined by their monocytic differentiation and the absence of the above-mentioned fusion proteins, were distributed among the different clusters of the array forming a heterogeneous group. One of the samples harboring a *CBFβ-MYH11* rearrangement, with a minimally differentiated phenotype (M0 subtype), segregated from two other cases with the same molecular alteration but presenting with a myelomonocytic phenotype.

Supervised analysis. We applied supervised methods based on the six different AML categories (clusters 1-5 and a sixth group containing monocytic-lineage leukemias) drawn from the unsupervised analysis. First, the analysis with a random-variance F test yielded 1,205 genes with a significant different expression level among AML subgroups. A hierarchical cluster done with this set of genes is presented in Fig. 1*B*. Afterwards, we selected 63 genes

[5] http://linus.nci.nih.gov/BRB-ArrayTools.html.

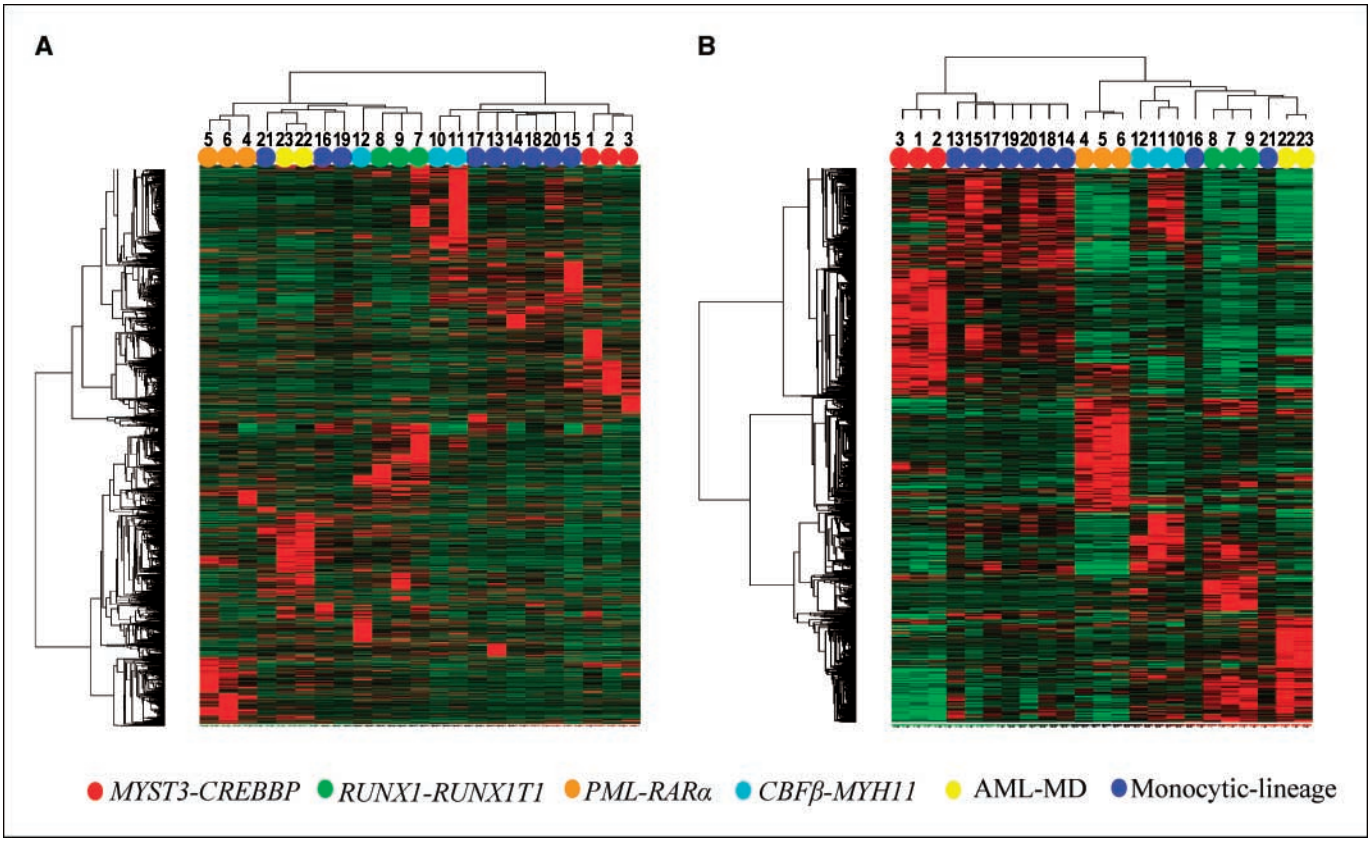

Figure 1. High-density array study done in 23 AML cases (subset A of patients). *A,* visualization of the results of the unsupervised analysis. The 2,683 genes obtained after applying a variation filter were visualized by hierarchical clustering method. Two main branches were distinguished according basically to the myeloid or monocytic differentiation lineage of blast cells. Five clusters were recognized, constituted by cases with well-defined rearrangement [i.e., *MYST3-CREBBP* (cluster 1), *RUNX1-RUNX1T1* (cluster 2), *CBFβ-MYH11* (cluster 3), and *PML-RARα* (cluster 4), and, on the other hand, AML with multilineage dysplasia (cluster 5)]. The remaining samples (nos. 13-21), defined by their monocytic differentiation and the absence of the above-mentioned fusion proteins, were distributed among the different clusters of the array forming a heterogeneous group. *B,* visualization of genes with a differentiated expression in the different AML subgroups. Hierarchical clustering visualization of the 1,205 genes with a significant different expression level ($P \leq 0.01$) among the six different AML categories (clusters 1-5 and a sixth group containing monocytic-lineage leukemias) using a random-variance *F*-test method.

