

## RESEARCH ARTICLE

# Aberrant Expression of HOXA5 and HOXA9 in AML

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### Abstract

**Background:** Aberrant expression of HOX gene expression has been observed in cancer. The purpose of this study was to investigate the alteration of HOXA5 and HOXA9 expression and their clinical significance in acute myeloid leukemia (AML). **Materials and Methods:** The expression of HOXA5 and HOXA9 genes of bone marrow samples from 75 newly diagnosed AML patients and 22 healthy controls for comparison were examined by Real-time quantitative PCR (RQ-PCR) assay. Statistical analysis was conducted to evaluate HOXA5 and HOXA9 expression as possible biomarkers for AML. **Results:** The results showed that the complete remission rate (52.6%) of the patients who highly expressed HOXA5 and HOXA9 was significantly lower than that (88.9%) in patients who lowly express the genes ( $P=0.015$ ). Spearman correlation coefficients indicated that the expression levels for HOXA5 and HOXA9 genes were highly interrelated ( $r=0.657$ ,  $P<0.001$ ). Meanwhile, we detected significant correlations between HOXA9 expression and age in this limited set of patients ( $P=0.009$ ). **Conclusions:** The results suggest a prognostic impact of increased expression of HOXA5 and HOXA9 in AML patients.

**Keywords:** Acute myeloid leukemia - HOXA5 - HOXA9

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### Introduction

Homeobox (HOX) genes are members of a transcription factor family and play a crucial role in embryonic development and in the control of differentiation of adult hematopoietic cells (Cillo et al., 2001; Abramovich and Humphries, 2005; Spencer et al., 2015). Homeobox genes encode the class of transcription factors in vertebrates and are found in clusters called A, B, C, and D on four separate chromosomes. The HOXA genes are associated not only with the development of hematologic malignancies but also with the prognosis of these conditions. Some recent studies have shown that increased expression of HOXA genes is correlated to the cytogenetic findings associated with poor prognosis in AML and mixed lineage leukemia (Drabkin et al., 2002; Golub et al., 1999). It has been reported that HOXA5 and HOXA9 are relevant to the development of myelomonocytes (van Oostveen et al., 1999; Cooks et al., 1999; Fuller et al., 1999). The abnormal expression of HOXA9 was reported to affect the proliferation, differentiation, and apoptosis of hemopoietic cells (Golub et al., 1999), or even lead to leukemia (Nakamura et al., 1996; Borrow et al., 1996). In the present study, we sought to further investigate the HOXA5 and HOXA9 gene expression in 75 patients with acute myeloid leukemia (AML) to determine the relationship between HOXA5 and HOXA9 gene expression and AML pathogenesis and prognosis. Here, we show that HOXA5

and HOXA9 mRNA were highly expressed in 38/75 (50.7%) AML patients. Spearman correlation coefficients indicated that the expression levels for the two genes were highly interrelated. Importantly, the complete remission rate in these patients was lower than that of HOXA5 and HOXA9 non-expressors following the chemotherapy.

### Materials and Methods

#### *Patients' samples*

This study included 75 patients who had a diagnosis of de novo AML at the First Affiliated Hospital of Guangzhou Medical University. The diagnosis and classification of AML patients were based on French-America-British (FAB) and World Health Organization (WHO) criteria (Sabattini et al., 2010; Bennett et al., 1985). Treatment protocol was described as reported previously (Li et al., 2013). Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of the First Affiliated Hospital of Guangzhou Medical University. The main clinical and laboratory features of the patient cohort were summarized in Table 1. A total of 22 healthy donors were collected as controls.

#### *Cytogenetic analysis*

Conventional cytogenetic analysis was performed in the cytogenetics laboratory of the First Affiliated Hospital

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of Guangzhou Medical University. Chromosomes were prepared routinely by the direct method or 24 h short-term culture of bone marrow cells. Karyotypes were analyzed on R-banded metaphases. Chromosome abnormalities were described according to the International System for Human Cytogenetic Nomenclature (Gonzalez et al., 2006).

