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Promoter DNA methylation and expression levels of *HOXA4*, *HOXA5* and *MEIS1* in acute myeloid leukemia

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Abstract. *HOXA* genes encode transcription factors, which are crucial for embryogenesis and tissue differentiation and are involved in the early stages of hematopoiesis. Aberrations in *HOXA* genes and their cofactor *MEIS1* are found in human neoplasms, including acute myeloid leukemia (AML). The present study investigated the role of *HOXA4*, *HOXA5* and *MEIS1* promoter DNA methylation and mRNA expression in AML. Samples from 78 AML patients and 12 normal bone marrow (BM) samples were included. The levels of promoter DNA methylation were determined using quantitative methylation-specific polymerase chain reaction (PCR; qMSP) and the relative expression levels were measured using reverse transcription quantitative PCR in Ficoll-separated BM mononuclear cells and in fluorescent activated cell sorting-sorted populations of normal hematopoietic progenitors. In total, 38.1 and 28.9% of the patients exhibited high methylation levels of *HOXA4* and *HOXA5,* respectively, compared with the control samples, and *MEIS1* methylation was almost absent. An inverse correlation between *HOXA4* methylation and expression was identified in a group of patients with a normal karyotype (NK AML). An association between the genes was observed and correlation between the DNA methylation and expression levels of the *HOXA* gene promoter with the expression of *MEIS1* was observed. Patients with favorable chromosomal aberrations revealed a low level of *HOXA4* methylation and decreased expression levels of *HOXA5* and *MEIS1* compared with the NK AML and the adverse cytogenetic risk patients. The NK AML patients with *NPM1* mutations exhibited elevated *HOXA4* methylation and expression levels of *HOXA5* and *MEIS1* compared with the *NPM1* wild-type patients. Comparison of the undifferentiated BM-derived hematopoietic $CD34^+CD38^{low}$, $CD34^+CD38^+$ and $CD15^+$ cells revealed a gradual decrease in the expression levels of these three genes and an increase in *HOXA4* promoter methylation. This differentiation-associated variability was not observed in AML, which was classified according to the French-American-British system.

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Introduction

Acute myelogenous leukemia (AML) is a heterogeneous disease caused by the uncontrolled proliferation of myeloid precursor cells with abnormal maturation.

Genes encoding transcription factors (TF), involved in normal hematopoiesis, are among the most common targets of genetic aberrations in AML (1). This involves chromosomal translocations, including the most prevalent t (8;21) (RUNX1-ETO), inv (16) (CBF-MYH11) and 11q23 *MLL* rearrangements and point mutations in key regulators of hematopoietic cell differentiation, termed class II mutations. Among these point mutations, the most frequent are changes in *CEBPA*, *NPM1*, *RUNX1* and *MLL* (2). Recurrent chromosomal translocations, together with *CEBPA* and *NPM1* mutations, constitute the basis of the current World Health Organization (WHO) classification and are included in the guidance for AML patients risk stratification (3).

In addition to genetic alterations, epigenetic aberrations are also involved in leukemogenesis. DNA methylation is one of the most widely described epigenetic elements involved in regulating gene expression. Aberrant DNA methylation in the regulatory regions of the genes encoding hematopoietic TFs has been observed in AML patients, including early-acting TFs, such as HOX cluster genes (4).

The present study focused on the role of DNA methylation and the expression levels of three selected early-acting hematopoietic TFs genes: *HOXA4*, *HOXA5* and *MEIS1* in AML.

HOXA genes are clustered at the 7p15.2 chromosomal region and these encode TFs that are crucial for embryogenesis and tissues differentiation (5). HOXA proteins are also involved in the early stages of hematopoiesis and lineage specification (6).

HOXA4 controls hematopoietic stem cell (HSC) self-renewal and the expansion of either myeloid- and lymphoid-primed progenitors (7). In addition to its paralogs, HOXB4 and HOXD4, HOXA4 is also involved in the inhibition of cell differentiation (8).

Another HOXA cluster gene, *HOXA5,* governs the specification of myeloid and erythroid lineages. Its constitutive expression inhibits erythropoiesis and promotes myelopoiesis (9,10). MEIS1 is a transcription activator-like effector protein, which functions as a HOX protein cofactor. This protein exerts its function by enhancing HOXA4 and HOXA5 DNA binding specificity (11).

