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High expression of HOXA5 is associated with poor prognosis in acute myeloid leukemia

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A B S T R A C T

Background: HOXA5 is considered as an oncogene in many tumors. This study in- vestigated the HOXA5 expression in Chinese acute myeloid leukemia (AML) patients and evaluated the predictive significance of HOXA5 with a single-center retrospective study. *Methods*: We investigated the expression pattern and prognostic value of HOXA5 in patients with AML through by using a series of databases and various datasets, including the ONCOMINE, TCGA, and STRING datasets. The bone marrow samples of 53 newly diagnosed AML patients (non-M3 subtype) and 19 benign individuals were collected in our center. HOXA5 mRNA expression levels were detected by real-time qPCR, HOXA5 protein expression levels were detected by Western Blot. Clinical data was obtained from inpatient medical records. *Results*: Two microarrays in Oncomine showed that the expression level of HOXA5 was significantly upregulated in AML. Our data revealed that AML patients had higher HOXA5 mRNA and protein expression levels than the controls ($P < 0.001$). The blast percentage in bone marrow of HOXA5 high-expression group was higher that of HOXA5 low-expression group ($P < 0.05$). Higher expression level of HOXA5 revealed a worse OS in AML

Abbreviations: : SHL, Shuanghuanglian; AML, acute myeloid leukemia; MLL, mixed-lineage leukemia; ESC, embryonic stem cells; HSCT, allogeneic hematopoietic stem cell transplantation; TCGA, The Cancer Genome Atlas; FAB, The French, American, and British; PPI, The protein-protein interaction; TALE, three amino acid loop extension.

☆ Conflict of interest: The authors declare no conflicts of interests.

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($P < 0.05$). *Conclusion*: Our findings suggested that HOXA5 might have the potential ability to act as a diagnostic biomarker and potential therapeutic target for AML.

ARTICLE INFO

Keywords: Acute myeloid leukemia; HOXA5; bioinformatics analysis; prognosis

Introduction

Acute leukemia is the most common hematological malignancy in the adolescent and young adult population. It is an aggressive hematological malignancy and is characterized by abnormal proliferation and differentiation of the immature bone marrow cells.[1] In adults, acute myeloid leukemia (AML) is the most prevalent form of acute leukemia.[2] Currently, targeted treatment for AML is in routine clinical practice which includes about 10 drugs against specific molecular targets and allogeneic hematopoietic stem cell transplantation (HSCT). However, excluding M3, all other types of AML have problems such as more treatment difficulties, poorer prognosis, higher relapse rate, and lower survival rate. Although the exact causes of AML remain elusive, it is believed that both genetic and environmental factors,[3] including previous cancer treatment.[4] exposure to certain chemicals, smoking and family history[5] are considerd to be the causes of disease progression. Therefore, in addition to ensuring a good living environment, finding the exact genetic-related causes is of great importance for understanding and treatmenting AML.

The *HOX* gene family includes HOXA, B, C, and D. The HOX gene encodes a family of transcription factors containing homologous domains and plays a significant role in early embryonic development, including the establishment of cell and tissue characteristics, and the regulation of cell proliferation, differentiation, and survival.[6] Heterodimers can be formed between MEIS1/PBX and HOX proteins in both a DNA-independent and DNA-dependent manner.[7] HOXA5 has play important roles in multiple tumors, including gastric cancer,[8] glioma,[9] esophageal cancer,[10] and so on. It was reported that *HOXA5* gene was up-regulated in regulate ordinary hematopoietic stem and progenitor cell (HS/PC), and was lowly expressed during terminal differentiation.[11] HOXA5 silencing has been reported to reduce cell proliferation and induce apoptosis in patients with AML.[12] However, the relationship between HOXA5 expression in AML and prognosis has been rarely described, so we selected *HOXA5* gene for the further exploration. Here, we provide evidence that HOXA5 is involved with AML based on bioinformatics analysis,mainly by exploring its expression in AML, its correlation with other HOX members, and its relationship with the overall survival of AML.

Materials and methods

Bioinformatics analysis

Oncomine database

The mRNA expression values of HOXA5 in AML and normal organs were analyzed by using the Oncomine platform, which collects publicly available cancer micro-array data. Downloaded the raw data on December 31, 2019, and statistical significance was calculated by Oncomine.