*RNA isolation, reverse transcription and real-time quantitative PCR*

Mononuclear cells from bone marrows of AML patients at initial diagnosis and healthy donors were enriched by Ficoll-Hypaque gradient. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. cDNA was synthesized using two micrograms of total RNA, dNTPs, RNase inhibitor (RNasin), MMLV reverse transcriptase (MBI Fermentas, Hanover, MD, USA), and random hexamers. The system of reverse transcription was incubated for 10 min at 25°C, 60 min at 42°C, and then stored at -20°C. Real-time quantitative PCR (RQ-PCR) assay was performed for each sample in duplicate in a final reaction volume of 20µL. HOXA5 was amplified using the primers 5’GCGCAAGCTGCACATAAGTCA3’ (forward) and 5’TGCTCTCGGAGAGGCAAAGA3’ (reverse) with expected products of 174bp. HOXA9 was amplified using the primers 5’ATCGATCCCAATAACCCAGCA3’ (forward) and 5’TGGTGT TTTGTATAGGGGCAC3’ (reverse) with expected products of 80bp. Reaction system consisted of 20ng of cDNA, 0.8µM of primers, 10µM AceQTMqPCR SYBR Green Master Mix (Vazyme Biotech Co, Piscataway, NJ, USA), and 0.4µM ROX Reference Dye1 (Invitrogen). Reaction conditions were as follows: initial denaturation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing and elongation at 58°C for 1 minute. Reverse transcription was performed in triplicate. The amount of target was calculated by the 2<sup>-ΔΔCt</sup> method (Arocho et al., 2006).

*Statistical analysis*

Statistical analysis was performed using SPSS 19.0 (SPSS, Armonk, NY). Pearson  $\chi^2$  analysis or Fisher exact test was employed to compare the difference of categorical variables. Mann-Whitney’s U-test was used to compare the difference of continuous variables. Receiver operating characteristic curve (ROC) were used to set cut-off value of HOXA5 and HOXA9 expression in AML patients. Spearman rank correlation analysis was used to analyze the correlation of HOXA5 expression with expression of HOXA9. For all analyses, two-tailed p-values of 0.05 or less were determined statistically significant.

**Results**

*HOXA5 and HOXA9 Gene Expression in de novo AML and Healthy Individuals*

We evaluated the level of HOXA5 and HOXA9 expression in AML and healthy controls. The relative expression levels of HOXA5 and HOXA9 mRNA levels were significantly overexpressed in 75 de novo AML patients in comparison with the controls ( $P<0.05$ )

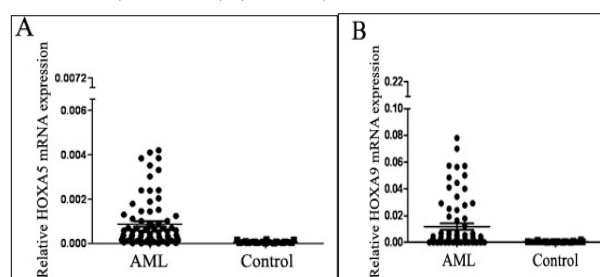
(Figure1). The expression of HOXA5 and HOXA9 was significantly higher in AML patients compared with normal subject.

*Correlation between HOXA5 and HOXA9 expression and clinical characteristics*

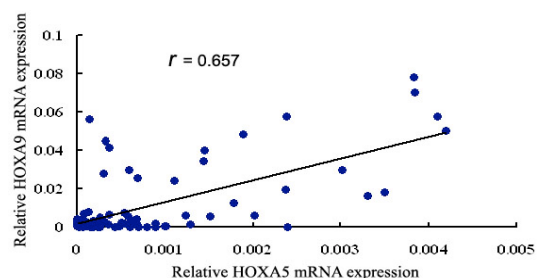
Overall, we studied 75 AML patients aged between 16 and 79 years (median 45 years) in this study (Table 1). AML subtypes, according to the French–American–British classification, were M2 (n=28, 37.3 %), M3 (n=20, 27%) non M2 and M3 (n=26, 34.7%). Flow cytometric analysis of bone marrow cells revealed (2–93.5) % (average 62.3%) immature cells in the patients.

The relative expression levels of the HOXA5 and HOXA9 gene in 75 de novo AML patients according to the various clinical and pathological parameters are shown in Table1. Expression analysis regarding to the FAB type showed significant overexpression of HOXA9 in non M2 and M3 types ( $P < 0.05$ ), low expression of HOXA9 were found in patients with M2, M3 and younger patients, especially in AML patients with t (8;21) (q22;q22) and t (15;17) (q22;q21).