Aberrations in the *HOXA* and *MEIS1* genes have been identified in human neoplasms, including AML (5). The *HOXA4*, *HOXA5* and *MEIS1* genes are frequently hypermethylated in different types of leukemia, including adult AML (4,12). Previous studies have demonstrated the prognostic value of *HOXA4* and *HOXA5* methylation in leukemia patients (4,13).

Materials and methods

Patients. Bone marrow (BM) samples from 78 AML patients were used and 12 BM samples were obtained from healthy donors as controls. The patients were classified based on their French-American-British (FAB) subtypes and their cytogenetic statuses. The BM mononuclear cells (BMMC) were isolated from the BM samples using Ficoll density gradient centrifugation at 400 x g for 30 min at room temperature. The patient characteristics are shown in Table I. The present study was approved by the committee of M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology (Warsaw, Poland).

Cell sorting. The $CD34^+$ progenitor cells were purified from normal BMMC samples using a Dynabeads® CD34 Positive Isolation kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The $CD34^+$ fraction was subsequently labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 and APC-conjugated anti-CD38 antibodies. The depleted fraction (CD34-) was labeled with FITC-conjugated anti-CD15 antibodies (all antibodies were purchased from BD Biosciences, Franklin Lakes, NJ, USA). The $CD34^+CD38^-$, $CD34^+CD38^+$ and $CD15^+$ BM cell populations were sorted using a BD FACSAria (BD Biosciences). The results are presented in Fig. 1.

Quantitative DNA methylation analysis. The DNA was extracted from the BMMC using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and subsequently subjected to bisulfite conversion using an EpiTect kit (Qiagen). The cells isolated by fluorescence-activated cell-sorting were subjected to direct bisulfite conversion using an EpiTect Lyse All kit (Qiagen). The levels of DNA methylation were determined by quantitative methylation-specific PCR (qMSP). Each region of interest was amplified with methylation-specific primer pairs using reverse transcription (RT) qPCR in parallel to a reference *ACTB* region containing no CpG dinucleotides. The RT-qPCR

Table I. Characteristics of patients with acute myeloid leukemia in the present study.

Patient characteristic	Number of patients[b]
Gender	
Male	38/78
Female	40/78
Age (years)	
<60	64/77
≥60	13/77
FAB classification	
M0	6/76
M1	16/76
M2	16/76
M3	10/76
M4	15/76
M5	13/76
Cytogenetics	
Inv (16)	7/76
T (8;21)	9/76
T (15;17)	8/76
3q/11q abnormalities	6/76
Complex karyotype	4/76
Normal karyotype	31/76
Other	11/76
Cytogenetic risk	
Favorable	24/76
Intermediate	33/76
Adverse	19/76
CEBPA mutations[a]	
Negative	25/29
Positive	4/29
NPM1 mutations[a]	
Negative	12/29
Positive	17/29
FLT3-Itd	
Negative	57/76
Positive	19/76
MLL-Ptd	
Negative	56/61
Positive	5/61
WBC count at diagnosis (10^3/Ml)	
Median	23,15
Range	0.3-331.1
Blast percentage in bone marrow	
Median	75
Range	6.3-98

[a]Assessed in patients with a normal karyotype. FAB, French-American-British; WBC, white blood cell. [b]Number of patients with a trait/total number of patients with a given characteristic assessed.

Table II. Sequences of polymerase chain reaction primers and probes used for quantitate methylation-specific analysis.

Gene	Forward	Reverse	Probe
HOXA4	5'-GTAGTATTTATT ACGTATTCGCGC-3'	5'-CCGTACCCC ACGTACAACG-3'	5'Fam-CCCCACCAATAA ACGCACCGCG-Tamra-3'
HOXA5	5'-AATGGGTTGTAA TTTTAATTCGATTTC-3'	5'-CGTTCAACC GAACTCGAACG-3'	5'Fam-AAAACAAAACTC ATCGCCCAACTTCCG-Tamra-3'
MEIS1	5'-TGCGGTTAG AGTTCGTTTCGC-3'	5'-CATAACAAATCG CGTCTTACACAA-3'	5'-CATTAAACTACAACAAAT AAACTCCTCGAC-Tamra-3'
ACTB	5'-TGGTGATGGAGG AGGTTTAGTAAGT-3'	5'-AACCAATAAAAC CTACTCCTCCCTTAA-3'	5'Fam-ACCACCACCCAACAC ACAATAACAAACACA-Tamra-3'

