OncoTargets and Therapy

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ORIGINAL RESEARCH

Higher expression levels of the *HOXA9* gene, closely associated with *MLL-PTD* and *EZH2* mutations, predict inferior outcome in acute myeloid leukemia

This article was published in the following Dove Press journal:
OncoTargets and Therapy
9 February 2016
Number of times this article has been viewed

Li Gao[1,*]
Junzhong Sun[2,*]
Fang Liu[2,3,*]
Hui Zhang[1]
Yigai Ma[1]

[1]Department of Hematology, China–Japan Friendship Hospital, [2]Department of Hematology and Oncology, The First Affiliated Hospital of Chinese PLA General Hospital, [3]Department of Oncology, Chinese PLA General Hospital, Beijing, People's Republic of China

*These authors contributed equally to this work

Background: Although the biological insight of acute myeloid leukemia (AML) has increased in the past few years, the discovery of novel discriminative biomarkers remains of utmost value for improving outcome predictions. Systematical studies concerning the clinical implications and genetic correlations of *HOXA9* aberrations in patients with AML are relatively promising.

Materials and methods: Here, we investigated mutational status and the mRNA levels of the *HOXA9* gene in 258 patients with AML. Furthermore, hematological characteristics, chromosome abnormalities, and genetic mutations associated with AML were analyzed, followed by the assessment of clinical survival. Besides, the expression level and mutational status of *MEIS1*, a cofactor of *HOXA9*, were also detected in patients with AML with the aim of a deeper understanding about the homeodomain-containing transcription factors associated with hematological characteristics.

Results: *HOXA9* and *MEIS1* mutations were detected in 4.26% and 3.49% AML cases, respectively. No correlations were detected between mutation status and clinical characteristics, cytogenetic and genetic aberrations, and clinical survival. Higher *HOXA9* expression levels were correlated with white blood cell count and closely associated with unfavorable karyotype as well as *MLL-PTD* and *EZH2* mutations, whereas, there was an inverse correlation with the French–American–British M3 subtype. Compared with patients with lower *HOXA9* expression levels, those with higher *HOXA9* expression levels had a lower complete remission rate and inferior survivals in both AML and cytogenetically normal AML.

Conclusion: *HOXA9* expression may serve as a promising biomarker to ameliorate a prognostic model for predicting clinical outcome and consummating individualized treatment in patients with AML.

Keywords: acute myeloid leukemia, *HOXA9*, expression, clinical survival

Introduction

Recent cytogenetic and genetic findings have described the molecular mechanisms underpinning hematological cell malignant transformation and progression.[1] There are a number of well-established models for the cytogenetic and genetic classification of acute myeloid leukemia (AML) into different prognostic groups.[2,3] Although the biological insight of AML has increased in the past decade, the discovery and assessment of novel discriminative biomarkers remain of utmost value for improving outcome predictions.[4,5]

A sizable amount of new data points to a vital role for the *HOX* family of homeobox genes in hematopoietic development.[6,7] Increasing evidence and theoretical basis

Correspondence: Yigai Ma
Department of Hematology, China–Japan Friendship Hospital, Yinghua East Road, Beijing 100029, People's Republic of China
Tel +86 10 8420 5522
Fax +86 10 8420 5379
Email dr_myg@163.com

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http://dx.doi.org/10.2147/OTT.S95279

promulgated and attested that aberrant *HOXA* expression is functionally significant in myeloid transformation. One of them, *HOXA9*, part of the cluster on chromosome 7p15, is expressed under specific physiological and molecular environments in primitive hematopoietic cells of human origin and encodes a DNA-binding transcription factor that may participate in the regulation of gene expression, thus coordinate morphogenesis and differentiation.[8,9] The expression pattern of *HOXA9* in hematopoietic cells is specific to both lineage and differentiation stage. This expression is downregulated as blood cells differentiate, indicating a distinctive function in early hematopoiesis.[10,11] An increasing amount of evidence demonstrated that *HOXA9* overexpression maintains the self-renewal capacity of leukemic stem cells and blocks their differentiation, thus contributing to leukemogenesis and frequently occurring in association with 11q23 translocations and 11p15 translocations.[12–15]

Considering various expressions of *HOXA9* in leukemic and normal bone marrow cells and its crucial role in the regulation of hematopoietic development and differentiation, clinical impacts of the *HOXA9* transcript level and mutational status in adult AML, as well as the correlations with genetic aberrations, are not yet fully and systematically understood. In this study, we sought to characterize the clinical relevance of the *HOXA9* transcript level and mutational status, followed by the correlation between *HOXA9* aberrations with cytogenetic data, mutation status, and clinical prognosis. Besides, various AML studies have elaborated dysregulation of *HOXA9*, almost always in association with abnormal expression of its cofactor Meis homeobox 1 (*MEIS1*).[16,17] In addition, a wide range of data suggests that *HOXA9* and *MEIS1* play a synergistic causative role in AML, although the molecular mechanisms leading to transformation by *HOXA9* and *MEIS1* remain elusive.[18–20] Hence, in this study, the expression level and mutational status of *MEIS1* were also detected in patients with AML with the aim of a deeper understanding about the homeodomain-containing transcription factors contributing to leukemogenesis.

Materials and methods

Patient samples

A total of 258 patients with newly diagnosed AML and 25 healthy donors attending China-Japan Friendship Hospital, Chinese PLA General Hospital, and the First Affiliated Hospital of Chinese PLA General Hospital between July 2006 and March 2015 were enrolled in this study. The study was approved by the ethics committees of the China-Japan Friendship Hospital, Chinese PLA General Hospital, and the First Affiliated Hospital of Chinese PLA General Hospital. Written informed consent was obtained from each patient for sample preservations and aenetics analyses. Diagnosis and classification of AML were made according to French–American–British (FAB) cooperative group's criteria. Available clinical characteristics were age, sex, FAB subtype, white blood cell and platelet counts, amount of bone marrow blasts, and hemoglobin levels. All patients with non-M3 AML received intensive induction therapy with daunorubicin and cytarabine or mitoxantrone and cytarabine or decitabine (demethylating treatment) followed by consolidation therapy with cytarabine-based therapy. For M3 patients with t(15;17), all-trans retinoic acid and arsenic trioxide-based treatment was given for induction and consolidation therapy, of which five patients were treated with cytarabine-based therapy as a part of consolidation for high-risk diagnosis. Twenty-nine patients underwent allogeneic hematopoietic stem cell transplantation and 26 patients received autologous hematopoietic stem cell transplantation. The clinical characteristics of the patients are described in Table 1.

