

## The Relationship between Mutation in *HOXB1* Gene and Acute Myeloid Leukemia

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### Abstract

**Background:** HOX genes are an exceedingly preserved family of homeodomain-involving transcription factors. They are related to a number of malignancies, comprising acute myeloid leukemia (AML). This study aimed to evaluate the effect of *HOXB1* 7bp deletion mutation on *HOXB1* gene expression in 36 individuals.

**Materials and Methods:** The present cross-sectional study was done on a large Iranian family. In this experimental study, 5 homozygous 7bp deletion individuals along with their unaffected siblings and their parents were investigated. The candidate gene, *HOXB1* was screened and analyzed in blood samples of these participants. After RNA extraction, cDNA was synthesized according to manufacturer's protocol. *HOXB1* expression level was analyzed by  $2\Delta\Delta CT$  method. All laboratory procedures used in this experimental study were carried out in genetic laboratory of Shahid Sadoughi University of Medical Sciences.

**Results:** Sequence analysis of *HOXB1* gene by ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) revealed a family with 5 homozygous ( $22\pm 17$  years) and 22 healthy heterozygous carriers ( $42\pm 19$  years) for 7bp deletion in *HOXB1* gene along with 9 healthy wild type ( $55\pm 41$  years). Gene expression analysis by RT-qPCR demonstrated that expression level of *HOXB1* gene in wild type and heterozygous carriers specimens had similar levels ( $p=0.05$ ).

**Conclusion:** Although *HOXB1* mutations has been reported in AML, but association between *HOXB1* mutation and AML was not found in our study. Additionally, *HOXB1* expression levels showed no significant difference between wild type and heterozygous carriers. So, *HOXB1* gene expression cannot provide a powerful tool to differentiate wild type from heterozygous carries.

**Keywords:** Acute Myeloid Leukemia, Gene expression, *HOXB1*

### Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous group and phenotypically an aggressive diseases in which the differentiation of hematopoietic progenitor cells are lost, resulting in increment of their self-renewal capacity and dysregulation in cell proliferation(1). In terms of maturation stage and morphology, AML is categorized into eight groups (M0-M7). Besides, AML divides into four major groups based on immune phenotyping, genetics, and clinical characteristics. Recurrent genetic abnormalities, AML with myelodysplasia-

related changes, therapy-related AML and myelodysplasia syndrome (MDS) are four types of AML which there is no agreement about AML classification in these four groups.

To date, cytogenetic and molecular examinations are frequently used to classify AML. For instance, the translocations *inv* (16), *t*(8;21), and *t*(15;17) harbor a helpful prognosis although other cytogenetic aberration indicate leukemia with mild or high risk of relapse (2-4). However, the categorization of AML on the basis of karyotyping is still far from convincing. Non-random

chromosomal alterations, such as balanced translocations, monosomies, trisomies, inversion, and deletions have been found in the leukemic cells of almost 55% of AML patients, and until recently they are considered to be the most crucial prognostic factors for complete remission (CR), likelihood of relapse, and overall survival (5, 6).

Initiation of AML is associated with two distinct genetic changes, referred to Class I and Class II. Class II mutations aim hematopoietic transcription factor genes leading to a block in myeloid differentiation and conferring the self-renewal ability of hematopoietic precursors (7). The most affected and mutated transcription factors are homeobox (*HOX*) genes (8). The clustered *HOX* family of homeobox genes (class I homeobox genes) is an evolutionarily highly conserved set of genes that encode DNA-binding transcription factors that were first identified as key regulators of positional identity along the anterior–posterior body axis of animal embryos (9). Thirty nine *HOX* genes placed on four different chromosomes (Table I) have been found in mammals. By considering homeobox sequence similarity, mammals *HOX* genes comprising of 13 paralogous groups, with no cluster containing a full set (10, 11). The previously reported *HOXB1* mutation, substitution of arginine to cysteine in the homeodomain of *HOXB1* at 207 residue, modifies binding capability of *HOXB1* for transcriptional co-regulators and DNA (12). *HOXB1* has a characteristic helix-turn-helix DNA binding motif with three alpha helical regions where the specificity increased by heterodimerization with *PBX1* (13). NMR experimental studies displayed that the conserved hexapeptide of *HOXB1* (TFDWMK) stabilizes binding of *PBX1* and *HOXB1* to DNA (14). Recently, mutations in ten *HOX* genes have been found to cause different human disorders (15). The first report was done by Goddard et al., in 1996, who showed a nonsense

mutation in *HOXB1* gene in mice caused impaired development of facial nerve but no similar cases were reported in human (16). Additionally, In previous studies indicated that a set of seven downregulated *HOX* genes, including *HOXA3*, *A4*, *A5*, *A7*, *B1*, *B9* and *C9* as significantly clustered in pediatric AMLs. To analysis gene expression for mRNA sequences, precise, sensitive, and reproducible measurements are needed. To date, the most sensitive technique for the detection of low abundance mRNAs is Real Time PCR. It has various applications, such as clinical diagnostic and gene expression analysis in animals and plants (18). Real Time PCR is well known as an internal control gene whose conditions of the experiment should not affect internal control gene expression (19). However, several studies revealed that internal standards, mainly reference genes used for the quantification of mRNA expression, could be influence by experimental situations (20). The most common reference genes are actin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal genes, cyclophilin, 18s rRNA, and elongation factor 1-a (21).