Survival analysis

Downloaded the raw data from Oncomine on December 31, 2019. Based on the mean of HOXA5 expression, patients were divided into high expression of HOXA5 group (HOXA5-high) and low expression of HOXA5 group (HOXA5-low).

STRING database analysis

The STRING database has collected, scored, and integrated all publicly available sources of protein-protein interaction (PPI) information, and supplemented these with computational predictions (https://string-db.org/.). The goal is to build an exhaustive and objective global network, including physical as well as functional interactions. This study retrieved the data with the following settings: HOXA5; Homo sapiens; high confidence (0.700); the maximum number of interactors displayed: no more than 50 interactions.

Gene coexpression with HOXA5 in TCGA dataset

We used The Cancer Genome Atlas (TCGA) which had both sequencing and pathological data on 30 different cancers. The AML (TCGA, PanCancer Atlas) dataset including data from 173 cases with mRNA expression z-scores (RNA Seq V2 RSEM) was selected for further analyses of HOXA5 using by cBio- Portal (http://www.cbioportal.org). Gene coexpression with HOXA5 in PPI network were calculated according to the cBioPortal. The search date was December 31, 2019.

Patients and specimens

Fifty-three newly-diagnosed AML patients (non-M3 subtype) were finally enrolled in Affiliated Hospital of Southwest Medical University from January 2019 to May 2020 were selected, and 19 participants with benign hematological diseases including anemia and thrombocytosis were selected as control group. AML patients were diagnosed based on WHO 2008 criteria and classified according to FAB classification. The treatment of AML patients is mainly based on the Chinese guidelines for diagnosis and treatment of adult AML (not APL) (2017)[13] at the time of admission. ELN Criteria were used for prognostication and of AML patients. Factors influencing survival were incorporated in the EPI score applicable to adults aged between 15 and 60 years. Poor outcome is associated with shorter CR1 duration, increasing age at the time of relapse, non-favorable karyotype at initial diagnosis, or history of prior allogeneic HCT.[14] A total of 43 mutant genes were detected by next-generation sequencing, including NPM1, N-RAS, c-KIT, TET2, WT1, FLT3, DNMT3A, PTPN11, CBFβ-MYH11, AML-ETO, TEL-JAK2, C ABL-BCR, EBPA and p53. According to cytogenetics and molecular genetic standards, patients with non-M3 subtypes of AML are divided into good, moderate and poor prognosis groups. The study was approved by the Affiliated Hospital of Southwest Medical University was conducted in accordance with the Declaration of Helsinki.

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using RZ solution (Transgen, China). cDNA was synthesized using a TranScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR kit (Transgen, China), and TranStart Tip Green Qpcr SuperMix(Transgen, China) was used to assay the HOXA5 mRNA levels. The amplification proceeded as follows: 94°C, 30 seconds, 1 cycle; 94°C, 5 seconds, 60°C, 20 seconds, 40 cycles. The primers were HOXA5- forward, 5′-TTTTGCGGTCGCTATCC-3′, HOXA5-reverse, 5′-CTGAGATCCATGCCATTGTAG-3′, β-Actin- forward, 5'-GGCGGCACCACCATGTACCC-3', β-Actin - reverse, 5'- CCACACGGAGTACTTGCGC -3'. The $2^{-\triangle\triangle CT}$ method was used to calculate relative mRNA expression. Statistical analysis was performed with SPSS (Version 19.0, IBM Corp. Armonk, NY) and GraphPad Prism (Version 7.01, GraphPad Software). Taking the median as the node, AML patients were divided into HOXA5 high expression group and low expression group. We used Mann−Whitney test to compare differences between 2 groups. It was considered statistically significant when P values <0.05.