At the set cut-off value of 0.000102 and 0.00212 according to ROC curves, this cohort of AML patients was divided into four groups: high HOXA5 expression ( $\geq 0.000102$ ) and low HOXA5 expression ( $< 0.000102$ ); high HOXA9 expression ( $\geq 0.00212$ ) and low HOXA9 expression ( $< 0.00212$ ). There were no significant differences between low HOXA5 and high HOXA5 expressing patients with respect to age, white blood cells (WBCs), the percentage of blasts in bone marrow, FAB and karyotypes (Table1). Significant difference was seen between low HOXA5 and HOXA9 and patients with high HOXA5 and HOXA9 in the rates of complete remission after induction therapy ( $P = 0.015$ ). However, significant differences can be seen between low HOXA9 and high HOXA9 expressing patients with respect to age ( $P = 0.009$ ) and FAB ( $P = 0.034$ ) (Table 1).



**Figure 1. Relative Expression levels of HOXA5 and HOXA9 mRNA in AML and Control**



**Figure 2. Results of qRT-PCR Performed for 75 Newly Diagnosed AML Patients, Expression of HOXA5 Correlated with HOXA9 ( $r = 0.657, P < 0.001$ )**

**Table 1. Relationship between Relation Gene Expression Level and Standard Clinical and Biological Factors**

| Grouping variable            |                        | HOXA5 n (%) |             |         | HOXA9 n (%) |             |         |
|------------------------------|------------------------|-------------|-------------|---------|-------------|-------------|---------|
|                              |                        | Low (n=37)  | High (n=38) | p value | Low (n=37)  | High (n=38) | p value |
| Age (years)                  | ≤60                    | 33 (88.9%)  | 24 (63.2%)  | 0.070   | 35 (94.4%)  | 22 (57.9%)  | 0.009   |
|                              | >60                    | 4 (11.1%)   | 14 (36.8%)  |         | 2 (5.56%)   | 16 (42.1%)  |         |
| WBC                          | <30×10 <sup>9</sup> /L | 29 (77.7%)  | 32 (84.2%)  | 0.629   | 31 (83.3%)  | 30 (78.9%)  | 0.934   |
|                              | ≥30×10 <sup>9</sup> /L | 8 (22.2%)   | 6 (15.8%)   |         | 6 (16.7%)   | 8 (21%)     |         |
| Blasts in BM                 | ≤70%                   | 21 (55.6%)  | 20 (52.8%)  | 0.641   | 16 (44.4%)  | 22 (57.9%)  | 0.260   |
|                              | >70%                   | 16 (44.4%)  | 18 (47.4%)  |         | 21 (55.6%)  | 16 (42.1%)  |         |
| Karyotype                    | Normal                 | 8 (22.2%)   | 16 (42.1%)  | 0.309   | 16 (44.4%)  | 16 (42.1%)  | 0.393   |
|                              | Abnormal               | 23 (62.5%)  | 18 (47.4%)  |         | 16 (44.4%)  | 20 (52.6%)  |         |
| Karyotype t (8;21) (q22;q22) | Absent                 | 29 (77.8%)  | 34 (88.9%)  | 0.348   | 27 (72.2%)  | 36 (94.7%)  | 0.074   |
|                              | Present                | 8 (22.2%)   | 4 (10.5%)   |         | 10 (27.8%)  | 2 (5.3%)    |         |
| FAB                          | M2                     | 16 (44.4%)  | 12 (31.6%)  | 0.186   | 19 (51.3%)  | 10 (26.3%)  | 0.034   |
|                              | M3                     | 12 (33.3%)  | 8 (21%)     |         | 12 (33.3%)  | 8 (21%)     |         |
|                              | Other                  | 8 (22.2%)   | 18 (47.4%)  |         | 6 (16.7%)   | 20 (52.6%)  |         |
| CR (-)                       |                        | 33 (88.9%)  | 20 (52.6%)  | 0.015   | 33 (88.9%)  | 20 (52.6%)  | 0.015   |

#### Relation between HOXA5 and HOXA9 expression

Spearman correlation coefficients indicated that the HOXA5 expression correlated with expression of HOXA9 ( $r = 0.657$ ,  $P < 0.001$ ; Figure 2).