overexpressed and 60 genes underexpressed in *MYST3-CREBBP* samples with a ratio of mean expression equal or higher than twice the observed in each of the other groups. The 63 genes specifically overexpressed in *MYST3-CREBBP* samples (Supplementary Table D; Supplementary Fig. *A*1) included the oncogene *RET*, genes involved in chromatin remodeling and transcription (*HOXA10* and *PPARG*), and genes with a known function in DNA damage repair (*DDB2*) and apoptosis (*DAP*). Other genes such as *IRAK1*, expressed in response to cell injury, *NICAL*, implied in neuronal development, and prolactin (*PRL*), involved in signal transduction, were also up-regulated. In addition, 60 genes specifically down-regulated in *MYST3-CREBBP* leukemias (Supplementary Table D; Supplementary Fig. *A*2) included genes involved in cell cycle regulation, such as cyclin D2 (*CCND2*) and two members of the RAS oncogene family (*RAB6A* and *RAB8A*).

As an additional method to study possible shared patterns of gene expression between *MYST3-CREBBP* and other AML, we selected those genes with high or low expression in *MYST3-CREBBP* leukemias and a similar pattern of expression in one of the other AML categories (Supplementary Tables E and F; Supplementary Fig. *B*). This analysis revealed that *MYST3-CREBBP* leukemias had 33 genes overexpressed in common with AML of monocytic lineage (i.e., *AKR7A2, CHD3*, and *AK2*) and 19 with *PML-RARα*, but only a minority of genes in common with other AML subtypes.

Finally, when *MYST3-CREBBP* cases were compared with the other samples as a group using a *t* test, 237 genes (53 overexpressed and 184 underexpressed) showed a differential expression (Supplementary Tables G and H). Using this analysis, a high expression of the aforementioned gene *RET* could be observed. Additionally, an overexpression of genes with a role in transcription (*SATB1*), genes involved in apoptosis (*OPTN*), and *CEBPα*, a crucial gene in the myeloid differentiation process, was observed.

Low-density Array Analysis

Supervised analysis. Forty-six genes were selected according to their differential expression in *MYST3-CREBBP* samples in the high-density array and/or their relevant oncogenic role in leukemia. The relative expression of these genes was analyzed in 40 AML patients (subset B, Supplementary Table B). To define the group of genes characteristic of *MYST3-CREBBP* subtype, a *t* test with two groups was used to compare the gene expression in *MYST3-CREBBP* samples and the remaining AML patients. Twenty-two genes were significantly overexpressed in *MYST3-CREBBP* samples whereas three genes were underexpressed in this group of leukemias (Table 1). Subsequently, an ANOVA test was used to compare the gene relative expression among the seven AML categories defined by their underlying molecular abnormality (A, *MYST3-CREBBP*; B, *PML-RARα*; C, *RUNX1-RUNX1T1*; D, *CBFβ-MYH11*;

Table 1. Genes with a significant differential expression level (overexpressed and underexpressed) in *MYST3-CREBBP* samples according to the low-density array study

t test		ANOVA	
Symbol	*P*	Symbol	*P*
Genes overexpressed			
C20ORF103	0.000000	*C20ORF103*	0.000000
GGA2	0.000000	*ICSBP1*	0.000010
ICSBP1	0.000000	*FLT3*	0.000087
FLT3	0.000000	*GGA2*	0.000094
LMO2	0.000005	*AKR7A2*	0.000245
ITGA7	0.000013	*SURF1*	0.002103
AKR7A2	0.000014	*LMO2*	0.002979
PPARG	0.000016	*HIST1H2A*	0.004580
SURF1	0.000024	*ITGA7*	0.005785
ADAMTS2	0.000036	*PPARG*	0.006282
DAP	0.000092	*ADAMTS2*	0.014015
IRAK1	0.000093	*CEBPA*	0.014782
PRL	0.000096	*IRAK1*	0.019370
HIST1H2A	0.000134	*LGALS3*	0.021620
RET	0.000260	*DDB2*	0.023266
CHD3	0.000264	*DAP*	0.025509
LGALS3	0.000749	*PRL*	0.027868
DDB2	0.000829	*SATB1*	0.039598
SATB1	0.001823		
HOXA9	0.003608		
S100A11	0.015956		
CEBPA	0.021236		
Genes underexpressed			
STAT5B	0.036902	*WT1*	0.000061
CREBBP	0.041165	*CCND2*	0.001073
STAT5A	0.049642	*STAT5B*	0.003404
		STAT5A	0.027617