Clinical end points

Complete remission (CR) was defined as recovery of morphologically normal bone marrow and blood counts and no circulating leukemic blasts or evidence of extramedullary leukemia. Relapse was defined as ≥5% bone marrow blasts, circulating leukemic blasts, or the development of extramedullary leukemia. Overall survival (OS) was calculated from the date of diagnosis until the date of death and was censored for patients who were alive at the last follow-up. Relapse-free survival (RFS) was defined as the time from the date of CR until the date of relapse or death regardless of cause and was censored for patients who were alive at the last follow-up.

Karyotype analysis

Cytogenetic analysis was performed in bone marrow samples that were obtained during diagnosis using a direct method or short-term culture. Cytogenetic reports were independently reviewed by two expert cytogeneticists who were blinded to the patients' clinicopathological information. Metaphase chromosomes were banded by G-banding techniques, while chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature.[21] The diagnosis of normal cytogenetics or cytogenetic aberrations was based on the analysis of ≥20 metaphases in specimens. Complex cytogenetic abnormalities were defined as the presence of at least three unrelated cytogenetic abnormalities in one clone. According to the published criteria adopted by Southwest Oncology Group, cytogenetic

Table 1 Comparison of clinical manifestations and laboratory features between patients with AML by *HOXA9* expression level

Variables	Total (n=258)	Lower *HOXA9* expression (n=194)	Higher *HOXA9* expression (n=64)	*P*-value
Sex (male/female)	258 (139/119)	194 (102/92)	64 (37/27)	0.466
Age (range, years)	43.8 (12–91)	43.6 (12–91)	44.6 (14–85)	0.678
>60	48	35	13	0.686
≤60	210	159	51	0.686
WBC count (range, $\times10^9$/L)	24.1 (0.4–86.3)	22.5 (0.4–82)	28.9 (2.3–86.3)	0.015
Hemoglobin (range, g/dL)	76.7 (31–131)	76.7 (31–131)	76.4 (34–131)	0.920
Platelet (range, $\times10^9$/L)	37.3 (2–146)	38.3 (5–146)	34.4 (2–94)	0.302
Blast (range, %)	68.7 (50–98)	68.6 (50–98)	69.0 (50–98)	0.843
HOXA9	0.5058 (0–3.05)	0.2540 (0–0.8365)	1.2692 (0.8450–3.05)	<0.001
FAB				
M0	3	1	2	0.107
M1	10	9	1	0.243
M2	78	62	16	0.293
M3	18	17	1	0.036
M4	47	34	13	0.616
M5	53	36	17	0.169
M6	7	7	0	0.132
M7	5	3	2	0.363
Undetermined	37	27	10	0.735
De novo	212	161	51	0.550
Secondary	46	33	13	0.550
Induction therapy				
DA	106	80	26	0.931
MA	69	51	18	0.773
IA	70	50	20	0.393
Allo-PBSCT	29	21	8	0.713
Auto-PBSCT	26	19	7	0.792
CR after one cycle	211	165	46	0.018
1-year relapse	49	33	16	0.049

Note: The 75th percentile of *HOXA9* expression in the total population was used as the cutoff point to define lower and higher expression groups.
Abbreviations: AML, acute myeloid leukemia; WBC, white blood cell; FAB, French–American–British cooperative group; DA, daunorubicin and cytarabine; MA, mitoxantrone and cytarabine; IA, idarubicin and cytarabine; Allo-PBSCT, allogeneic peripheral stem cell transplantation; Auto-PBSCT, autologous peripheral stem cell transplantation; CR, complete remission.

abnormalities were grouped as favorable, intermediate, and unfavorable.[22] Patients with chromosome 5 or 7 abnormalities and inv(16)/t(16;16) and 11q23 abnormalities were confirmed by fluorescence in situ hybridization (FISH).

Reverse transcription and real-time quantitative polymerase chain reaction

Bone marrow mononuclear cells were purified by density centrifugation using the standard Ficoll-Hypaque method. Total RNA was isolated from bone marrow mononuclear cells using Qiazol isolation reagent (Qiagen NV, Venlo, the Netherlands) and subsequently reverse transcribed to cDNA using a reverse transcription kit (Promega Corporation, Fitchburg, WI, USA).

We performed real-time quantitative polymerase chain reaction (qPCR) to quantify *HOXA9* and *MEIS1* transcripts in samples from all patients using the *HOXA9, MEIS1*, and *ABL1* primers and probes shown in Table S1. The qPCR protocol was performed in a 40 µL volume with TaqMan Universal Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.25 µM appropriate primers and probes, and 20 ng of cDNA. The qPCR protocol included 40 cycles of denaturation for 15 seconds at 95°C and annealing for 60 seconds at 60°C. A standard curve was produced for the *HOXA9* and *MEIS1* gene by tenfold serial dilutions of five different plasmid concentrations. Relative *HOXA9* and *MEIS1* expression levels were calculated as the ratio of copies of *HOXA9* or *MEIS1* and *ABL1*. For all qPCR assays, a reference dilution was analyzed, and the standard curve was loaded over this reference dilution range.

Detection of gene mutations

All coding exons of the longest known *HOXA9* and *MEIS1* genes were amplified from genomic DNA by PCR and were analyzed by direct sequencing. Other mutation analyses were performed for hyperfrequency mutational sites of *ASXL1, CEBPA, FLT3, IDH1, NRAS, RUNX1, TET2, DNMT3A, SF3B1, U2AF1, SRSF2, SETBP1, TP53, NPM1,*

EZH2, MLL, UTX, and *KIT*, with the exons studied being as follows: *ASXL1* exon 12, *CEBPA* exon 1, *FLT3* exons 14, 15, and 20, *IDH1* exon 4, *NRAS* exons 1 and 2, *RUNX1* exons 1–8, *TET2* exons 3–11, *DNMT3A* exons 22 and 23, *SF3B1* exons 3–16, *SRSF2* exons 1–2, *SETBP1* exon 4, *TP53* exons 2–8, *NPM1* exons 3–10, *EZH2* exon 14–19, *MLL* exons 2–6, *UTX* exons 2–12, and *KIT* exons 8–17.[23–30] The primers for sequencing are listed in Table S2. PCR fragments were sequenced directly and analyzed using Sequencing Analysis Software Version 5.3.1 (Thermo Fisher Scientific). Abnormal sequencing results were confirmed by at least two repeated analyses.