This study aimed to evaluate the effect of 7bp deletion mutation on *HOXB1* gene expression in an extended Iranian family by using Real Time PCR.

## Materials and Methods

### Sample collection

At first, 36 blood samples, including 5 affected individuals as well as 31 healthy individuals (22 heterozygous for 7bp deletion), were obtained. Participants' clinical and pathological data were collected. The study was approved by the Human Studies Committee at the Shahid Sadoughi University of Medical Science (IRB No. 1394.239). Sample taking was done at Shahid Sadoughi Hospital, Shahid Sadoughi University of Medical sciences.

### Mutation analysis

Genomic DNA was extracted from blood samples using QIAmp DNA mini kit

(Qiagen, Hilden, Germany), based on the manufacturer's protocol. Then, all DNA samples were kept at  $-20^{\circ}\text{C}$ . Mutation analysis by PCRs were done in 25 ml reaction volumes: 12.5  $\mu\text{l}$  of PCR master mix 2X (Sinaclon, Iran), 3  $\mu\text{l}$  of DNA, 1  $\mu\text{M}$  of each primer (primers listed in Table II), and 7.5  $\mu\text{l}$  dd H<sub>2</sub>O. PCR amplification was done under the following thermal protocol: 95  $^{\circ}\text{C}$  for 3 min, followed by 38 cycles at 65  $^{\circ}\text{C}$  45 s, 75  $^{\circ}\text{C}$  for 1 min, and 95  $^{\circ}\text{C}$  for 45 s and a final extension cycle at 78  $^{\circ}\text{C}$  for 4 min. We loaded 5 ml of total PCR product on a 2% agarose gel electrophoresis in order to determine the presence and size of the amplified of all exons as well as exon-intron boundaries of *HOXB1* gene. The PCR amplicons were purified and both sense and antisense direction sequenced in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

#### RNA extraction and cDNA synthesis

Total RNA was extracted from blood samples using RNX<sup>TM</sup>-Plus solution (SinaClon, IRAN) based on the instructions presented by the manufacturer, except for an extended 1-h treatment with DNase I. RNA was analyzed by Thermo Scientific NanoDrop<sup>TM</sup> 1000 Spectrophotometer to evaluate its purity and concentration, and electrophoresed on 2% agarose gel to confirm its integrity. One microgram of RNA was applied for complementary DNA (cDNA) synthesis using random hexamer priming and Prime Script<sup>TM</sup>-RT reagent kit (TaKaRa, Japan).

#### Real Time PCR

All the samples were placed in a rotor gene 6000 Corbett detection system. Real Time PCR was done by SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> II kit (Takara, Japan) based on the manufacturer's instructions. Thermal cycling conditions were an initial activation step for 5 min at 95  $^{\circ}\text{C}$ , which were followed by 40 cycles at 95  $^{\circ}\text{C}$  for 5 s and 65  $^{\circ}\text{C}$  for 30 s. Forward and reverse primers sequences have been shown in Table I. Melting curve analysis was done to confirm the specificity of PCR products.

To confirm product sizes and specificity, PCR products were electrophoresed on 2 % agarose gel. All samples were normalized to GAPDH for Real Time PCR analysis. The average value in each triplicate was used to calculate  $\Delta\text{CT}$ . Expression fold changes were calculated using  $2^{-\Delta\Delta\text{Ct}}$  methods. The qPCR assays were done in triplicate, and the data were shown as the mean  $\pm$ standard error of the mean (SEM).

#### Statistical analysis

For statistical analyses of clinical and demographic data, SPSS version19 (SPSS Inc., Chicago, IL) was used. One-way ANOVA with post-hoc Tukey test were used to detect any significant differences in *HOXB1* expression levels between three groups. Significance threshold was defined as  $p < 0.05$ .

## Results

#### General statistical information

Average age for homozygous, heterozygous carriers, and wild type groups were  $22\pm 17$ ,  $42\pm 19$ , and  $55\pm 41$  years old, respectively. Data were analyzed based on the information taken from laboratory tests, clinical findings, interviews, and questionnaires. Autosomal recessive was considered for mode of inheritance based on the phenotypic presentation in this family.