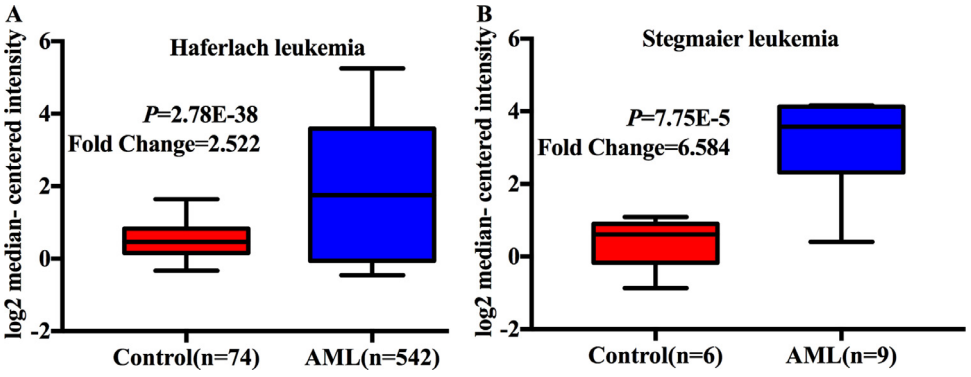

Fig. 1. *HOXA5* gene mRNA expression in AML in the Oncomine leukemia database. (A) HOXA5 mRNA expression in Haferlach leukemia dataset, analyzed by Oncomine. (B) HOXA5 mRNA expression in Stegmaier leukemia dataset, analyzed by Oncomine.

Western blotting analyses

BMMCs were lysed in RIPA buffer. Protein concentration was determined using the Bradford dye binding assay with bovine serum albumin as the standard. Total proteins were resolved on SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Then, the membrane was incubated with HOXA5(1:1000) (abcam, Britain) and antibactin (1:1000) antibodies (CST,USA), followed by incubating with secondary antibodies overnight. The bands were visualized using the enhanced chemiluminescence system (Beijing 4A Biotech Co., Ltd, China).

Statistical analysis

Statistical analysis was performed with GraphPad Prism (Version 7.01, GraphPad Software). We used *t* test or Mann−Whitney test to compare differences between 2 groups. OS was estimated by the Kaplan-Meier method with Log Rank. Univariate analysis, multivariate analysis, hazard ratio and 95% confidence interval were calculated by Cox regression. Based on the mean of HOXA5 expression, patients were divided into high expression of HOXA5 group (HOXA5-high) and low expression of HOXA5 group (HOXA5-low). It has statistical significance ($P < 0.05$).

Results

HOXA5 is highly expressed in acute myeloid leukemia(Oncomine leukemia database and AML patients)

We investigated HOX mRNA expression in tumors and it showed that HOXA5 was higher expressed in leukemia than the corresponding normal tissues (Fig. S1). Next, we analyzed NAT10 levels in AML patients in our center. In total, 40 of 48 AML patients were non-M3 subtype and 8 were acute promyelocytic leukemia (APL, M3 subtype). NAT10 was upregulated in AML patients ($P < 0.01$, Fig. 1C). There were also no significant differences in NAT10 expression among different age, gender, or among patients with good, intermediate, or poor prognosis (Table 1). We investigated HOXA5 expression in AML from Oncomine database including Haferlach leukemia dataset (n = 542, $P = 2.78E-38$, fold change = 2.52)[15] and Stegmaier leukemia dataset (n = 9, $P = 7.75E-5$, fold change = 6.584).[16] The results showed that HOXA5 levels in AML patients were higher than in the normal ones ($P < 0.01$, Fig. 1A and B). Next, we analyzed HOXA5 levels in

Table 1
Relationships between baseline characteristics and HOXA5 mRNA expression.

			HOXA5-low(%)	HOXA5- high (%)	*P* value
Age	<60	43	22	21	0.7643#
	≥60	15	7	8	
Gender	Male	34	20 (69%)	20 (69%)	0.1097#
	Female	24	9 (31%)	15 (52%)	
Immunophenoyping	M0	6	2	4	
	M1	4	3	1	
	M2	19	7	12	
	M4	19	13	6	
	M5	10	4	6	
Gene mutation/fusion	FLT3	11	4	7	
	N-RAS	6	2	4	
	c-KIT	2	0	2	
	NPM1	3	1	2	
	WT1	3	1	2	
	IDH1	1	0	1	
	DNMT3A	3	1	2	
	CEBPA	3	1	2	
	AML-ETO	5	2	3	
	TEL-JAK2	3	1	3	
	ABL-BCR	1	0	1	
Risk	Good	5	3 (60%)	2 (40%)	
	Intermediate	14	8 (57%)	8 (57%)	
	Poor	39	18 (46%)	18 (46%)	
Lab examinations	WBC		42.53 ± 10.22	32.79 ± 10.27	0.5042*
	Hb		84.66 ± 5.043	84.17 ± 4.816	0.9451*
	PLT		50.79 ± 11.58	91.03 ± 34.13	0.2689*
	Blast in peripheral blood		53.69 ± 3.368	48.10 ± 4.01	0.3614*
	Blast in bone marrow		41.75 ± 3.572	55.17 ± 4.271	0.0193*
Complete remission		28	16(53%)	12(47%)	

#Chi-square test.