reaction was performed in 7.5 µl (1.5 µl bisulfite-converted DNA template, 2X SensiMix II Probe mastermix (Bioline, London, UK), 2.25 pmol of each primer (4.5 pmol of each primer for *ACTB*) and 1.88 pmol of the probe. The RT-qPCR amplification was performed using an Applied Biosystems 7900 HT Sequence Detection system (Applied Biosystems, Foster City, CA, USA) in 384-well plates. The sequences of the primers and probes used are shown in Table II.

The standard curves of known DNA template concentrations were used to quantify the resulting PCR product. These were prepared using serial dilutions of plasmid DNA containing PCR product inserts of each amplified region. The plasmid constructs were obtained by amplification of standard methylated genomic DNA (Qiagen), with the use of each primer pair and cloning of the PCR products using a Strataclone kit (Agilent, Santa Clara, CA, USA). The plasmid DNA was amplified in bacteria, purified using a Plasmid Mini kit (A&A Biotechnology, Gdynia, Poland) and quantified using Quanti-iT Picogreen (Invitrogen Life Technologies, Carlsbad, CA, USA).

The levels of DNA methylation, determined by the percentage of the methylated reference (PMR), was calculated by dividing the 'gene of interest':*ACTB* ratio of a patient sample by the 'gene of interest':*ACTB* ratio of the methylated DNA in the control sample (Qiagen) and multiplying by 100.

An EpiTect Control DNA and Control DNA set (Qiagen) containing human methylated and unmethylated DNA served as positive and negative control samples, respectively.

Expression analysis. RNA was isolated from the BMMC using an RNeasyMini kit (Qiagen) and quantified using a NanoDrop 2000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). Each RNA sample (500 ng) was subjected to RT using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). The RNA from the cells, sorted by flow cytometry, was extracted using RNAqueous-Micro (Ambion Life Technologies, Carlsbad, CA, USA) and subjected to RT, as previously.

The expression levels of *HOXA4*, *HOXA5* and *MEIS1* were assessed using gene expression assays: Hs00269972_s1, Hs00270931_s1 and Hs00357657_m1 (Applied Biosystems). The ubiquitin gene assay (Hs00824723_m1) (Applied Biosystems) was used as a reference. RT-qPCR was performed using the following cycling conditions: 95˚C for 10 min followed by 45 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Gene expression was calculated using the $2^{-\Delta Ct}$ method.

Statistical analysis. The gene expression levels and DNA methylation levels (PMR) were analyzed using a two-sided Mann-Whitney U-test, Kruskal-Walis test and Spearman's correlation. For descriptive statistics, the quantitative methylation results were also categorized into binary data. The samples were classified as methylation-positive/methylation-high or methylation-low based on the assessment of DNA methylation level in the normal BM samples. The mean value ± two standard deviations of the BM results was used as a threshold for each gene independently. A two-sided exact Fisher's test was used for the comparison of proportions. $P<0.05$ was considered to indicate a statistically significant difference. The statistical evaluation and visualization was performed using GraphPad Prism software 5.03 (GraphPad, La Jolla, CA, USA).

Results

Promoter DNA methylation and gene expression levels in AML patients and controls. The levels of DNA methylation were assessed using qMSP in 78 AML patient samples and 12 normal BM samples. The promoter regions of *HOXA4* and *HOXA5* revealed variable degrees of methylation in the patient-derived samples and the normal BM cells. AML samples with a methylation level exceeding the threshold value, described previously, were classified as methylation-high. According to these criteria, high levels of *HOXA4* and *HOXA5* promoter DNA methylation were observed in 38.1 (30/76) and 28.9% (21/76), respectively, in the AML patients.

A degree of *HOXA4* and *HOXA5* promoter methylation in the three sorted hematopoietic progenitors populations was also observed. The level of *HOXA4* methylation was higher in the immature BM-derived $CD15^+$ granulocytes compared with the early $CD34^+$ progenitors. The *HOXA5* methylation level, however, did not vary between the sorted progenitor fractions, but was higher compared with the total BMMC. DNA methylation was not observed in the *MEIS1* promoter in either the patient or normal samples. The results are shown in Fig. 1C.