Statistical analysis

The statistical analysis was performed using SPSS 20.0 software (IBM Corporation, Armonk, NY, USA). Chi-squared analysis and Fisher's exact test (for categorical variables) were used to compare patient groups. The correlation between the frequency of *HOXA9* expression levels and clinical parameters was analyzed with Pearson's and Spearman's rank correlations. OS curves were plotted using the Kaplan–Meier method and compared using the log-rank test. The median time between visits was 42 months (range, 8–89 months). Hazard ratios and 95% confidence intervals were estimated by Cox proportional hazards regression models to determine the independent risk factors associated with survival in multivariate analyses. For all analyses, *P*-values were two-tailed, and $P<0.05$ value was considered statistically significant.

Results

Associations of *HOXA9* and *MEIS1* expression with hematological parameters

We measured the expression of *HOXA9* and *MEIS1* transcripts in a total of 258 patients with AML using qPCR. Occasionally, 25 healthy donors were also detected for expression of *HOXA9* and *MELSI*. The mean expression levels of *HOXA9* and *MEIS1* in the AML samples were 50.58% and 60.34%, respectively, while those in the healthy bone marrow samples were only 3.74% and 2.23%, respectively. *HOXA9* and *MEIS1* expressions were significantly higher in patients with AML than in healthy bone marrow donors (Figure S1A and B; both $P<0.001$). In addition, *HOXA9* expression levels were lower in de novo AML compared with those in secondary AML (Figure 1A; $P=0.041$), whereas this difference did not occur concerning *MEIS1* expression levels in de novo AML compared with secondary AML (Figure 1B). Moreover, as shown in Figure 1C, *HOXA9* expression levels were not equally distributed among the FAB subtypes. The patients with M3 had lower transcript levels than the individuals with the other FAB subtypes ($P=0.043$). However, *MEIS1* expression levels did not demonstrate equivalent significant difference between FAB subtypes (Figure 1D). Among the whole group, Spearman's rank correlation showed that white blood cell count was significantly correlated with *HOXA9* expression (Figure 1E; $P=0.001$ and $R=0.199$). Nevertheless, no significant correlation was discovered of *MEIS1* expression with white blood cell count (Figure 1F; $P=0.086$ and $R=0.116$).

Prevalence and clinical associations of *HOXA9* and *MEIS1* mutations

Of 258 patients, variations in the *HOXA9* and *MEIS1* coding sequence in eleven and nine patients, respectively, were identified. Mutations predicted to result in missense changes occurred most frequently and were distributed throughout all coding exons. Analyses of remission bone marrow samples showed that all evaluable mutations were conquered. Thus, the frequencies of *HOXA9* and *MEIS1* mutations in our cohort were 4.26% and 3.49%, respectively. The details of *HOXA9* and *MEIS1* mutations are shown in Tables S3 and S4. Furthermore, associations of *HOXA9* and *MEIS1* mutations with pretreatment demographic, clinical, and molecular characteristics were analyzed, and no significant differences were exhibited (data are not shown). Moreover, Kaplan–Meier analysis indicated that impacts of *HOXA9* and *MEIS1* mutations on clinical outcome with non-M3 patients did not denote significant differences (RFS: $P=0.119$ and $P=0.263$, respectively; OS: $P=0.113$ and $P=0.141$, respectively). The data are shown in Figure 2.

Patients with higher *HOXA9* transcript levels had specific clinical features

To further appraise the clinical impact of *HOXA9* expression levels, the patients were divided into two groups according to the 75th percentile of the initial transcript levels. Clinical information such as age, platelet count, bone marrow blast count, and FAB classification was completely available for 258 patients (Table 1). Higher *HOXA9* expression levels were underrepresented in FAB-M3 and closely associated with high white blood cell count, while there were no significant differences in age, sex, hemoglobin, platelet, or marrow blast levels between these two groups. The CR rate after one-cycle chemotherapy and 1-year relapse rate also exhibited remarkable differences between patients with higher *HOXA9* expression levels and those with lower expression levels (85.1% vs 71.9% and 17.5% vs 29.6%, respectively).

