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 2ΔΔCT 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±17 years) and 22 healthy heterozygous carriers (42±19 years) for 7bp deletion in HOXB1 gene along with 9 healthy wild type (55±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 carriers.

**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 place
ded 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,
some 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 °C. Mutation analysis by PCRs were done in 25 ml reaction volumes: 12.5 μl of PCR master mix 2X (Sinaclon, Iran), 3 μl of DNA, 1 μM of each primer (primers listed in Table II), and 7.5 μl dd H2O. PCR amplification was done under the following thermal protocol: 95 °C for 3 min, followed by 38 cycles at 65 °C 45 s, 75 °C for 1 min, and 95 °C for 45 s and a final extension cycle at 78 °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™-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™ 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™-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® Premix Ex Taq™ II kit (Takara, Japan) based on the manufacturer’s instructions. Thermal cycling conditions were an initial activation step for 5 min at 95 °C, which were followed by 40 cycles at 95 °C for 5 s and 65 °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 ΔCT. Expression fold changes were calculated using $2^{-\Delta\Delta C_{T}}$ methods. The qPCR assays were done in triplicate, and the data were shown as the mean ±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±17, 42±19, and 55±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 ($p=0.05$).
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Table I: Human HOX genes and their respective location

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

Table II: HOXB1 gene oligonucleotide primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>sequence</th>
<th>Area</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxb1</td>
<td>PCR</td>
<td>Forward:  CGGGTGATTGAAGTGTCTTTG  Reverse: CCCAGAGGGTAGTACTGAGAAG</td>
<td>Exon1 579</td>
</tr>
<tr>
<td>Hoxb1</td>
<td>PCR</td>
<td>Forward:  TGACAGCTATGCAAGCGAGG  Reverse: GAAAGAGAAGAACCCAGCCC</td>
<td>Exon1 575</td>
</tr>
<tr>
<td>Hoxb1</td>
<td>PCR</td>
<td>Forward:  CCCTGGGAACTTTGACAACTG  Reverse: GCAGCTCTAAACTGCACTTTC</td>
<td>Exon2 600</td>
</tr>
<tr>
<td>Hoxb1</td>
<td>Real</td>
<td>Forward:  GACAGCGAAGGTCAGAGAAG  Reverse: GCAATCTCCACCCTCCGG</td>
<td>exon–exon junction 156</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Real</td>
<td>Forward:  CCTAGATTATTCTCTGATTTGG  Reverse: ATGTTGGAGGTCATGAAG</td>
<td>673-835 162</td>
</tr>
</tbody>
</table>
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 abberation 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.

Reference
8. Alharbi RA, Pettengell R, Pandha HS, Morgan R. The role of HOX genes in normal