E, *MLL*-translocated samples; F, *MLL* partial tandem duplication; and G, miscellaneous). The ANOVA test yielded five different gene expression profiles. First (profile 1, Fig. 2*A*), a group of nine genes (*PRL, C20orf103, RET, GGA2, ICSBP1, ITGA7, DAP, IRAK1* and *PPARG*) were found to be highly expressed in *MYST3-CREBBP* AML with absent or low expression in the remaining samples. Genes such as *FLT3, HOXA9, MEIS1, AKR7A2, CHD3*, and *APBA2* composed a second pattern of expression (profile 2, Fig. 2*B*), with high expression level in both *MYST3-CREBBP* and AML with monocytic phenotype. In the third profile observed (profile 3, Fig. 2*C*), some genes had a high expression level in *MYST3-CREBBP* AML and were variably expressed in other AML categories. This group included *SATB1, LMO2, CEBPα, SURF1, HIST1H2A, DDB2*, and *LGALS3* genes.

Another group of genes were characteristically down-regulated in the *MYST3-CREBBP* leukemia subtype compared with other AML categories, including *CCND2, STAT5A*, and *STAT5B* (profile 4, Fig. 3*A*). A decrease in expression levels of *CREBBP* was seen in *MYST3-CREBBP* subtype, but the difference did not reach statistical significance in the ANOVA test comparing the AML subgroups. Finally, a low or absent expression of Wilms' tumor 1 (*WT1*) gene was distinctly observed in both *MYST3-CREBBP* and *RUNX1-RUNX1T1* cases (profile 5, Fig. 3*B*).

Mutational Status of *RET* Gene

Due to the distinctly overexpression of *RET* gene observed in *MYST3-CREBBP* samples, mutations of this gene were screened by direct sequencing of exons 8 to 16, where somatic and germ-line mutations associated with human diseases have previously been described (24). None of the five *MYST3-CREBBP* samples that could be analyzed harbored mutations of *RET* gene, and only neutral polymorphisms at codons 769 and 836 were found (data not shown).

Expression of Homeobox Genes

Given the high expression of several homeobox (*HOX*) genes observed in *MYST3-CREBBP*, we did an unsupervised analysis on the whole series of patients focused on the relative level of expression of the *HOX* genes present in the array. Several patterns of *HOX* genes expression were seen in different AML categories (Fig. 4*A*). Thus, overexpression of 5′-*HOX* genes (*HOXA9, HOXB9*, and *HOXA10*) was characteristically found in all *MYST3-CREBBP* samples and most cases of monocytic AML. Other *HOX* genes (*HOXA2-A7* and *HOXB2-B7*) showed a high expression in the group of monocytic leukemias whereas their expression was low in *MYST3-CREBBP* AML. In contrast, *HOX* genes were underexpressed in good-risk cytogenetic AML.

Ten genes of the *HOX* family were subsequently studied by low-density arrays. With this method, the distinctive pattern of *HOX* expression according to AML subtypes was confirmed (Fig. 4*B*). Thus, good-prognosis AML showed a lower expression of all *HOX* analyzed. On the contrary, a high expression of all the *HOX* genes studied was detected in *MLL*-rearranged, partial tandem duplication, and monocytic-phenotype AML. In addition, *MYST3-CREBBP* AML showed a high expression of *HOXA9* and *MEIS1* but a lower expression of the remaining *HOX* genes.

Discussion

AML with *MYST3-CREBBP* rearrangement is an infrequent leukemia subtype resulting from the fusion of two genes with chromatin-modifying properties. In this regard, inhibition of *RUNX1*-mediated transcription, possibly through a disturbed histone acetyltransferase activity, has been hypothesized to be the main mechanism of leukemogenesis (10). Nevertheless, the signaling pathways disrupted by the chimerical protein MYST3-CREBBP are mostly unknown and, to the best of our knowledge, no previous studies have focused on the genomic profile of this AML variety. With this purpose, we used high-density microarrays to study a series of 23 AML samples that included three *MYST3-CREBBP* cases. The unsupervised analysis of the results identified a distinctive gene expression signature associated with the *MYST3-CREBBP* rearrangement. Thereafter, a group of genes was selected and analyzed using a quantitative approach with low-density arrays. This technology allowed us to study four additional *MYST3-CREBBP* samples with no appropriate material for the genome-wide assay. Interestingly, this approach confirmed the existence of a characteristic gene expression profile in *MYST3-CREBBP* AML, clearly distinguishable from that of other well-defined AML subtypes.