The RNA extraction and assessment of relative gene expression levels were successfully performed in 70 patient and 8 BM samples. All three genes of interest exhibited homogenous, low levels of expression in the normal BM samples. Marked heterogeneity was observed in the AML patient expression levels of *HOXA4*, *HOXA5* and *MEIS1*, with a notable proportion of patients having high expression levels compared to the BM cells (Fig. 1A-C).

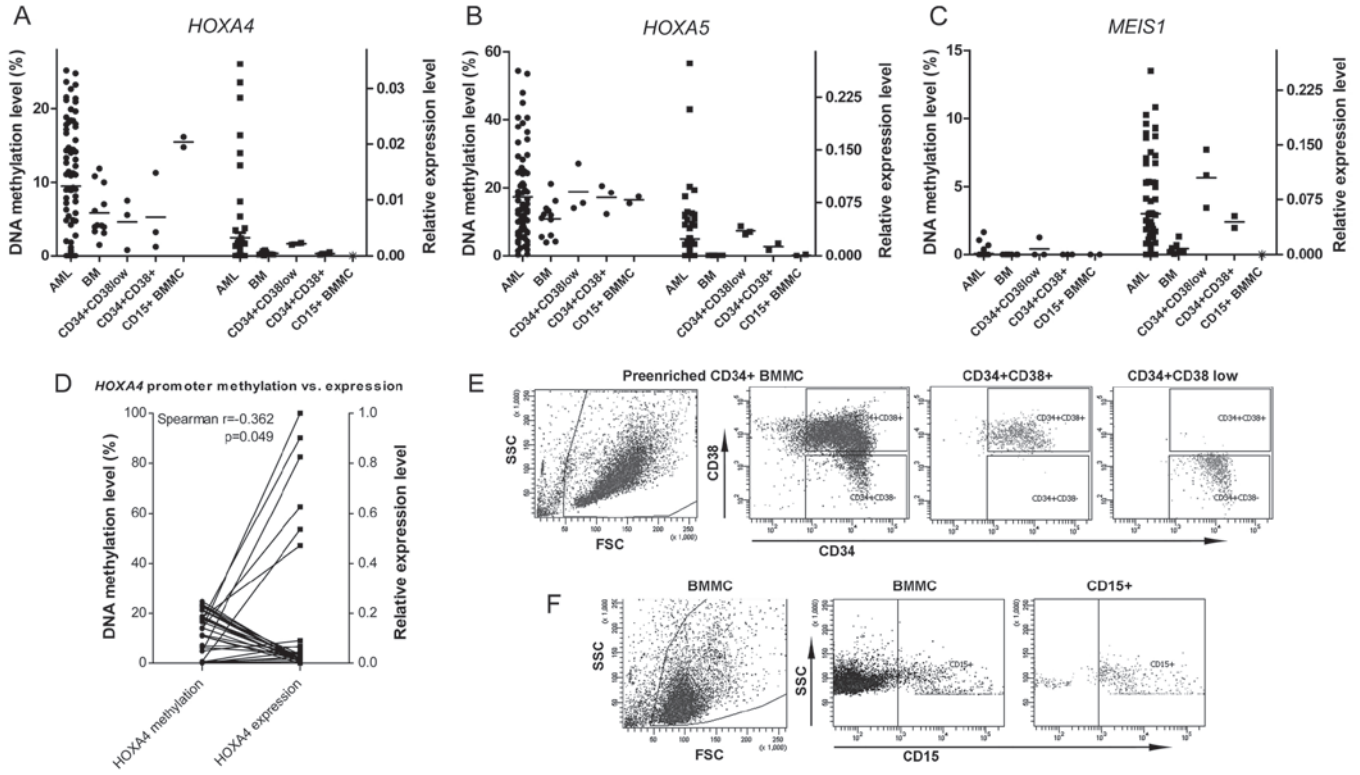

Figure 1. Comparison between the promoter DNA methylation and the relative expression levels of (A) *HOXA4*, (B) *HOXA5* and (C) *MEIS1* in AML and normal BM and $CD34^+CD38^{low}$, $CD34^+CD38^+$ and $CD15^+$ cells isolated from normal BMMC. The horizontal lines represent the mean value. (D) Correlation of *HOXA4* promoter DNA methylation and expression levels in the NK AML. Representative example of pre- and post-sort analysis of (E) normal BM $CD34^+CD38^{low}$, $CD34^+CD38^+$ and (F) $CD15^+$ cells. The cell populations were sorted using BD FACSAria (BD Bioscience). AML, acute myeloid leukemia; BM, bone marrow; BMMC, bone marrow mononuclear cells; NK AML, normal karyotype acute myeloid leukemia; SSC, side scatter; FSC, forward scatter.