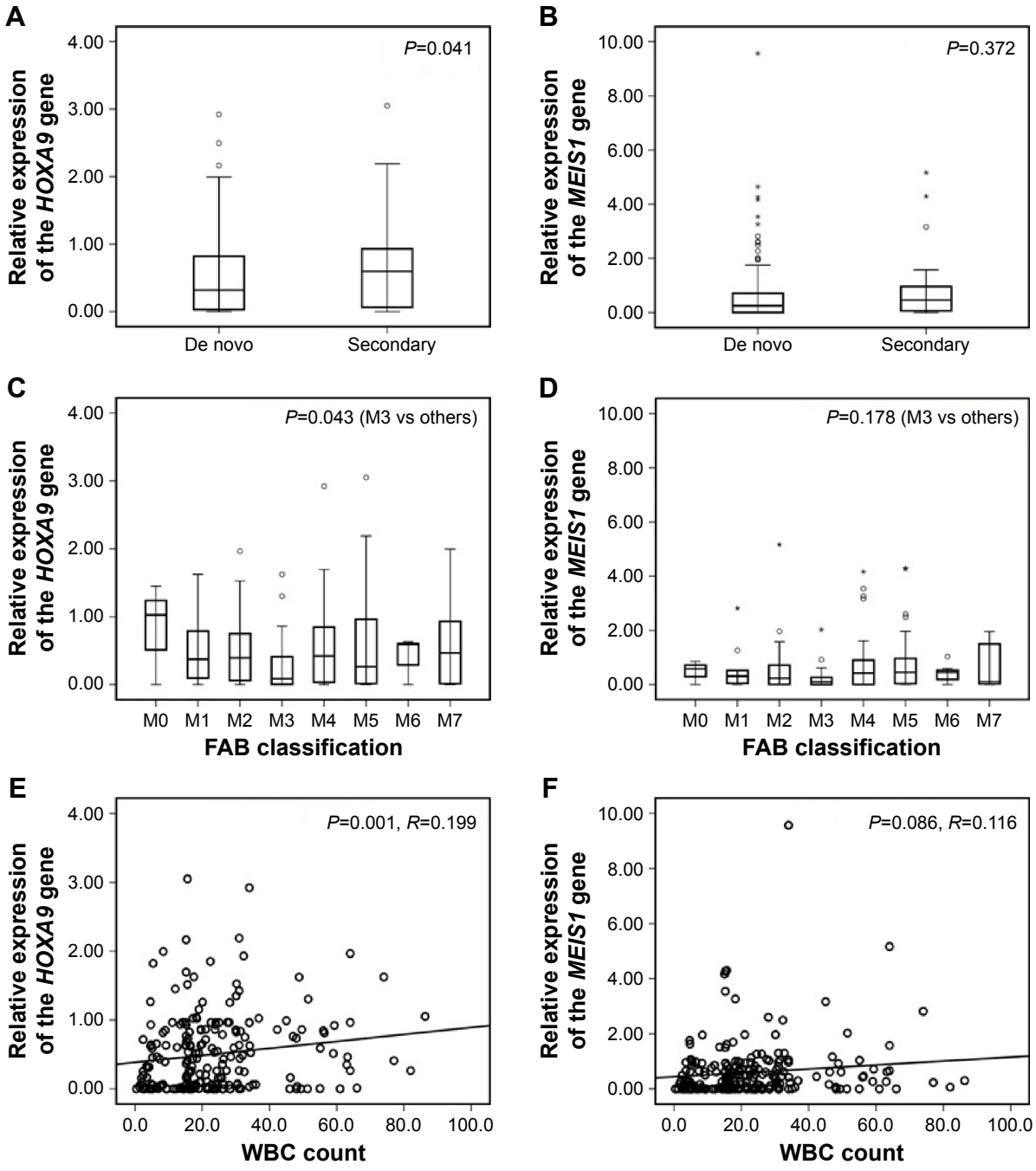

Figure 1 Relative expression of *HOXA9* and *MEIS1* in AML.

Notes: (**A**) *HOXA9* expression levels were higher in de novo AML than those in secondary AML (*P*=0.041); (**B**) *MEIS1* expression levels did not demonstrate significant differences between de novo AML and secondary AML (*P*=0.372); (**C** and **D**) *HOXA9* and *MEIS1* expression levels and FAB classification. The lowest median expression levels of *HOXA9* were detected in patients with the M3 subtype (*P*=0.043), while no difference occurred in *MEIS1* expression between FAB subtype; (**E** and **F**) among the whole group, Spearman's rank correlation showed that WBC counts were significantly correlated with *HOXA9* expression in patients with AML (*P*=0.001 and *R*=0.199). However, no significant correlation was discovered in *MEIS1* expression (*P*=0.086 and *R*=0.116). *Singular value.

Abbreviations: AML, acute myeloid leukemia; FAB, French–American–British; WBC, white blood cell.

Higher *HOXA9* expression levels were highly associated with unfavorable chromosome aberrations, *MLL-PTD* and *EZH2* mutations

Chromosomal data were available in all 258 patients at diagnosis, and clonal chromosomal abnormalities were detected in 116 patients (45%). The karyotype comparison of patients with lower and higher *HOXA9* expression is shown in Table 2. Patients harboring higher *HOXA9* expression levels had higher frequencies of unfavorable chromosome aberrations, while the levels were underrepresented in the cytogenetic favorable group. In addition, higher *HOXA9*