The combination of several methods of comparative analysis allowed the identification of groups of genes with a differential expression in distinct AML categories. First, a subset of genes seemed to be highly characteristic of *MYST3-CREBBP* AML, being up-regulated in these cases and showing a low or absent expression

in the remaining AML categories. Thus, genes such as *PRL, C20orf103, RET, GGA2, ICSBP1, ITGA7, DAP, IRAK1*, and *PPARG* were overexpressed almost exclusively in *MYST3-CREBBP* cases. Among those genes, *PRL* and *RET* have been occasionally reported to be involved in leukemogenesis (24–33). In this regard, an increased expression of prolactin protein in blast populations has been observed in anecdotal cases of monocytic-lineage leukemia as well as in the eosinophilic cell line Eol-1 (25–29). In a recent study, prolactin expression in Eol-1 cells was shown to involve different signaling pathways induced by cyclic AMP whereas inhibition of Jak-STAT5 pathway resulted in up-regulation of prolactin (28). Interestingly, *STAT5A* and *STAT5B* genes were found to be significantly underexpressed in our *MYST3-CREBBP* AML samples, suggesting a negative regulatory effect between prolactin and STAT5 proteins. On its turn, *RET* gene is a proto-oncogene that encodes a tyrosine kinase receptor expressed during normal myelomonocytic differentiation and, accordingly, has been found to be predominantly expressed in AML of monocytic phenotype (30–32). Moreover, RET mRNA levels are typically low among immature $CD34^+$ hemopoietic progenitors whereas overexpression

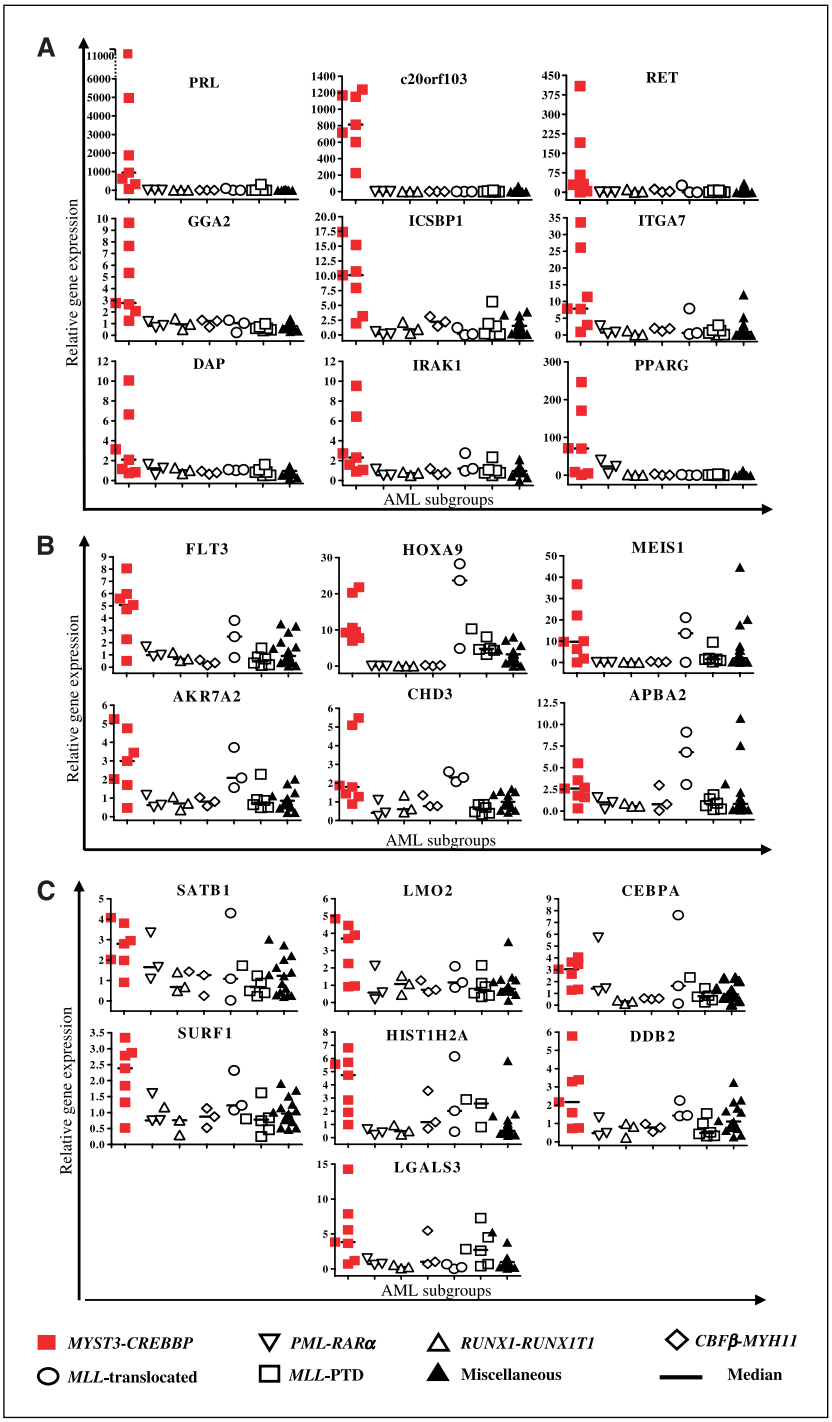

Figure 2. Low-density array study: genes significantly overexpressed in *MYST3-CREBBP* samples (ANOVA test). *A,* profile 1. Genes specifically overexpressed in *MYST3-CREBBP* samples, with absent or low expression in the remaining AML categories. *B,* profile 2. Genes overexpressed in *MYST3-CREBBP* and monocytic AML, either *MLL*-rearranged or non-rearranged variants. *C,* profile 3. Genes overexpressed in *MYST3-CREBBP* samples with variable expression in other AML categories.