The three examined genes were differentially expressed when the hematopoietic progenitors and immature $CD15^+$ cells were compared. The highest level of expression was observed in the earliest $CD34^+CD38^{low}$ progenitors, the expression level decreased in the more mature $CD34^+CD38^+$ cells and expression either further decreased or was lost in the $CD15^+$ cells (Fig. 1E). The decreased expression of *HOXA4* in the three sorted populations corresponded to an increase of gene promoter DNA methylation.

Correlation between the *HOXA4* and *HOXA5* promoter DNA methylation levels was observed in AML (Spearman r=0.3020; P=0.0080). Correlation was also observed between the expression levels of *HOXA4* and *HOXA5* and the expression of their cofactor *MEIS1* (Spearman r=0.3182; P=0.0068 and Spearman r=0.800; P<0.0001, respectively).

The analysis of the association between promoter methylation and expression levels in the entire group of AML patients revealed no statistically significant correlation. This was also preformed separately in cytogenetically normal AML patients and an inverse correlation was observed between the expression of *HOXA4* and the levels of gene methylation (Spearman r=-0.362; P=0.049; Fig. 1D).

Gene promoter DNA methylation, expression levels and cytogenetic risk. The DNA methylation and expression levels were compared in the AML patients, grouped according to their cytogenetic status, which constitutes the basis of the current WHO classification and risk assessment.

The patients in the favorable prognostic group demonstrated a lower level of *HOXA4* methylation compared with those in the intermediate and high risk groups. The NK patients and those carrying unfavorable translocations exhibited the highest variability in levels of *HOXA4* methylation (Fig. 2A). No significant differences were detected in the levels of *HOXA5* methylation between the patients with distinct cytogenetic risk or status.

The gene expression analysis revealed differences in the expression levels of *HOXA5* and *MEIS1* between patients with different cytogenetic statuses (Fig. 2B and C). All the patients carrying the favorable translocations, t(8;21), inv (16) and t(15;17), exhibited low expression levels of *HOXA5* and *MEIS1* and the expression levels of *HOXA5* and *MEIS1* were generally lower in the favorable prognostic group compared with the intermediate and high risk patients. Notably, all the patients with 3q aberrations revealed low expression levels of the two genes compared with the favorable risk patients. However, the 3q group consisted of only three patients.

No significant variation was observed in the *HOXA4* expression level between distinct cytogenetic risk and status groups.

Promoter DNA methylation/expression levels in patients grouped according to FAB. The FAB classification system has been commonly used to classify AML patients on the basis of leukemic cell morphology, cytochemistry and maturation (14). In the present study, the patients were grouped into