AML patients in our hospital. 58 AML patients were non-M3 subtype. NAT10 mRNA and protein were upregulated in AML patients ($P < 0.01$, Fig. 2). The blast percentage in bone marrow had great significant differences in HOXA5 high-expression group and low-expression group (Table 1, Fig. 3). There were also no significant differences in HOXA5 expression among different age, gender, or among patients with good, intermediate, or poor prognosis (Table 1). The white blood cell count, hemoglobin, and thrombocyte count had no differences between HOXA5 high-expression group and low-expression group (Table 1). We also analyzed the relationship between HOXA5 mRNA levels and some mutant genes. However, the number clinical cases of gene mutation and fusion in this study is too small (Table 1), the statistical significance of HOXA5 expression and their relationship is meaningless.

The expression of HOXA5 in AML FAB subtypes

French, American, and British (FAB) classification systems are widely,[17] and traditionally it applied to classify AML as discrete types of M0-M7. The expression of HOXA5 mRNA in different FAB subsets of AML was analyzed in the Oncomine database. Three datasets meet the search criteria were selected for further analysis, including the TCGA dataset (http://tcga-data.nci.nih.gov/tcga/), the Valk Leukemia dataset,[18] and the Wouters leukemia dataset.[19] HOXA5 was significantly over-expressed in FAB subtype M5 and under-expressed in FAB subtype M4Eo compared with other FAB subtypes (Fig 4).

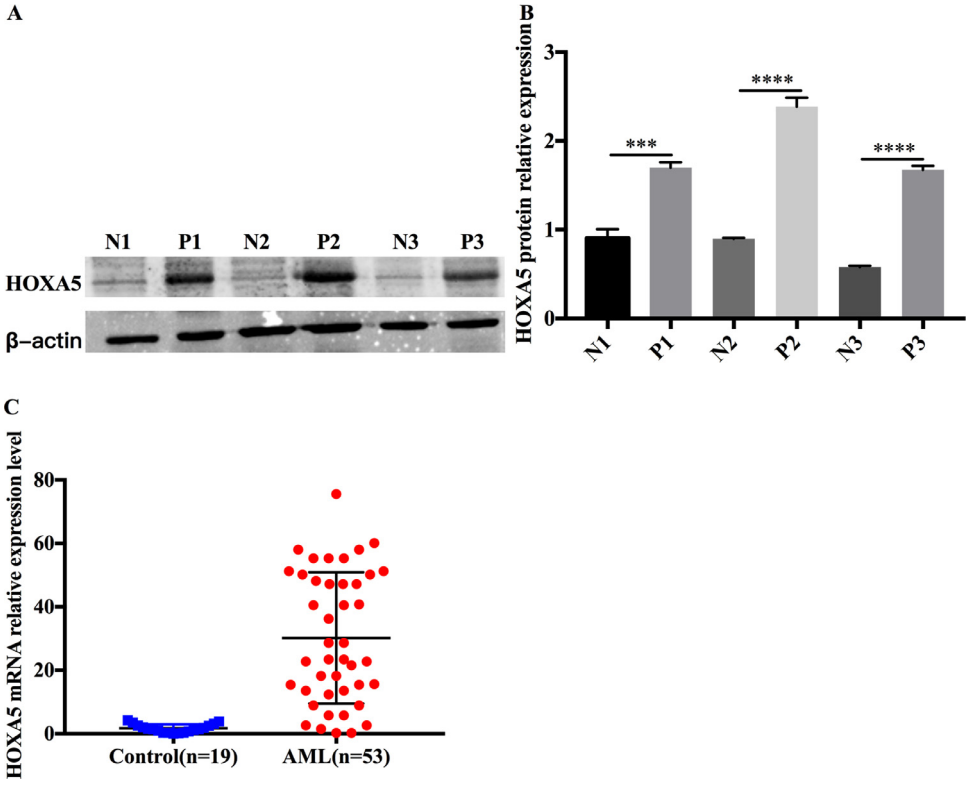

Fig. 2. HOXA5 expression in AML patient (A) HOXA5 protein expression in AML patients and controls was assayed by Western blot. N1, 2, 3: Normal 1, 2, 3; P1,2,3: Patient 1, 2, 3. (B): The histogram of HOXA5 protein in AML and controls. Statistical significance was calculated by. (*** <0.0005, **** <0.0001, t-Test). (C) HOXA5 mRNA expression in AML patients and controls was assayed by RT-qPCR (****P* < 0.0001, *t* test).