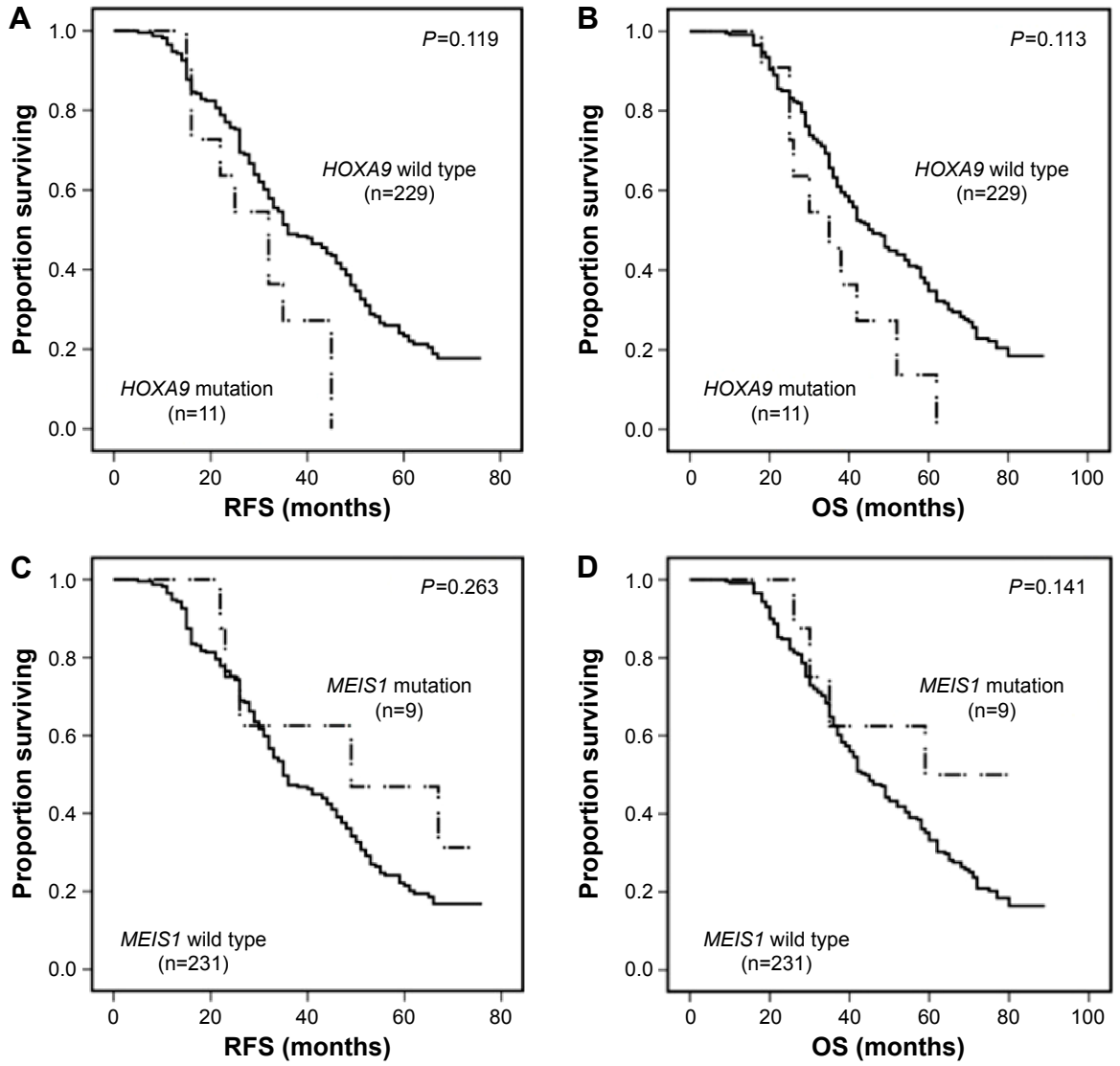

Figure 2 Kaplan–Meier survival curves for RFS and OS stratified by *HOXA9* and *MEIS1* mutation status in patients with AML.
Notes: (**A** and **B**) Kaplan–Meier plots of *HOXA9* mutated compared with *HOXA9* wild type showing RFS and OS. In non-M3 patients with AML, no significant differences were detected in RFS and OS (P=0.119 and P=0.113, respectively); (**C** and **D**) In non-M3 AML cases, there were no differences between the patients with *MEIS1* mutation and wild type in RFS and OS (P=0.263 and P=0.141, respectively).
Abbreviations: RFS, relapse-free survival; OS, overall survival; AML, acute myeloid leukemia.

transcript levels were associated with specific 11q23 abnormalities. In particular, of the nine cases with 11q23 translocations, eight cases were among the patients with higher expression levels.

Next, to investigate the relationship of *HOXA9* expression and gene aberrations associated with leukemogenesis, *ASXL1, CBL, FLT3, IDH1, NRAS, RUNX1, TET2, DNMT3A, SF3B1, U2AF1, SRSF2, SETBP1, TP53, NPM1, EZH2*, and *KIT* were detected. The spectrum of gene mutations in two groups is shown in Figure 3. Significantly, patients with higher *HOXA9* transcript levels had higher frequencies of *MLL-PTD* and *EZH2* mutations than those with lower transcript levels (P=0.001 and P=0.048, respectively; Table 2). Eleven patients harbored *MLL-PTD*, including eight patients in the higher-level group and three patients in the lower-level group. Furthermore, 12 patients had an *EZH2* mutation, six patients in the group with higher *HOXA9* expression levels.

Prognostic value of *HOXA9* expression in the context of other predictive molecular markers

We analyzed RFS and OS in patients with higher *HOXA9* transcript levels versus those with lower *HOXA9* transcript levels. Kaplan–Meier analyses and log-rank test showed that higher *HOXA9* transcript levels in AML indicated shorter RFS and OS compared with lower transcript levels

Table 2 Comparison of cytogenetic and genetic alterations in patients with AML by *HOXA9* expression level

Variant	Whole cohort (n=258)	Lower *HOXA9* expression (n=194)	Higher *HOXA9* expression (n=64)	*P*-value
Cytogenetic risk[a]				
Favorable	44	39	5	0.023
Intermediate	169	131	38	0.234
Unfavorable	45	24	19	0.001
Cytogenetic characteristics[b]				
t(8;21)	19	17	2	0.134
t(15;17)	16	14	2	0.193
Inv16	9	8	1	0.300
Normal karyotype	142	110	32	0.350
+8	11	8	3	0.542
11q23	9	1	8	<0.001
Complex	19	11	8	0.067
–5(q)/–7(q)	11	9	2	0.458
Mutation status				
CEBPA	17	14	3	0.353
FLT3-ITD	23	16	7	0.513
NPM1	38	28	10	0.833
MLL-PTD	11	3	8	0.001
NRAS	13	9	4	0.409
KIT	5	4	1	0.637
RUNX1	15	11	4	0.535
ASXL1	19	14	5	0.531
DNMT3A	21	15	6	0.677
TET2	20	14	6	0.373
IDH1	20	17	3	0.220
SF3B1	14	10	4	0.473
EZH2	12	6	6	0.048
SRSF2	15	9	6	0.137
UTX	7	6	1	0.447
SRSF2	15	9	6	0.137
TP53	12	10	2	0.392
SETBP1	14	11	3	0.527

Notes: [a]Favorable risk: t(15;17), t(8;21), inv(16)/t(16;16); unfavorable risk: inv(3)/t(3;3), t(6;9), 11q23 translocations other than t(9;11), –5 or del(5q), –7, abn(17p), complex karyotype; intermediate risk: all chromosome abnormalities not classified as favorable or unfavorable. [b]Patients may be counted more than once owing to the coexistence of more than one cytogenetic abnormality in the leukemic clone.
Abbreviations: AML, acute myeloid leukemia; t, translocation; inv, inverse; del, deletion; abn, abnormality.