#### Expression and sequence analysis of *HOXB1* gene

Sanger sequencing was done for *HOXB1* gene in all the available individuals. Twenty-two and 9 out of 36 were heterozygous carriers and wild type, respectively, for 7bp deletion. The results of this study confirmed that the expression level of *HOXB1* gene in wild type and heterozygous carriers was similar. As shown in Figure 1, expression level of *HOXB1* gene did not differ significantly between samples ( $p=0.05$ ).

Table I: Human HOX genes and their respective location

| cluster     | Human chromosome | genes   |
|-------------|------------------|---|
| <b>HOXA</b> | Chromosome 7     | HOXA1, HOXA2, HOXA3,<br>HOXA4, HOXA5, HOXA6,<br>HOXA7, HOXA9, HOXA10,<br>HOXA11, HOXA13 |
| <b>HOXB</b> | Chromosome 17    | HOXB1, HOXB2, HOXB3,<br>HOXB4, HOXB5, HOXB6,<br>HOXB7, HOXB8, HOXB9,<br>HOXB13          |
| <b>HOXC</b> | Chromosome 12    | HOXC4, HOXC5, HOXC6,<br>HOXC8, HOXC9, HOXC10,<br>HOXC11, HOXC12, HOXC13                 |
| <b>HOXD</b> | Chromosome 2     | HOXD1, HOXD3, HOXD4,<br>HOXD8, HOXD9, HOXD10,<br>HOXD11, HOXD12, HOXD13                 |

Table II: HOXB1 gene oligonucleotide primer

| Gene         |               | sequence   | Area                      | Product size |
|--------------|---------------|--|---------------------------|--------------|
| <b>Hoxb1</b> | PCR           | Forward:<br>CGGGTGATTGAAGTGCTTTG<br>Reverse:<br>CCCAGAGGGTAGTACTGAGAAG   | <b>Exon1</b>              | <b>579</b>   |
| <b>Hoxb1</b> | PCR           | Forward:<br>TGACAGCTATGCAAGCGAGG<br>Reverse:<br>GAAAGAGAAGAACCCAGCCC     | <b>Exon1</b>              | <b>575</b>   |
| <b>Hoxb1</b> | PCR           | Forward:<br>CCCTGGGAACTTTGACAACTG<br>Reverse:<br>GCAGCTCTAAACTGGCATTTC   | <b>Exon2</b>              | <b>600</b>   |
| <b>Hoxb1</b> | Real Time PCR | Forward: GACAGCGAAGGTGTCAGA<br>Reverse: GCAATCTCCACCCTCCGG               | <b>exon-exon junction</b> | <b>156</b>   |
| <b>GAPDH</b> | Real Time PCR | Forward:<br>CCTAGATTATTCTCTGATTTGGT<br>Reverse:<br>ATGTAGTTGAGGTCAATGAAG | <b>673-835</b>            | <b>162</b>   |