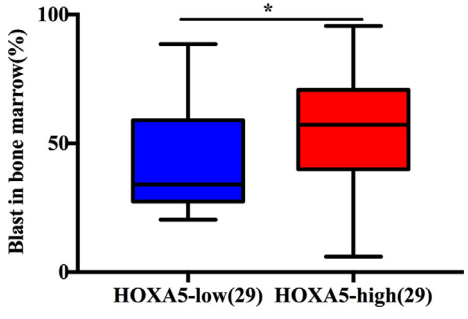

Fig. 3. The blast percentage in bone marrow of HOXA5 high-expression group and low-expression group. Statistical significance was calculated by *t* test. (* <0.05).

High level of HOXA5 is associated with poor prognoses in AML patients

HOXA5 is highly expressed in AML. To further analysis whether it could be a prognostic marker for AML, 4 datasets available with the clinical outcome information were selected for survival analysis with the Kaplan-Meier plotter. As shown in Fig. 5A, the increased HOXA5 mRNA is significantly correlated with shorter overall survival time of AML patients in the TCGA dataset

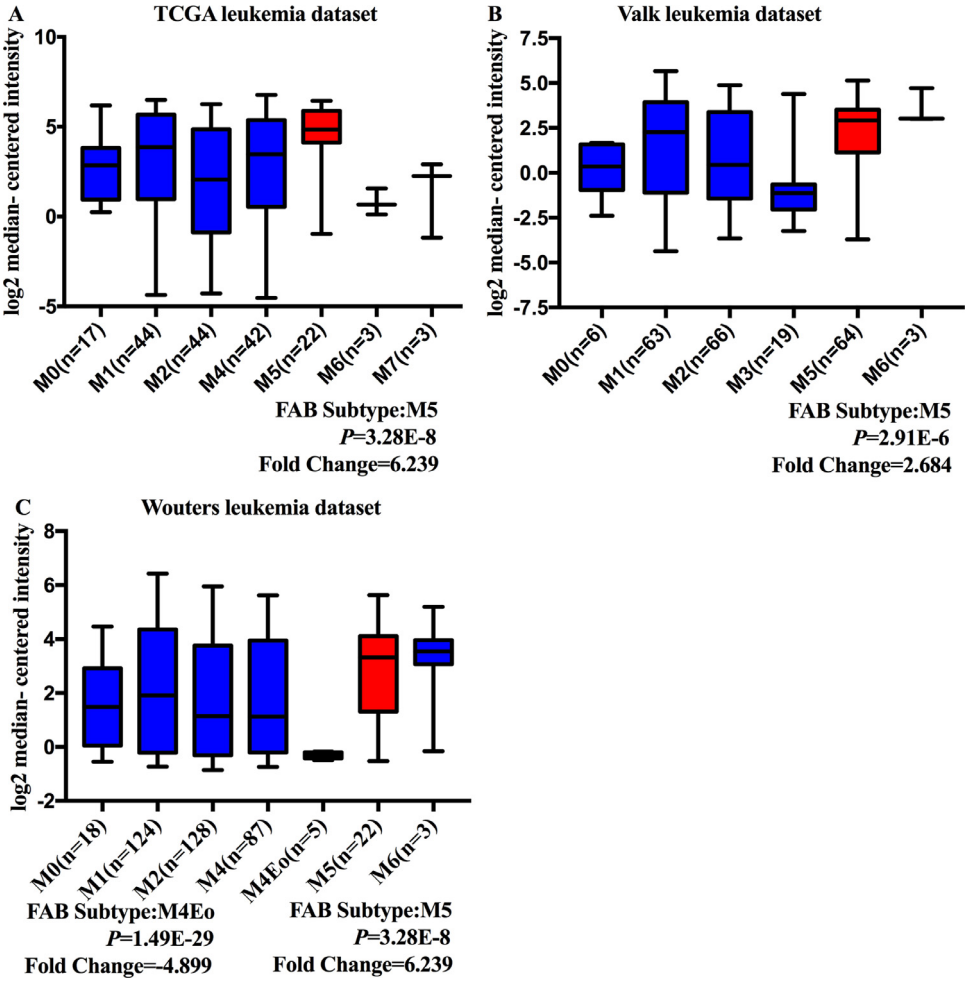

Fig. 4. HOXA5 mRNA expression in AML FAB subtypes .(A) HOXA5 mRNA expression in TCGA leukemia dataset, analyzed by Oncomine. (B) HOXA5 mRNA expression in Valk leukemia dataset, analyzed by Oncomine. (C) HOXA5 mRNA expression in Wouders leukemia dataset, analyzed by Oncomine.