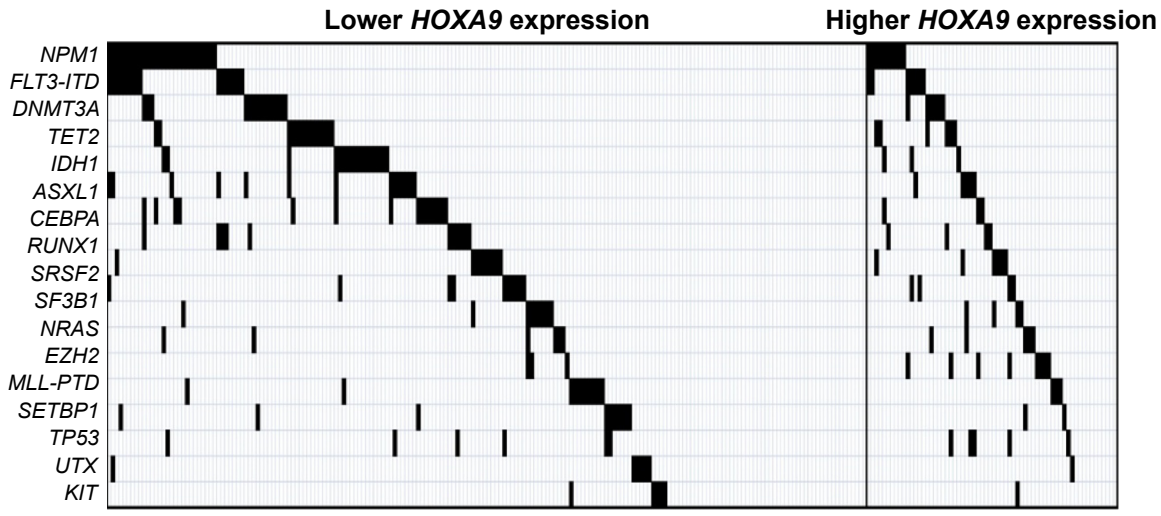

Figure 3 The spectrum of mutations in 258 patients with AML stratified by *HOXA9* expression levels (higher *HOXA9* expression group and lower *HOXA9* expression group).
Note: Each column represents an individual patient sample.
Abbreviation: AML, acute myeloid leukemia.

(*P*=0.014 and *P*=0.022, respectively; Figure 4A and B). Similarly, in non-M3 AML, patients with higher *HOXA9* transcript levels had inferior RFS and OS than those with lower transcript levels (*P*=0.015 and *P*=0.013, respectively; Figure 4C and D).

In multivariate analysis (Table 3), including variables significantly associated with clinical outcome, the independent poor risk factors for RFS and OS were age >60 years, secondary AML, unfavorable karyotype, higher *HOXA9* expression, *CEBPA* mutation, *RUNX1* mutation, *EZH2* mutation, *SRSF2* mutation, *FLT3-ITD*, and *MLL-PTD*. Among the patients with AML, higher *HOXA9* expression level was still an independent poor prognostic factor for OS but not for RFS. The other independent markers included secondary AML, *FLT3-ITD, MLL-PTD, EZH2* mutation, and *RUNX1* mutation.

Furthermore, we analyzed survival in 142 patients with cytogenetically normal AML (CN-AML); RFS and OS differed significantly between patients with higher levels (n=32) and those with lower levels (n=110; *P*=0.024 and *P*=0.018, respectively; Figure 5A and B). According to the prognostic value of *HOXA9* expression in 142 patients with a normal karyotype, higher *HOXA9* expression level was entered into a multivariate model in addition to factors significantly associated with prognosis on univariate analysis for this population. *HOXA9* higher expression levels and *FLT3-ITD,*