of this protein has been associated with coexpression of adhesion molecules such as CD56 (30). These observations resemble the characteristic phenotype of *MYST3-CREBBP* AML, defined by common CD34 negativity, high expression of monocytic antigens, and frequent coexpression of CD56 and NG2 (16). Of note, *RET* has recently been reported as one of the most characteristic genes in one of the 16 AML clusters defined according to its genomic profile in a recent study by Valk et al. (14). Although most of the cases forming that cluster were monocytic-differentiated leukemias (14), modulation of *RET* does not seem to be merely attributable to a monocytic differentiation process because, in the present study, the expression level of *RET* was significantly higher in *MYST3-CREBBP* cases than in other monocytic-lineage leukemia samples. Somatic and germ-line *RET* mutations leading to gene activation are responsible for several human diseases, including multiple endocrine neoplasia types 2A and 2B and papillary thyroid carcinomas (24). Nevertheless, mutations of *RET* as a mechanism of overexpression were discarded in the present *MYST3-CREBBP* series, in accordance with a previous study analyzing diverse AML subtypes (33).

One of the most striking findings of this study was the similarities observed between *MYST3-CREBBP* and *MLL*-rearranged leukemias. *MYST3-CREBBP* cases presented high levels of homeobox genes (*HOXA9* and *HOXA10*), their cofactor *MEIS1*, and the receptor with tyrosine-kinase activity *FLT3*, all of them typically upregulated in *MLL* leukemias (34–36). *HOX* genes are transcription factors required for a proper hematopoietic development and constitute downstream targets of *MLL* protein (37). In this regard, *MLL*-rearranged leukemias are typically characterized by an impaired pattern of *HOX* expression, and *HOXA9, HOXA10*, and the cofactor *MEIS1* are up-regulated in virtually all lymphoid, myeloid, and biphenotypic lineage *MLL*-rearranged leukemia subtypes (38). In addition to the unexpected *HOX* overexpression in *MYST3-CREBBP* cases, the results of the present study confirmed previous findings on the pattern of *HOX* expression in different AML subtypes (39–41). Thus, whereas an overall low expression of *HOX* genes was observed in cases of AML with favorable cytogenetics, high levels of the members of *HOX* family *HOXA9* and *HOXA10* were seen in *MYST3-CREBBP* and *MLL*-rearranged leukemias. In contrast, other *HOX* genes analyzed, such as *HOXA3, HOXA7*, and *HOXB5*, were expressed at different levels in *MLL*-translocated cases, *MLL* with partial tandem duplication, and other monocytic leukemias, but were not expressed in *MYST3-CREBBP* AML cases.

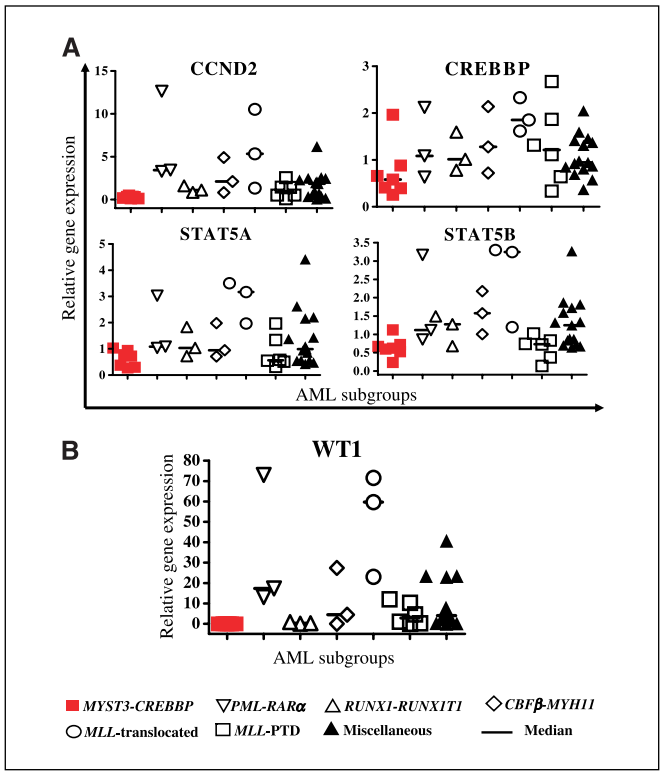

Figure 3. Low-density array study: genes significantly underexpressed in *MYST3-CREBBP* samples (ANOVA test). *A,* profile 4. Genes specifically underexpressed in *MYST3-CREBBP* samples. *B,* profile 5. *WT1* gene was found to be characteristically underexpressed in *MYST3-CREBBP* samples as well as in *RUNX1-RUNX1T1* cases.