($P = 0.0405$) (http://tcga-data.nci.nih.gov/tcga/). The similar trends, although not significant, were observed in the Metzeler leukemia dataset[20], and the Raponi leukemia dataset (Fig. 5B–C).[21] However, it was opposite in the Heuser leukemia dataset (Fig. 5D),[22] which may be due to the small sample size (n = 28).Taken together, our data imply that HOXA5 is a potential prognostic marker for AML patients with poor outcome.

The PPI network of HOXA5 protein

To achieve a comprehensive and objective global PPI network, HOXA5 was analyzed in the STRING database. The PPI enrichment *P*-value was < 1.0E-16 and the number of nodes was 23. Not unexpectedly, HOXA5 interacted with most other HOX family genes, including HOXA3/6/7/9, HOXB3/4/5/6/7/8), HOXC4/5/6/8/9) and HOXD4/9 through the co-expression, gene fusion, and gene co-occurrence manner (Fig 6). HOXA5 also interacted with transcriptional

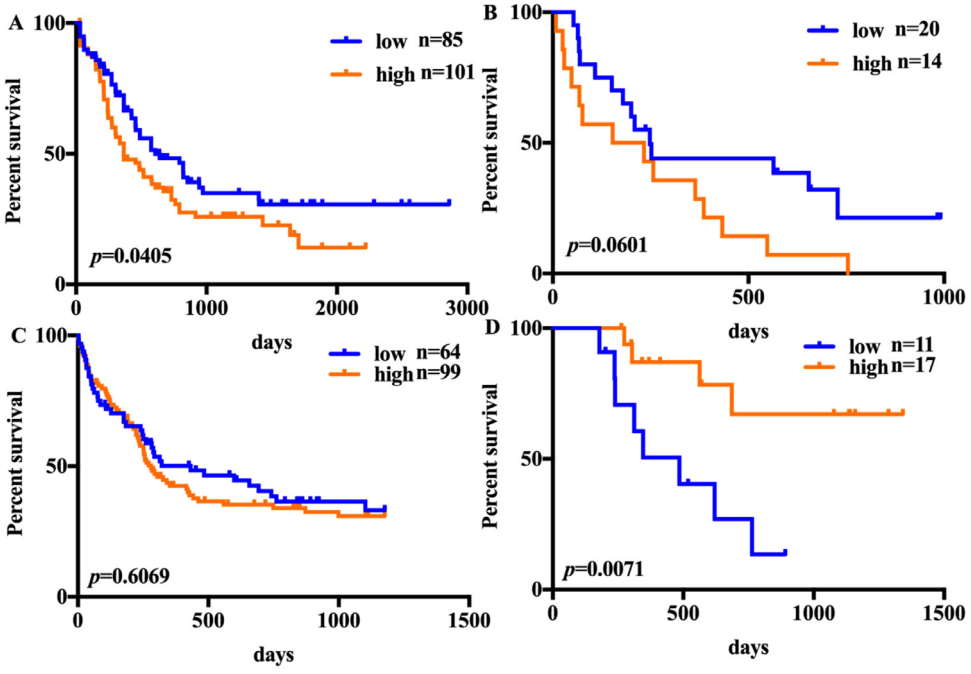

Fig. 5. The prognostic value of HOXA5 mRNA level in AML patients (OS in Kaplan-Meier plotter). (A)TCGA dataset; (B)Raponi leukemia dataset; (C) Metzeler leukemia dataset; (D)Heuser leukemia dataset. It has statistical significance (P <0.05).