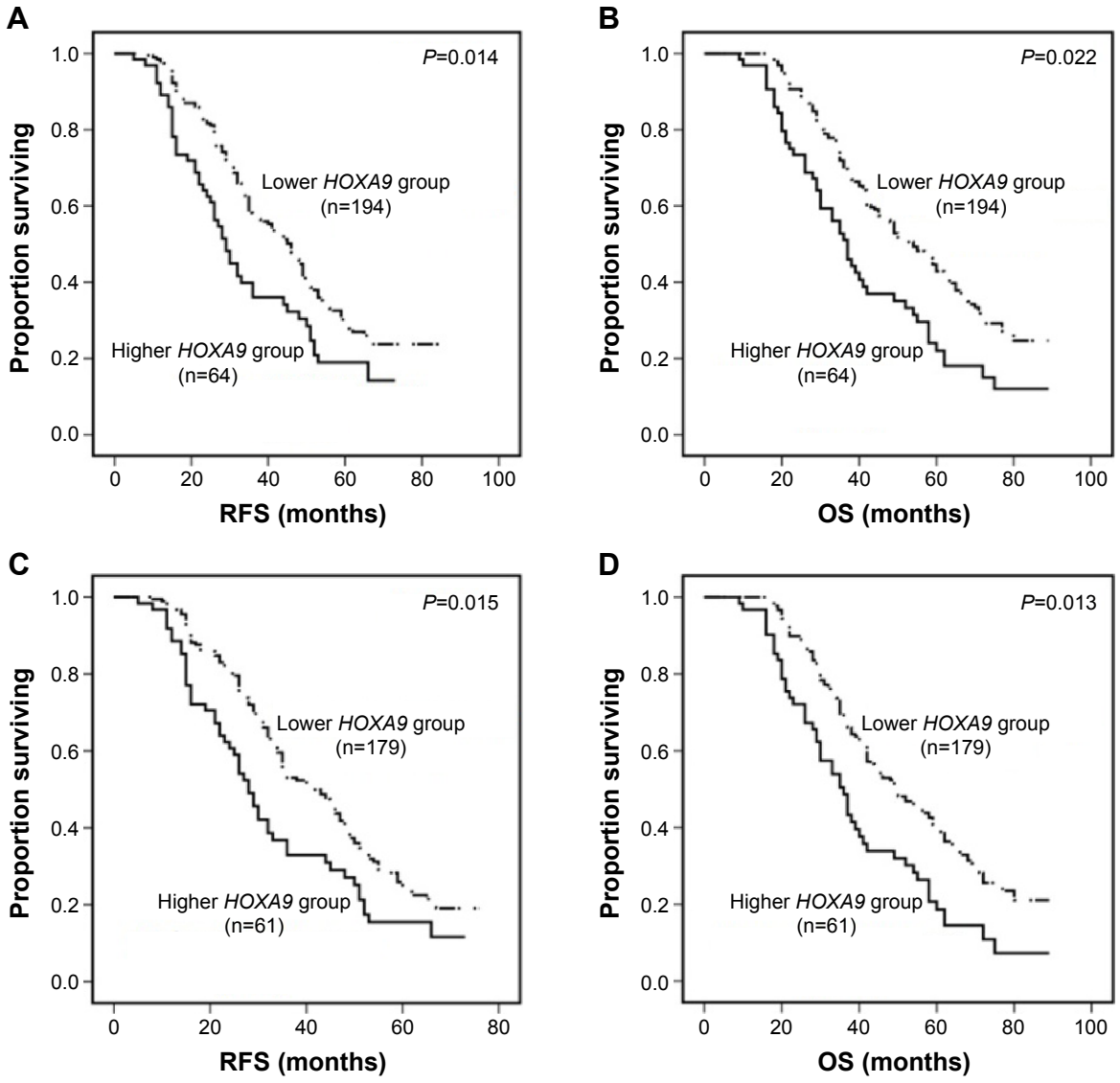

Figure 4 Kaplan–Meier survival curves for RFS stratified by *H0XA9* mRNA expression in patients with AML.
Notes: (**A** and **B**) The survival curves stratified by *HOXA9* mRNA expression into two groups (higher *HOXA9* expression group and lower *HOXA9* expression group) according to the 75th value. In patients with AML, the group with higher *HOXA9* mRNA levels had worse RFS and OS (*P*=0.014 and *P*=0.022, respectively); (**C** and **D**) In non-M3 AML cohort, patients with higher *HOXA9* mRNA levels had shorter RFS and OS (*P*=0.015 and *P*=0.013, respectively).
Abbreviations: RFS, relapse-free survival; AML, acute myeloid leukemia; OS, overall survival.

Table 3 Multivariate analysis (Cox regression) of survival in patients with AML

Variables	RFS			OS		
	Hazard ratio	95% confidence interval	*P*-value	Hazard ratio	95% confidence interval	*P*-value
AML group						
Age >60 years	0.922	0.756–1.125	0.425	0.889	0.732–1.081	0.238
Secondary AML	0.817	0.673–0.992	0.041	0.814	0.670–0.987	0.037
Unfavorable karyotype	0.883	0.715–1.091	0.248	0.856	0.694–1.056	0.146
HOXA9 higher expression	1.345	0.936–1.931	0.109	1.446	1.004–2.083	0.047
CEBPA mutation	1.186	0.877–1.603	0.269	1.329	0.957–1.846	0.089
FLT3-ITD	0.683	0.511–0.912	0.010	0.688	0.514–0.921	0.012
MLL-PTD	0.620	0.449–0.856	0.004	0.612	0.442–0.847	0.003
RUNX1 mutation	0.564	0.413–0.770	0.001	0.581	0.426–0.793	0.001
EZH2 mutation	0.712	0.527–0.963	0.027	0.734	0.543–0.992	0.045
SRSF2 mutation	0.747	0.557–1.001	0.051	0.581	0.426–0.793	0.040
CN-AML group						
Age >60 years	0.905	0.683–1.200	0.489	0.888	0.670–1.177	0.408
Secondary	0.810	0.637–1.029	0.085	0.771	0.607–0.981	0.034
HOXA9 higher expression	1.721	1.098–2.697	0.018	1.864	1.187–2.928	0.007
CEBPA mutation	1.185	0.848–1.657	0.120	1.349	0.929–1.959	0.116
NPM1 mutation	1.344	0.992–1.820	0.056	1.243	0.923–1.674	0.151
FLT3-ITD	0.518	0.345–0.778	0.002	0.561	0.373–0.846	0.006
MLL-PTD	0.535	0.328–0.873	0.012	0.533	0.323–1.187	0.013
RUNX1 mutation	0.597	0.390–0.913	0.017	0.590	0.384–0.906	0.016
EZH2 mutation	0.712	0.471–1.075	0.106	0.733	0.484–1.112	0.144

Notes: Only variables with a *P*-value <0.05 on univariate analysis were incorporated into the multivariate Cox proportional hazards regression analysis; unfavorable karyotype: inv(3)/t(3;3), t(6;9), 11q23 translocations other than t(9;11), –5 or del(5q), –7, abn(17p), complex karyotype.
Abbreviations: AML, acute myeloid leukemia; RFS, relapse-free survival; OS, overall survival; CN-AML, cytogenetically normal acute myeloid leukemia.

MLL-PTD, and *RUNX1* mutations were the independent prognostic parameters for RFS and OS.

Discussion

Genetics play an increasingly important role in the risk stratification and individualized therapy of AML patients.[31,32] During the past few years, *HOXA9* aberrations, as well as its cofactors *MEIS1* dysregulation, have been gradually investigated in AML, including their clinical intimation.[33–37] However, very few studies have systematically reported on the role of *HOXA9* mutational status and mRNA expression levels in adult AML, especially the correlation with the other

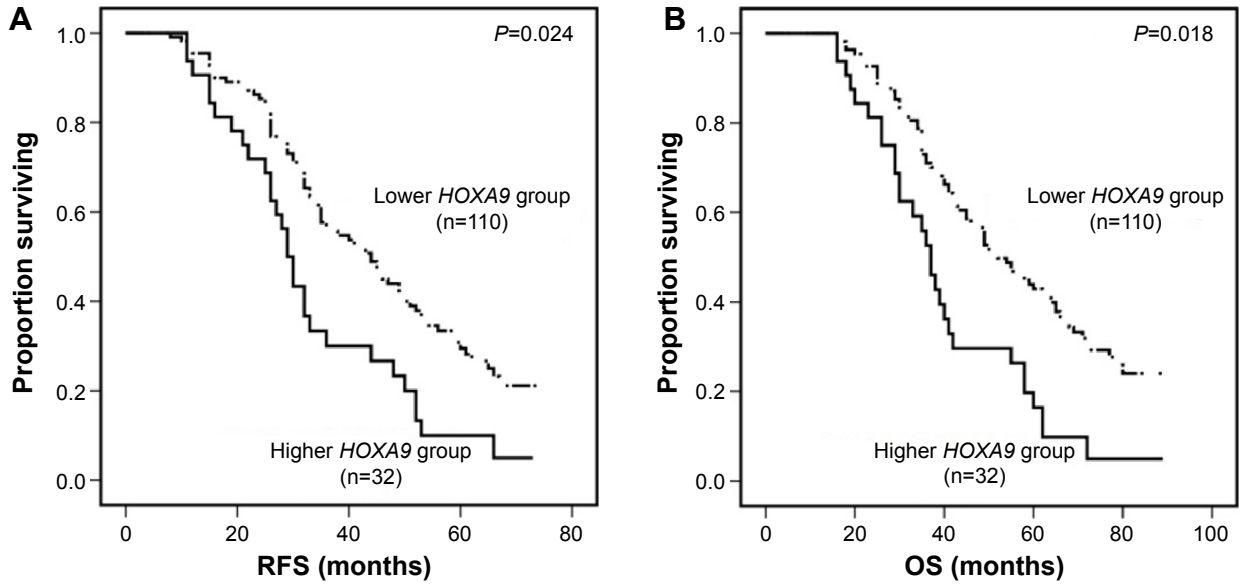

Figure 5 In the subgroup of 142 patients with CN-AML, those with higher *H0XA9* expression had shorter RFS and OS.
Note: The 75th percentile of *H0XA9* expression in the cohort of 142 patients with CN-AML was used as the cutoff point to define the lower and higher expression groups. (**A** and **B**) Patients with CN-AML and higher *HOXA9* expression levels exhibited significantly shorter RFS (*P*=0.024) and significantly shorter OS (*P*=0.013).
Abbreviations: CN-AML, cytogenetically normal acute myeloid leukemia; RFS, relapse-free survival; OS, overall survival.