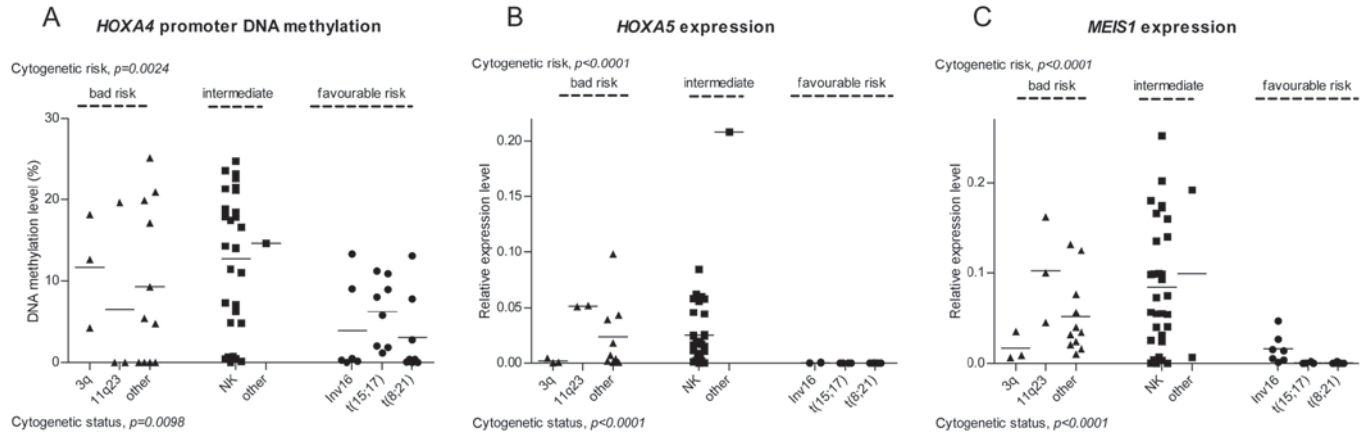

Figure 2. Comparison between the promoter methylation levels of (A) *HOXA4* and relative expression levels of (B) *HOXA5* and (C) *MEIS1* in AML, stratified according to cytogenetic status. Horizontal lines represent the mean. AML, acute myeloid leukemia; NK, normal karyotype.

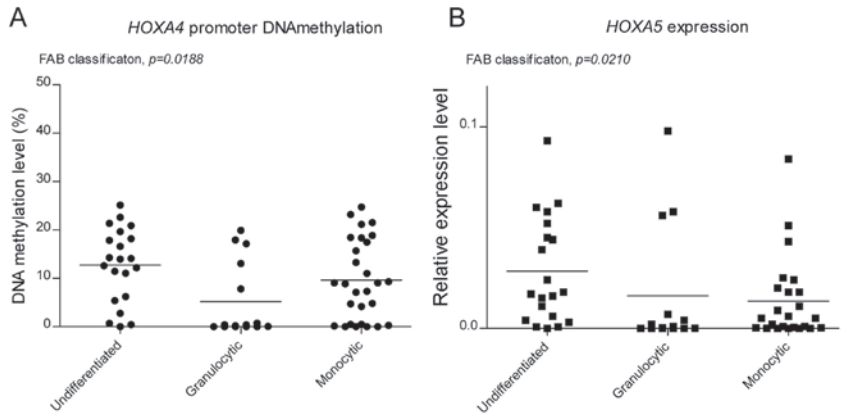

Figure 3. Differences in the (A) promoter methylation level of *HOXA4* and (B) relative expression level of *HOXA5* in AML, classified according to FAB into three categories: AML without differentiation, AML with granulocytic and AML with monocytic maturation. Horizontal lines represent the mean. AML, acute myeloid leukemia; FAB, French-American-British.

three categories based on the FAB classification system: AML without maturation (M0 and M1), AML with granulocytic maturation (M2) and AML with monocytic maturation (M4 and M5). These three groups were then compared in terms of *HOXA4*, *HOXA5* and *MEIS1* promoter methylation and expression levels. The M3 PML patients were excluded as they constitute a separate cytogenetic group defined by the presence of t(15:17), which is described above. The groups differed significantly in *HOXA4* promoter methylation and expression levels of *HOXA5* (Fig. 3A and B, respectively). The *HOXA4* DNA methylation level was lowest in the granulocytic AMLs and highest in the AMLs without maturation. This differed to the previous observations in the normal hematopoietic progenitors (Fig. 1A), where the undifferentiated $CD34^+$ cells exhibited lower *HOXA4* methylation compared with the immature granulocytes (BMMC $CD15^+$). The patients with granulocytic characteristics revealed the lowest expression levels of *HOXA5*, whereas the highest mean value was observed in patients without maturation.

In AML without maturation, high expression levels of *HOXA5* were observed compared with the AML with granulocytic differentiation, which was concordant with the observed difference between the isolated $CD34^+$ progenitor and the BMMC $CD15^+$ immature granulocytes (Fig. 1B).

DNA promoter methylation and mRNA expression analysis in the AML patients according to the FLT3-ITD and NPM1 mutation status in NK AML. The promoter methylation and expression levels of *HOXA4*, *HOXA5* and *MEIS1* were compared in the entire group of AML patients, stratified according to *FLT3*-ITD, which is a poor prognostic factor. The patients with *FLT3*-ITD (*FLT3*-ITD+) demonstrated significantly elevated expression levels of *HOXA5* and *MEIS1* compared with those without mutation with a 3.57-fold (P=0.0043) and 2.38-fold (P=0.0048) change in mean relative expression, repectively. No differences were observed in DNA promoter methylation levels depending on the presence of FLT3-ITD.