Similarities between *MYST3-CREBBP* and *MLL* leukemias comprised other genes. Thus, overexpression of A*KR7A2, PBX3, NICAL*, and *IRAK1B* genes, observed in *MYST3-CREBBP* subtype, was also found in AML with *MLL* rearrangement, as reported in a recent study by Kohlmann et al. (36). Moreover, a coincidental expression of several genes (*RET, C20orf103, GGA2, GAGED2, AKR7A2*, and *AK2*) was observed between our *MYST3-CREBBP* samples and the above-mentioned cluster no. 16 of the study of Valk et al. (14). Of note, 5 of the 11 patients included in this cluster had 11q23 abnormalities.

As a common mechanism of leukemogenesis, chimeric protein *PML-RARα* in acute promyelocytic leukemia and *RUNX1-RUNX1T1* and *CBFβ-MYH11*, associated to core binding factor leukemias, respectively, induce a constitutive transcriptional repression, leading to a blockage of the normal myeloid differentiation program (42). This contrasts with the presumed function of fusion gene products derived from the rearrangement of *MLL* with different partners, thought to produce a constitutive transcriptional activation through a gain-of-function mechanism resulting in an inappropriate expression of target genes such as *HOX* (42). In this context, the gene expression signature of *MYST3-CREBBP* rearrangement obtained in this study seemed to be similar to that of *MLL*-rearranged leukemia and markedly different from those of CBF-AML and acute promyelocytic leukemia. Therefore, the leukemogenic effect of *MYST3-CREBBP* fusion gene could rely on a deregulated modulation of downstream targets, resembling that of *MLL* chimeras, probably due to impaired histone acetyltransferase activity of the proteins involved in this translocation. Moreover, the adverse prognosis classically associated with this entity differs from that of CBF-AML and acute promyelocytic leukemia and is similar to that of *MLL*-rearranged AML.

With regard to the underexpressed genes, the *CREBBP* expression was decreased in *MYST3-CREBBP* samples, although not reaching a significant difference, thus suggesting a negative regulation of the chimerical protein *MYST3-CREBBP* over native *CREBBP* transcript. Additionally, down-regulation of *WT1* was observed in *MYST3-CREBBP*, as well as in *RUNX1-RUNX1T1*, as compared with the other AML categories. *WT1* gene has been shown to be overexpressed in >90% of AML cases and it has recently been proposed as a molecular marker for minimal residual disease studies in AML (43). However, the low levels of *WT1* in *MYST3-CREBBP* leukemias observed in our study would hamper this strategy in the follow-up of the minimal residual disease in this group of leukemia.

In summary, the double strategy followed, based on the selection of a group of genes according to their differential expression in the high-density array assay and further analysis by real-time PCR in an additional set of patients, allowed the