mutation status.[38] In this study, we sequenced the coding region of *HOXA9* and *MEIS1* genes from 258 AML cases, and only eleven cases were detected with *HOXA9* mutations and nine cases with *MEIS1* mutations. Although no significant associations were unearthed with clinical characteristics and survivals, it at least gave another new light for the roles of *HOXA9* and *MEIS1* in AML. The relative low frequencies of *HOXA9* and *MEIS1* mutations provided the clue that they may be a rare molecular mechanism for the development of AML. Studies comprising a large number of cases for the roles of *HOXA9* and *MEIS1* mutations in AML would give a new interesting perspective.

Meanwhile, we analyzed *HOXA9* and *MEIS1* expression levels by qPCR in patients with AML as well as healthy donors. We investigated the disaccord of clinical correlations of these two gene expression levels with clinical parameters. This provided gradually verified knowledge that aberrant expression of *MEIS1*, as a cofactor of *HOXA9*, is involved in leukemia transformation. Because this study focused on the clinical impact and mutational associations of *HOXA9* expression, we have only a brief analysis concerning the correlations of the *MEIS1* gene with clinical characteristics. It is worth noting that *HOXA9* expression differed according to cytomorphological AML type, with the lowest expression occurring in M3. The low *HOXA9* expression in AML M3 remains unexplained. *HOXA9* is expressed in the hematologic stem cell and immature progenitor compartment but is transcriptionally downregulated upon the induction of terminal differentiation.[10,11] Thus, a possible explanation could be that AML M3 is characterized by a more differentiated blast population.

Similar to a previously mentioned study,[39] higher *HOXA9* transcription level was closely correlated with 11q23 translocation and *MLL-PTD* in our study. In this study, *MLL-PTD* was found in only 4.26% of the total AML cohort, which was consistent with a previous report.[40] Comparative analysis of gene expression profiles in human acute leukemia implicated upregulation of a series of *HOX* genes, including *HOXA9*, which were fully accredited as direct binding targets for *MLL* or *MLL* fusion protein as a pivotal mechanism of leukemic transformation.[41] Gene expression studies in murine models also demonstrated that *HOX* overexpression in the presence of *MLL* fusion initiated the occurrence of leukemia.[42] This gave a very important explanation regarding the *HOXA9* high expression being highly associated with 11q23 abnormalities and *MLL* mutations.

The finding that high *HOXA9* expression was closely associated with *EZH2* mutation in this study was interesting and suggestive. Within the AML cohort with higher *HOXA9* transcription levels, overrepresentation of the *EZH2* mutation was ascertained, which may indicate that *HOXA9* dysregulation coworked with *EZH2* mutation contributes to the pathogenesis of leukemia. Khan et al reported that *EZH2* mutations were characterized by decreased H3K27 trimethylation and increased chromatin relaxation at specific gene loci accompanied by higher transcriptional activity. One of the major downstream targets is *HOXA9*, which is being found to be overexpressed in cases with *EZH2* mutations.[43] Further in-depth study of the molecular association between *HOXA9* and *EZH2* is required to confirm its expected molecular basis for targeted therapy.

To correlate *HOXA9* expression levels with clinical outcome, we analyzed CR rate, relapse rate, RFS, and OS in the two groups with higher and lower *HOXA9* expression levels. Notably, higher *HOXA9* expression levels predicted a reduced one-cycle CR rate and an increased 1-year relapse rate. Mohamad Ismail et al found that *HOXA9* expression was a poor molecular indicator in chronic myelogenous leukemia, and a few other studies demonstrated that *HOXA9* overexpression was a poor indicator.[11,44,45] It is inspiring that by multivariate analyses, higher *HOXA9* expression level indicated shorter OS but not RFS in the entire AML cohort. Moreover, it is worth noting that high *HOXA9* expression independently predicted inferior RFS and OS in patients with CN-AML. This is the first study for analyzing *HOXA9* expression levels coordinated with gene mutations associated with AML. A growing number of reports ascertained that *RUNX1* mutations and *FLT3-ITD* are frequently mutated in de novo AML and had strong adverse prognostic effects in AML.[46–49] In this study, *RUNX1* mutation and *FLT3-ITD* were still the independent poor prognostic factors for survival in AML and CN-AML. With continued advances in understanding clinical associations of the *HOXA9* gene, there is a wealth of opportunity for developing individualized therapy.

An increasing body of evidence supports the notion that *HOXA9* aberrations are frequent and critical, which is crucial to the induction and maintenance of the malignant phenotype. Although no significant correlations of *HOXA9* mutations with clinical characteristics and survivals were found in this study, this provided a little more perspective about *HOXA9* in leukemogenesis. Furthermore, this study gave logical evidence that higher *HOXA9* expression level correlated with specific clinical characteristics and gene mutation status and functioned as a strong prognostic marker for therapy response as well as survival in patients with AML. Therefore,

pretreatment screening for *HOXA9* transcript level should be considered in patients with newly diagnosed AML to better guide risk assessments and therapeutic approaches.

Acknowledgment

This study was supported by the National Natural Science Foundation of China (Nos 81300425 and 81300450), Major State Basic Research Development Program of China (No 2005CB522400), Key Research Program for Clinical High-Tech Research of People's Liberation Army (No 2010gxjs091), and Key Program of Capital Development Foundation (No 2007-2040).

Disclosure

The authors report no conflicts of interest in this work.

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