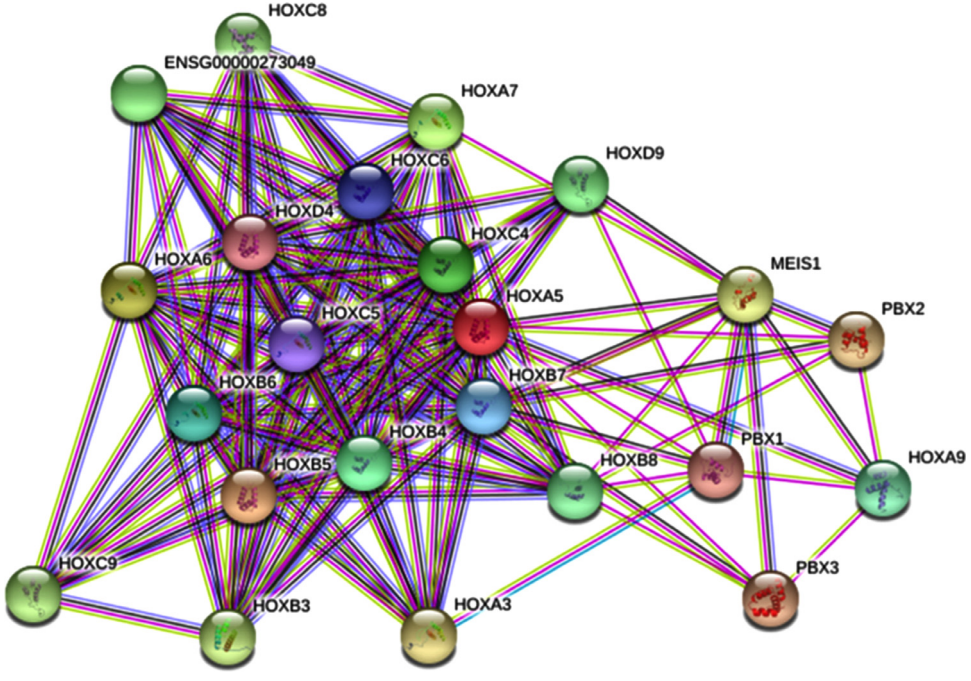

Fig. 6. HOXA5 PPI network complex and modular analysis.

cofactors PBX1/2/3 and MEIS1, which belong to the highly conserved TALE ("three amino acid loop extension") family of non-HOX HD cofactors and are required for the HOX family proteins to form a synergistic DNA-binding complex.[23] PBX cofactors supervise and guarantee normal hematopoiesis, the pluripotency of embryonic stem cells (ESC) and tissue regeneration, similar to HOX proteins in hematopoiesis.[24] Meis1 is highly expressed in mixed-lineage leukemia (MLL) and plays an important role in establishing leukemic stem cell potential and frequency by quantitatively regulating the extent of self-renewal, differentiation arrest, and cycling.[25] The interactions of HOXA5 with other HOX proteins and the TALE cotranscriptional factors were further proved by the coexpression in AML patients (Fig. S3)), implying the potential role of HOXA5 in AML pathogenesis.

Known associations: blue lines indicate protein interactions confirmed by the source database, pink lines indicate experimentally validated protein interaction relationships. Predicted associations: green lines refer to gene neighbors, red lines refer to gene fusion, purple lines refer to gene co-occurrence. others: cyan lines refer to protein associations by text mining, black lines represent co-expression, and lavender represents protein homology.

Discussion

HOX gene disorder play a significant role in leukemogenesis, and among the family members, HOXA5 is less researched. Here, by bioinformatics analysis, we highlighted the importance of HOXA5 in AML. We found that HOXA5 mRNA was differentially expressed in normal and tumor tissues (Fig. S1). However, it was significantly overexpressed in AML patients compared to normal subjects' peripheral blood mononuclear cell, monocytes or neutrophils. The increased HOXA5 mRNA was significantly associated with shorter over survival time in AML patients. It is true that some of the data reported in our study are actually conflicting (Fig 5). Out of 4 datasets used, 2 gave no differences but the similar trends were observed. The other 2 datasets indeed showed opposite results. But the number of sample in the TCGA dataset (n = 186) and the Heuser leukemia dataset (n = 28) had great difference. The opposite result in the Heuser leukemia dataset may be due to the small sample size.