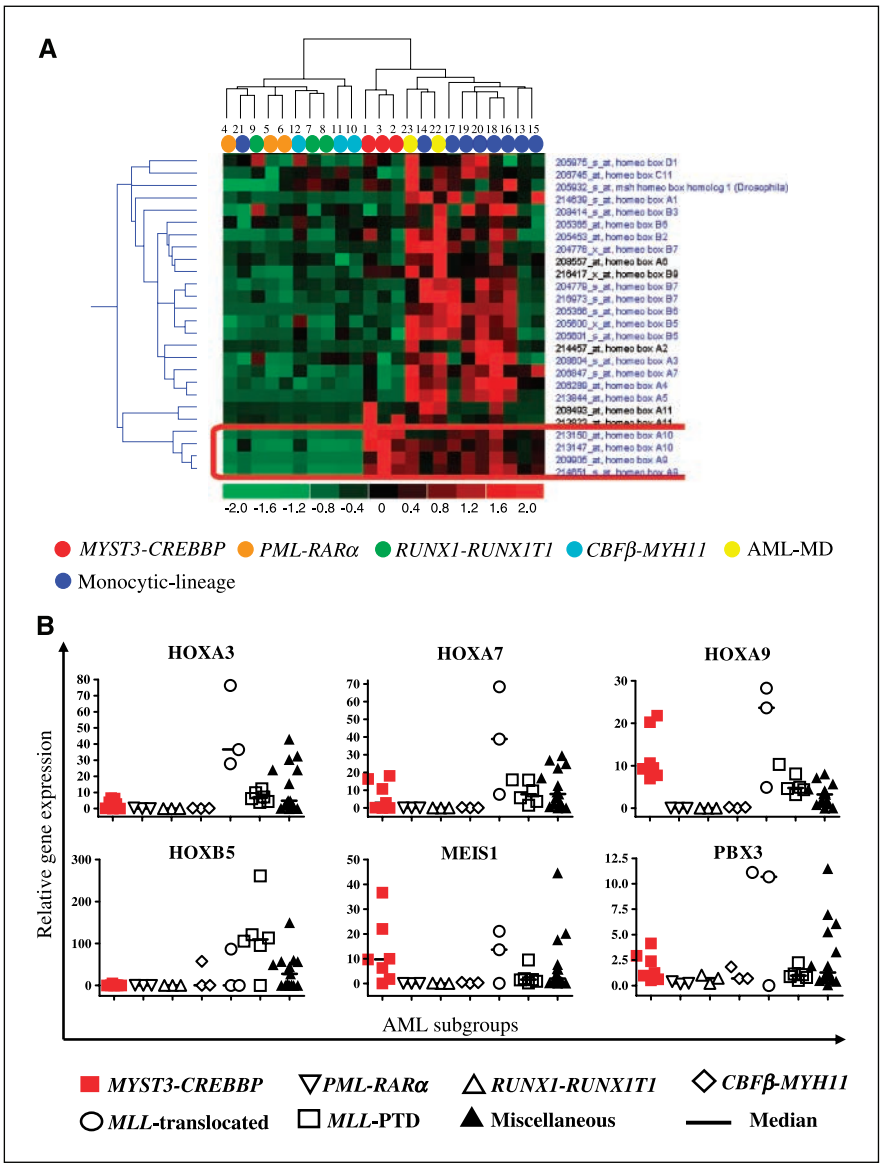

Figure 4. Gene expression signature of homeobox genes. *A,* high-density array study. Visualization of the homeobox genes in the unsupervised analysis using hierarchical clustering method. Several 5′-homeobox genes (*HOXA9, HOXB9*, and *HOXA10*) were overexpressed in *MYST3-CREBBP* samples and most cases of monocytic-lineage AML. In contrast, other *HOX* genes (*HOXA2-A7* and *HOXB2-B7*) showed a high expression level only in monocytic leukemias but not in *MYST3-CREBBP* samples. Overall, *HOX* expression was low in good-risk cytogenetic AML categories. *B,* low-density array study. Comparative expression of homeobox genes among the different AML categories using an ANOVA test confirmed the results of the high-density array study with high expression of *HOXA9* and *MEIS1* in *MYST3-CREBBP* cases and overall low expression in good-prognosis AML. In addition, *MLL*-rearranged cases showed the highest expression of all the *HOX* genes studied.

assessment of gene profile in a group of seven *MYST3-CREBBP* patients. Of note, a distinctive gene expression signature of *MYST3-CREBBP* leukemias was observed, characterized by the overexpression of homeobox genes *HOXA9, HOXA10*, and their cofactor *MEIS1*; the up-regulation of the oncogenes *RET* and *PRL*; and the decreased expression of genes such as *CCND2, STAT5*, and *WT1*. This profile harbors some similarities with that of *MLL*-rearranged leukemias, thus suggesting a partially common leukemogenic pathway.

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References

1. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1612 patients entered into the MRC AML 10 Trial. Blood 1998;92:2322–3.
2. Byrd JC, Mrózek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with *de novo* acute myeloid leukemia: results from Cancer and Leukemia Group (CALGB 8461). Blood 2002;100:4325–36.
3. Hanslip JL, Swansbury GJ, Pinkerton R, Catovsky D. The translocation t(8;16)(p11;p13) defines an AML subtype with distinct cytology and clinical features. Leuk Lymphoma 1992;6:479–86.
4. Borrow J, Stanton VP, Andresen JM, et al. The translocation t(8;16)(p11;p13) of acute myeloid leukemia fuses a putative acetyltransferase to the CREB-binding protein. Nat Genet 1996;14:33–41.
5. Sun T, Wu E. Acute monoblastic leukemia with t(8;16): a distinct clinicopathologic entity; report of a case and review of the literature. Am J Hematol 2001;66:207–12.
6. Bernasconi P, Orlandi E, Cavigliano P, et al. Translocation (8;16) in a patient with acute myelomonocytic leukemia, occurring after treatment with fludarabine for