## Discussion

Acute myeloid leukemia (AML), the most common type of hematological malignancies in adults, is characterized by differentiation arrest and accumulation of myeloid blasts in bone marrow that leads to the insufficiency of normal hematopoiesis (Estey and Döhner, 2006; Shahab et al., 2013). Some markers have been shown to predict the clinical outcome and provide potential targets for molecular therapies, which are <30% of de novo AML (Cancer Genome Atlas Research Network, 2013; Hou et al., 2014; Kumar, 2014). Therefore, the molecular pathogenesis of AML has not yet been completely defined, new molecular markers are warranted to identify those who are at the risk of poor outcome and to optimize treatment strategies in patients with a normal karyotype and no date. In cancer, deregulation of HOX gene expression and HOX alterations have been most convincingly demonstrated in leukemia (Nakamura et al., 1996; Nakamura et al., 2009). In this report, we have used real-time RT-PCR to study HOXA5 and HOXA9 expression in defined cases of AML with clinical follow-up. The loss of HOXA5 function limits leukaemia associated with specific chromosomal translocations, which is a key regulator of myeloid differentiation (Boucherat et al., 2009). Overexpression of HOXA5 in CD34+progenitor cells induced increased levels of granulocytic/monocytic differentiation and inhibited erythroid/megakaryocytic differentiation. Inhibition of HOXA5 inhibited granulocytic/monocytic differentiation but increased erythroid/megakaryocytic differentiation (Cooks et al., 1999). In this study, we quantified the level of HOXA5 mRNA by real-time RT-PCR and examined its correlation with the clinical outcome. We found HOXA5 expression was significantly up-regulated in AML compared to healthy controls, especially in non-remission patients. High HOXA5 expression was observed in 50.7% of AML patients if the cut-off value 0.000102 was used

according to ROC curve. Our study further demonstrated that high HOXA5 expression was significantly associated with CR rate of AML patients, although we did not find the correlation of HOXA5 expression with other clinical parameters. HOXA9 gene is localized on the human chromosome 7p15, which is relevant not only to the function of the early hemopoietic stem cells, it also gets involved at the late differentiation and orientation stages of hemopoietic stem cells (Golub et al., 1999). Some data indicated that HOXA9 gene has medullary system specificity, its expression was relevant to the immature leukemia cells, and it might play a role in the pathogenesis of AML (Thorsteinsdottir et al., 2001). Here we found HOXA9 expression was significantly upregulated in AML compared with healthy controls, especially in non-M2 and M3 AML patients. High HOXA9 expression was observed in 50.7% of AML patients if the cut-off value 0.00212 was used according to ROC curve. Lower relative amounts of HOXA9 were detected in patients with t (8;21) (q22;q22) and cases with prognostically favorable cytogenetic features, but extended studies have not been able to reveal any significant associations with complete response rates of event-free survival (Drabkin et al., 2002). Our study further demonstrated that high HOXA9 expression was related with age and FAB. These findings are consistent with those of the previous study.

Regulation of HOXA5 expression requires multiple cis-acting regions, some encompassing coding sequences from neighboring genes (Boucherat et al., 2009). Our study showed that overexpression of HOXA5 closely correlated with high expression of HOXA9, which were related to the development of myelomonocytes and leukemogenesis, the correlation between HOXA5 and HOXA9 is a particularly interesting finding that calls for further research to reveal the underlying mechanism of the interaction.

In conclusion, our present study indicated that high HOXA5 expression in AML tended to increase in non-remission patients, while high expression of HOXA9 was found in patients with non-M2, M3 and AML patients with age older than 60 years, the expression of HOXA9 was low in AML patients with favorable karyotypes, such as M2 t (8;21) (q22;q22) and M3 t (15;17) (q22;q21).

Moreover, our results showed that HOXA5 correlated with a poor prognosis and HOXA9, which was correlated with leukemogenesis. However, the small sample size and the preliminary nature of this study are the limitations of our study, performing a larger study to clearly determine the relationships between HOXA5 and HOXA9, prognosis of AML patients is also needed.

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