Mutations in *NPM1* and *CEBPA* are favorable prognostic factors, which are used for the risk assessment in AML patients without recurrent chromosomal abnormalities (2). The present study compared the methylation and expression levels of the three genes of interest in NK AML, stratified according to *NPM1* mutation status. The results revealed elevated *HOXA4* methylation (2.03-fold change in the mean DNA methylation level; P=0.0178) and elevated expression levels of *HOXA5* and *MEIS1* in patients carrying the *NPM1* mutation (*NPM1*+) (3.27-fold change; P=0.0007 and 2.38-fold change; P=0.0048, respectively). Since *CEBPA* mutations were identified in only four AML patients, statistical evaluation was not performed.

Discussion

HOXA transcription factors and their cofactor *MEIS1* are among the key regulators of development and differentiation. They are involved in the initial stages of hematopoiesis and contribute to subsequent lineage specification (8-10). Aberrant *HOXA* expression is associated with numerous types of cancer and DNA hypermethylation has been identified as a mechanism partially responsible for the downregulation of these genes (5).

The present study focused on the role of promoter DNA methylation and expression levels of *HOXA4*, *HOXA5* and *MEIS1* in AML.

HOXA4 and *HOXA5* promoters have been previously described to be frequently methylated in AML (4). Analysis of these genes in the present study revealed promoter methylation in normal BM samples (mean 4.1 and 11.5% for *HOXA4* and *HOXA5*, respectively). Therefore a cutoff value for methylation-high samples was applied based on the results for the normal BM. The frequency of *HOXA4* and *HOXA5* hypermethylation observed in the present study differed from previous studies, possibly resulting from the use of different analytical techniques and threshold levels for 'methylated' sample classification. *HOXA4* hypermethylation was previously reported to occur in 64% (by combined bisulfite restriction analysis; COBRA) (4) and 77% (by methylation-sensitive melting curve analysis) (12) of patients compared with 39.4% of patients in the present study. *HOXA5* hypermethylation in AML was previously reported as 36% (13) by pyrosequencing and 60 and 59% by COBRA (4,15), compared to 27.6% in the present study. A slight correlation between the DNA methylation levels of *HOXA4* and *HOXA5* were observed in the AML samples in the present study, which may reflect the fact that the two *HOXA* genes are located closely within the same chromosomal region and are transcriptionally coregulated. This association was also observed in childhood leukemia (4). Previously reported results, indicating that *MEIS1* is hypermethylated in 15% of AML patients and frequently methylated in patients with t(8:21) (16) were not observed in the present study.

Gene expression is regulated through epigenetic mechanisms, which include DNA methylation. It has been proposed that *HOXA4* downregulation may be associated with promoter methylation status, which was supported by observations in chronic lymphoblastic leukemia (17) and AML (12). *HOXA5* promoter methylation regulates gene expression in myeloid leukemia cell lines (15).

AML is a heterogenous and complex disease, in which chromosomal aberrations are important in determining the leukemia biology and prognosis (3). The biological diversity of AML is the predominant factor, which may explain the lack of correlation between the promoter methylation and the expression levels of *HOXA4* and *HOXA5* in the entire group of patients. This association was additionally analyzed in NK AML and an inverse correlation of *HOXA4* methylation and expression was observed. The comparison of $CD34^+CD38^{low}$, $CD34^+CD38^+$ and $CD15^+$ BM derived cells also supported the involvement of *HOXA4* promoter methylation in regulating gene expression, as an increase in DNA methylation levels corresponded to a gradual decrease of RNA expression.

The role of *HOXA4* expression in myeloid leukemogenesis has been investigated previously. AML patients with low expression levels of *HOXA4* have a poor prognosis in terms of overall survival (18,19), however, a previous study identified *HOXA4* among the genes overexpressed in patients with poor outcome (20). The prognostic role is possibly more complex as it also dependent on the expression of *MEIS1* (12).