PPI network analysis indicated that HOXA5 was closely co-expressed, fused or interacted with the other HOX family members and transcriptional cofactors, most of which have been reported in the development AML.[26]

The main feature of AML is the abnormal proliferation of primitive and naive bone marrow cells in peripheral blood. The main clinical characteristics are anemia, bleeding, infection and fever, organ infiltration, abnormal metabolism, and so on. AML contributes to 15%-20% of the childhood leukemias.[27] Although the outcome has improved significantly in the past few decades, AML is still a malignant tumor that threatens the lives of children, with current OS rate of ~70%.[28] While, complete remission (CR) is achieved in 75%-80% of adult patients.[14] Whether children or adults, relapse is still the most common cause of treatment failure and the main obstacle to further improve the prognosis of AML.[29,30] Our finding is consistent with the previous report that patients with AML who had high expression of HOXA5 had significantly lower complete remission rates.[31] The 3-year overall survival rate in AML patients with high expression level of HOXA5 was much lower than that of the low populations (40.5% vs 82.5%).[32] It was also reported that the suppression of the expression of HOXA5 by short hairpin RNA can suppressed the proliferation of AML cells and enhanced the sensitivity of AML cells to cytarabine.[12] We found that HOXA5 was significantly over-expressed in FAB subtype M5 and under-expressed in FAB subtype M4Eo compared with other FAB subtypes (Fig 4). The subtype of M4eo also has better prognosis than other subgroups.[33] So, our study and the published data suggested that HOXA5 may be a potential prognostic biomarker of AML patients.

How HOXA5 is regulated in AML cells? Gene fusion and mutation are a common event in the development of leukemia, especially in the development of AML.[34] Nucleoporin 98 gene (NUP98) is able to fuse with NSD1, JARID1A, PHF23, and HOXA9/10/13, which has been reported to be associated with increased HOXA5 expression.[35] It has been reported that CALM-AF10 can enhance

the expression of HOXA5 by the regulation of H3K79 methylation,[36] which plays important roles in leukemogenesis.[37] Promoter methylation is another notable mechanism that regulates HOX mRNA expression and is generally negatively related to the expression of the gene.[38] However, it was reported that the HOXA5 promoter is highly methylated in more than 60% of AML samples.[39] This indicated that more than 60% of AML samples had low expression of HOXA5, which is contrary to our finding. It is probably due to the small sample size (n = 25) in this study. Aberrant HOX expression is found in nearly all AMLs that harbor a mutation in the Nucleophosmin (NPM1) gene, and FLT3 is concomitantly mutated in approximately 60% of these cases.[40] It was reported that the expression of HOXA5 mRNA level in AML patients with NPM1 mutations was high.[26,41]This may be caused by that NPM1 mutations result in abnormal cytoplasmic localization of the mutant protein (NPM1c) in AML cells; NPM1 mutation can immediately downregulate the expression of *HOX* family genes followed by differentiation.[42] However, in the previous study, the expression of HOXA5 in AML cells with NPM1 mutation was very low.[43] The reports are not entirely consistent. In addition, Previous study has reported that leukemia can be alone induced by overexpression of individual *HOXA* genes.[44] Study has found that NPMc+ leukemic cell survival required upregulation of *HOX* genes.[45] As reported, NPM1 mutation is an indicator of good prognosis for AML.[46] However, the study found the simultaneous presence of NPM1 mutation and high HOXA5 expression in AML patients had worse overall survival.[26] Mutations of the FMS-like tyrosine kinase 3 (*FLT3*) gene is a common driver mutation that presents with a high leukemic burden and confers a poor prognosis in patients with AML.[47] Flt3-ITD(+) AML bone marrow samples highly expressed *HOXA5* gene.[48] In our study, we found that the number of FLT3 mutations was higher in HOXA5-high expression than HOXA5-low expression. However, *HOXA* gene can regulate Flt3 in hematopoietic Progenitors.[49] FLT3 mutation had no significant effect on the expression of HOXA5.[50] It may be caused by the small number of the AML samples. There were not many cases of other mutations and gene fusion in the samples we collected (Table 1). But the complicated relationship among *HOXA5*, gene fusion and mutation in AML still needs to be explored in depth.

Taken together, this evidence implies that HOXA5 plays important roles in the pathogenesis of AML. However, because there are few clinical cases of gene mutation and fusion in this study, the statistical significance of HOXA5 expression and their relationship is meaningless. The detailed mechanisms and whether it could be a therapeutic target for AML still need further investigations.

Conclusions

In summary, the increased HOXA5 was associated with significantly shorter overall survival time in AML patients. This study indicates that HOXA5 might be a prognostic marker and a potential therapeutic target for AML.

Ethics committee approval

The study was approved by the Affiliated Hospital of Southwest Medical University was conducted in accordance with the Declaration of Helsinki (No. KY2020110).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.currproblcancer.2020.100673.

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