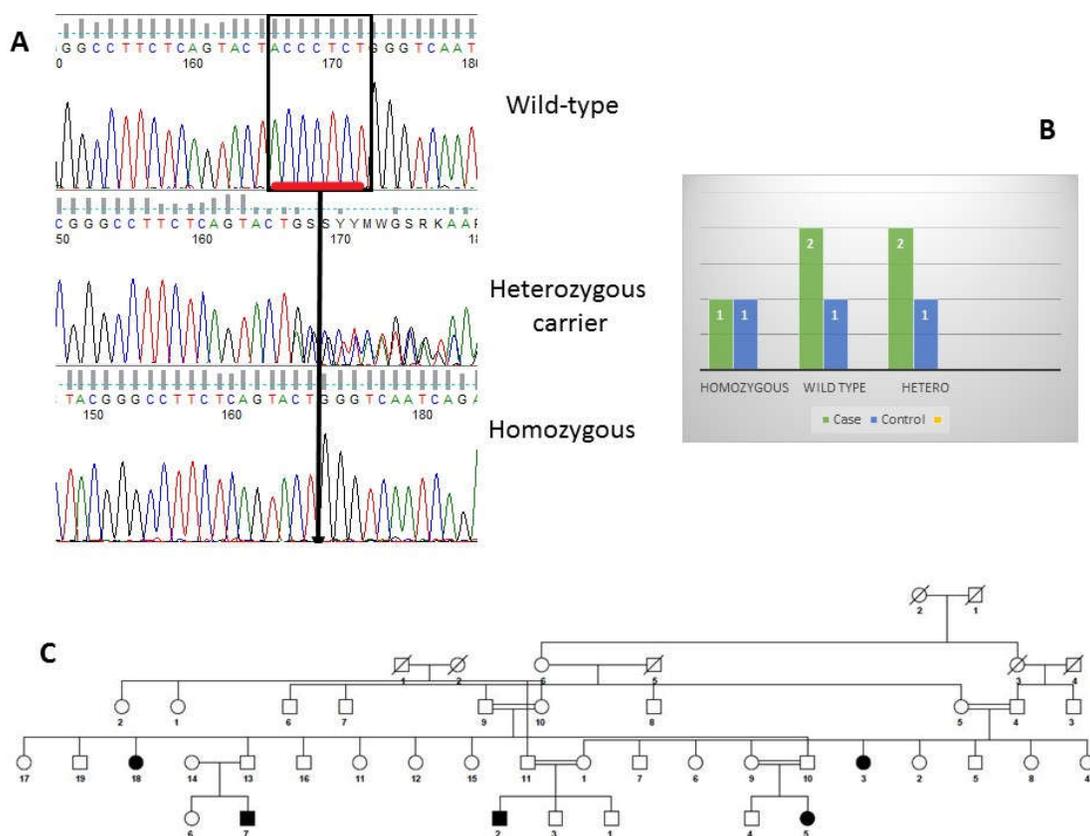


Figure 1: A) A section of Sanger sequencing Chromatograms for the wild type (top) heterozygous carrier (middle) and homozygous (below), also 7 base pairs deletion depicted in the box. B) Expression level of *HOXB1* gene. C) Pedigree of the Iranian family with five Homozygous for 7bp deletion (indicated as solid black symbols).

## Discussion

AML is a disease with molecular and phenotype heterogeneity which is the result of acquired mutations in hematopoietic progenitor cells. As the disease progresses, a large number of molecular abnormalities, including cytogenetic abnormality, transcriptomic changes, epigenetic alterations, and somatic mutations accumulate in affected progenitor cells (22). Mutations in genes involved in myeloid differentiation (CEBPA and RUNX1/AML1), cell proliferation (KIT, NRAS/KRAS) as well as up-regulation of *HOXB* gene expression are the major genetic alteration involved in AML (11).

Identifying molecular alterations can show a significant role in the treatment of AML. The first molecular model presented for AML was two-hit model proposed by Kelly and Gilliland. They demonstrated at least two broad classes of mutation in AML, namely class 1 and class 2 (23). However, today, with the advent of NGS technology, more molecular alterations can be identified in AML, paving the way for targeted treatment. Therefore, we evaluated *HOXB1* gene expression in homozygous, heterozygous carrier, and wild type as well as the association between *HOXB1* and AML to discover the role of 7bp deletion on *HOXB1* gene expression.

*HOX* genes show a high degree of homology to the clustered homeotic genes (*HOM-C*) of *Drosophila melanogaster*, which are placed in two gene clusters, namely Antennapedia (*Ant-C*) and bithorax complexes (*BX-C*). The changes of *HOX* gene expression can lead to abnormal morphological structures during the development of fetus, and can even cause tumorigenesis by changing the expression of apoptosis-related proteins and/or signaling pathways (24).

Recent intensive experimental studies displayed that aberration of *HOXB* expression can lead to cancer. For example, Oscar Lindblad (11) disclosed that *HOXB2* and *HOXB3* expression levels were up-regulated in AML patients, and they can be used as a powerful biomarkers in AML. *HOX* genes can be indirectly involved in AML and MLL through chromosomal rearrangements. MLL fusion proteins constitute about 10% of therapy-related AML (t-AML) and 3% of de novo AML (25). There are more than 64 translocation partner genes (TPG) that fuse with the MLL N-terminus (26).

Importance of *HOXB1* in cancers has also been shown in several studies. For example, in a study in 2015, Han et al. indicated that *HOXB1* expression is significantly downregulated in glioma tissues and cell lines, and that its expression may be closely associated with the degree of malignancy. In addition, *HOXB1* was shown experimentally to be a direct target of miR-3175 (27).

Furthermore, Petrini et al. suggested that the elevated expression of *HOXB1* gene reduced cell growth proliferation, leading to cell differentiation and apoptosis along the monocytes and granulocytic lineages. For that reason, gene expression analysis revealed the *HOXB1*-dependent down-regulation of some tumor promoting genes, paralleled by the up-regulation of apoptosis and differentiation related genes, thus assigning a tumor suppressor role for *HOXB1* in AML (28). In this research, *HOXB1* gene, as an additional member of

the *HOX* family with tumor suppressor properties, did not show any expression alterations in wild type and heterozygous carriers. This result emphasized that the gene sequence had no effect on gene expression. Additionally, we found no association between *HOXB1* mutation and AML in this Iranian family.

## Conclusion

In the present study, patient with 7bp mutation did not show any expression change in *HOXB1* gene from those who had not the same mutation. However, further studies with larger samples are needed to be performed to support the results of this study.

## Conflict of interest

The authors declared that there was no conflict of interest to publish this article.

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