a low-grade non-Hodgkin's lymphoma. Haematologica 2000;85:1087–91.
7. Champagne N, Pelletier N, Yang X-J. The monocytic leukemia zinc finger protein MOZ is a histone acetyltransferase. Oncogene 2001;20:404–9.
8. Jacobson S, Pillus L. Modifying chromatin and concepts of cancer. Curr Opin Genet Dev 1999;9:175–84.
9. Allard S, Masson J-Y, Côté J. Chromatin remodeling and the maintenance of genome integrity. Biochim Biophys Acta 2004;1677:158–64.
10. Kitabayashi I, Aikawa Y, Nguyen LA, Yokoyama A, Ohki M. Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. EMBO J 2001;20:7184–96.
11. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999;286:531–7.
12. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 2002;30:41–7.
13. Valk PJ, Delwel R, Lowenberg B. Gene expression profiling in acute myeloid leukemia. Curr Opin Hematol 2005;12:76–81.
14. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med 2004;350:1617–28.
15. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 2002;100:2292–302.
16. Rozman M, Camós M, Colomer D, et al. Type I *MOZ-CBP* (*MYST3-CREBBP*) is the most common chimeric transcript in acute myeloid leukemia with t(8;16)(p11;p13) translocation. Genes Chromosomes Cancer 2004;40:140–5.
17. Chomczynski P, Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
18. van Dongen JJ, Macintyre EA, Gabert JA, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 1999;13:1901–28.
19. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 2002;99:4326–35.
20. Boissel N, Renneville A, Biggio V, et al. Prevalence, clinical profile, and prognosis of *NPM* mutations in AML with normal karyotype. Blood 2005;106:3618–20.
21. Lo Coco F, Mandelli F, Breccia M, et al. Southern blot analysis of *ALL-1* rearrangements at chromosome 11q23 in acute leukemia. Cancer Res 1993;53:3800–3.
22. Kobayashi H, Espinosa R III, Thirman MJ, et al. Heterogeneity of breakpoints of 11q23 rearrangements in hematologic malignancies identified with fluorescence *in situ* hybridization. Blood 1993;82:547–51.
23. Caligiuri MA, Strout MP, Schichman SA, et al. Partial tandem duplication of ALL-1 as a recurrent molecular defect in acute myeloid leukemia with trisomy 11. Cancer Res 1996;56:1418–25.
24. Hubner RA, Houlston RS. Molecular advances in medullary thyroid cancer diagnostics. Clinica Chimica Acta. In press 2006.
25. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 1998;19:225–68.
26. Hatfill SJ, Kirby R, Hanley M, Rybicki E, Böhm L. Hiperprolactinemia in acute myeloid leukemia and indication of ectopic expression of human prolactin in blast cells of a patient of subtype M4. Leuk Res 1990;14:57–62.
27. Kooijman R, Gerlo S, Coppens A, Hooghe-Peters EL. Myeloid leukemic cells express and secrete bioactive pituitary-sized 23 kDa prolactin. J Neuroimmunol 2000;110:252–8.
28. Gerlo S, Verdood P, Hooghe-Peters EL, Kooijman R. Multiple, PKA-dependent and PKA-independent signals are involved in cAMP-induced PRL expression in the eosinophilic cell line Eol-1. Cell Signal 2005;17:901–9.
29. Ales N, Flynn J, Byrd JC. Novel presentation of acute myelogenous leukemia as symptomatic galactorrhea. Ann Intern Med 2001;135:303–4.
30. Gattei V, Degan M, Rossi FM, et al. The RET receptor tyrosine kinase, but not its specific ligand, GDNF, is preferentially expressed by acute leukemias of monocytic phenotype and is up-regulated upon differentiation. Br J Haematol 1999;105:225–40.
31. Gattei V, Degan M, Aldinucci D, et al. Differential expression of the RET gene in human acute myeloid leukemia. Ann Hematol 1998;77:207–10.
32. Muller-Tidow C, Schwable J, Steffen B, et al. High-throughput analysis of genome-wide receptor tyrosine kinase expression in human cancers identifies potential novel drug targets. Clin Cancer Res 2004;10:1241–9.
33. Visser M, Hofstra RM, Stulp RP, et al. Absence of mutations in the RET gene in acute myeloid leukemia. Ann Hematol 1997;75:87–90.
34. Armstrong SA, Golub TR, Korsmeyer SJ. Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. Cancer Cell 2003;3:173–83.
35. Libura M, Asnafi V, Tu A, et al. FLT3 and MLL intragenic abnormalities in AML reflect a common category of genotoxic stress. Blood 2003;102:2198–204.
36. Kohlmann A, Schoch C, Dugas M, et al. New insights into MLL gene rearranged acute leukemias using gene expression profiling: shared pathways, lineage commitment, and partner genes. Leukemia 2005;19:953–64.
37. Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. The pathophysiology of HOX genes and their role in cancer. J Pathol 2005;205:154–71.
38. Kasper LH, Brindle PK, Schnabel CA, Pritchard CE, Cleary ML, van Deursen JM. CREB binding protein interacts with nucleoporin-specific FG repeats that activate transcription and mediate NUP98-HOXA9 oncogenicity. Mol Cell Biol 1999;19:764–76.
39. Drabkin HA, Parsy C, Ferguson K, et al. Quantitative HOX expression in chromosomally defined subsets of acute myelogenous leukemia. Leukemia 2002;16:186–95.
40. Roche J, Zeng C, Baron A, et al. Hox expression in AML identifies a distinct subset of patients with intermediate cytogenetics. Leukemia 2004;18:1059–63.
41. Debernardi S, Lillington DM, Chaplin T, et al. Genome-wide analysis of acute myeloid leukemia with normal karyotype reveals a unique pattern of homeobox gene expression distinct from those with translocation-mediated fusion events. Genes Chromosomes Cancer 2003;37:149–58.
42. So CW, Cleary ML. Dimerization: a versatile switch for oncogenesis. Blood 2004;104:919–22.
43. Weisser M, Kern W, Rauhut S, et al. Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia. Leukemia 2005;19:1416–23.

Gene Expression Profiling of Acute Myeloid Leukemia with Translocation t(8;16)(p11;p13) and *MYST3-CREBBP* Rearrangement Reveals a Distinctive Signature with a Specific Pattern of *HOX* Gene Expression

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