The role of low expression levels of *HOXA4* as an adverse prognostic factor has been observed in a subgroup of cytogenetically normal patients (21) and also on an entire group of AML patients (18), despite the fact that low levels of *HOXA4* occur in patients with favorable translocations, particularly t(15:17) (22,23). The poor survival rates observed in AML with low *HOXA4* expression in a study by Tholouli *et al* (18) was possibly due to the high representation of NK AML patients and the fact that this group of patients is characterized by a high and heterogenous level of gene expression (18,21,24,25). The expression of *HOXA4* may therefore be involved, particularly in molecularly heterogenous cytogenetically normal patients, for which new prognostic markers are required.

In the present study, no statistically significant difference was observed in the expression of *HOXA4* between the AML cytogenetic groups, however different levels of promoter methylation were observed in this gene. A high, but variable *HOXA4* methylation level was observed in patients with NK AML and those with adverse cytogenetic risk compared with patients a with favorable prognosis. This was concordant with the previously reported differences in the frequency of *HOXA4* hypermethylation between the cytogenetic risk groups (12). Considering the observed association between *HOXA4* promoter methylation and expression levels in NK AML and the prognostic role of the expression of this gene in AML, it was suggested that the DNA methylation level of this gene may have a prognostic value. This is consistent with previously reported data, in which *HOXA4* was among the genes upregulated in NK AML patients with the *NPM1* mutation, a favorable prognostic factor (23,26). However, this is inconsistent with the observation in the present study of a higher *HOXA4* methylation level in a group of NK AML patients with the *NPM1* mutation.

Different levels of *HOXA4* promoter DNA methylation were observed when groups of patients, stratified according to the FAB classification system, were compared. However, by contrast to the results in normal hematopoietic precursors, the highest methylation level was observed in undifferentiated AMLs. Aberrant *HOXA4* methylation in leukemic BM samples may be associated with the neoplastic nature of leukemic cells rather than their differentiation stage.

HOXA5 promoter methylation was previously identified as an independent prognostic factor in a group of AML patients (13). As this study involved a small number of patients, the observed prognostic role was considered a possible result of higher levels of *HOXA5* methylation in the favorable cytogenetic group. In the present study, no difference in the methylation levels between the prognostic groups of patients was observed, which indirectly supported the previous finding. In the AML patients, no association was found between the *HOXA5* methylation and expression levels, as observed in myeloid leukemia cell lines (15).

Unlike *HOXA5* promoter methylation, diverse gene expression levels were observed when patients with different molecular profiles were analyzed. Similar to *HOXA4* promoter methylation, the expression levels of either *HOXA5* or *MEIS1* were lowest in the cytogenetically favorable patients and the

FLT3 wild-type patients, as reported previously (25). Notably, in NK AML, the two genes were expressed at higher levels in the patients with *NPM1* mutations, as has been observed in genome-wide expression profiling (27,28). This suggested that the *HOXA* and *MEIS1* genes may have slightly different roles in NK AML compared with AML patients with cytogenetic abberations.

Elevated expression levels of the three genes were observed in AML patients, compared with normal BM patients. The *HOXA* and *MEIS1* genes were expressed at relatively high levels in the primitive $CD34^+CD38^{low}$ cells, decreased in the $CD34^+CD38^+$ progenitors and were further decreased or absent in the BM derived $CD15^+$ cells. It is possible that the expression levels of the *HOXA* genes reflect the differentiation stage of leukemic blasts, however the regulation of these genes appeared to be generally impaired in AML. The expression levels of *HOXA4* and *HOXA5* may exceed their expression levels in normal cells, including normal early progenitors. The phenotypic features of the AML samples, according to the FAB classification, were not associated with *HOXA* or *MEIS1* methylation or expression, with the exception of higher expression levels of *HOXA5* in AML without differentiation compared with AML with granulocytic and monocytic differentiation. Similar observations have been reported previously, in which M1 patients revealed higher expression levels of *HOXA5* compared with other AML patients (29).

It appears that there is an association between *HOXA4*, *HOXA5* and *MEIS1* in AML. The two investigated *HOXA* genes are closely associated. They are located in the same gene locus and appear to be transcriptionally coregulated with *MEIS1*. The present study observed a correlation between the promoter methylation and gene expression of the *HOXA* genes and also observed a correlation between their expression and that of *MEIS1*. This association was explicit between *HOXA5* and *MEIS1* and the two genes exhibited corresponding mRNA levels in the distinct molecular groups of the AML patients.

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