Role of XmnIγ Polymorphism in Hydroxyurea Treatment and Fetal Hemoglobin Level at Isfahani Intermediate β-Thalassemia Patients

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ABSTRACT

Background: β-thalassemia is the most common monogenic disorder in human. The (C→T) polymorphism at -158 upstream region of the γ}-globin gene and pharmacological factors such as hydroxyurea have been reported to influence γ-globin gene expression and the severity of clinical symptoms of β-thalassemia. Methods: In the present study, 51 β-thalassemia intermediate patients were studied. XmnIγ polymorphism genotype was determined using Tetra-Primer ARMS-PCR technique. Hemoglobin (Hb) and fetal hemoglobin (HbF) levels were determined by gel electrophoresis. Results: Of 51 patients, 35 (68.6%) patients were heterozygous (CT) and 16 (31.4%) patients were homozygous (CC). Of 30 patients under treatment by hydroxyurea, 20 (66.7%) patients were heterozygous (CT) and 10 (33.3%) patients were homozygous (CC). Our results demonstrated that in the heterozygous (CT) genotype, the Hb (9.58 ± 1.25 gm/dl) and HbF (89.30 ± 21.87) levels were significantly higher in comparison with homozygous (CC) genotype (7.94 ± 1.34 gm/dl and 70.32 ± 40.56, respectively). Furthermore, we observed that after drug usage, the Hb and HbF levels in patients with heterozygous (CT) genotype (0.26 ± 1.43 gm/dl and 0.8 ± 1.31, respectively) raised more in comparison with patients carrying T allele are increased significantly, and they also response to hydroxyurea treatment. Conclusion: Hb and HbF levels in the patients carrying T allele are increased significantly, and they also response to hydroxyurea treatment. Iran. Biomed. J. 19 (3): 177-182, 2015

Keywords: Fetal hemoglobin (HbF), Hydroxyurea, Intermediate β-thalassemi

INTRODUCTION

The β-thalassemia, which is the most common human single-gene disorder, is caused by any of more than 200 mutations in the β-globin gene [1]. These mutations lead to imbalanced synthesis of α- and β-globin chains, resulting in precipitation of excess peptide chains and hemolysis [2]. β-thalassemia is classified into three types: 1) β-thalassemia minor (trait) that often goes undiagnosed because people with the condition have no real symptoms other than mild anemia; 2) β-thalassemia major that patients have varying effects from the disease [3].

Some genetic factors can influence the disease severity of β-thalassemia as follow: 1) The coinheritance of homozygosity or compound heterozygosity for mild β-thalassemia alleles; 2) coinheritance of α-thalassemia, which reduces the degree of α/β imbalance; 3) the genetic variation of patients that causes upregulation of γ-globin gene expression in adult life and therefore compensates the decreased levels of β-globin chain. The common genetic variant is C>T XmnI polymorphism (rs7482144) at the -158 site upstream region of the γ}-globin gene [4]. Temporal and tissue-specific transcription of the β-globin gene is regulated by the locus control region (LCR) consisting of five DNase I hypersensitive sites, which is located on 5' site of the β-globin gene. These sites are devoid of nucleosome formation and therefore are more accessible to interactions with transcription factors. Transcriptional activators are bound to the LCR hypersensitive sites and then recall RNA polymerase II to these sites. Finally, RNA polymerase is loaded onto the gene to be transcribed [5]. The hypersensitive sites are needed to form an active chromatin loop with looping the β-globin locus. This three-dimensional structure, which is termed an active chromatin hub, includes LCR elements interacting with transcriptional factors, downstream globin structural genes, and chromatin remodeling complexes necessary for hemoglobin (Hb) switching. These interactions in human adult and fetal cells activate the β-globin genes [6].

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The -158 C > T polymorphism is located near a nuclelease hypersensitive site at 50 to 150 bp upstream region of the $\gamma$-globin gene. Perhaps the -158 substitution reduces the binding of transcription factor(s) that silence(s) the $\gamma$-globin gene expression in adult cells. Therefore, the $\gamma$-globin gene is reactivated in adult lifen [2, 7]. Furthermore, several pharmacologic agents, such as 5-azacytidine, erythropoietin, butyrates, and hydroxyurea have been shown to stimulate $\gamma$-globin gene expression in vivo and therefore might reduce the severity of clinical symptoms in patients with intermediate thalassemia [8]. Moreover, one study on $\beta$-thalassemia patients treated with hydroxyurea has revealed a significant correlation between the presence of T allele in Xmn1 polymorphic site and the better treatment response. However, the molecular mechanisms responsible for this correlation have not been elucidated yet.

The main goal of this study is to investigate the association between Xmn1$^G$ polymorphism and the amount of total Hb and fetal hemoglobin (HbF) in adults with intermediate $\beta$-thalassemia disease in Isfahan population as well as to investigate the association between Xmn1$^G$ polymorphism and the effect of hydroxyurea.

MATERIALS AND METHODS

Patients and DNA extraction. In the present study, 51 $\beta$-thalassemia intermediate patients were identified and studied. Mean age of the patients was 23 years. Cases were collected from Omid Hospital (Isfahan, Iran) between October 2011 to November 2012. The Hb and HbF levels before and after treatment with hydroxyurea. The independent $t$-test was used to determine whether there was a significant difference between the average Hb and HbF levels before and after treatment with hydroxyurea. The independent $t$-test was also used to compare the average Hb and HbF levels between genotypes. In all cases, $P < 0.05$ was considered statistically significant.

Table 1. Tetra-Primer ARMS-PCR technique of the Xmn1 polymorphism

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner forward</td>
<td>5′ATGCAAATATCTGTCGAAACGTTTCTGAAATCTGCTCGAAACGTTC</td>
<td>54.3</td>
</tr>
<tr>
<td>Outer forward</td>
<td>5′GCCCTCAGTTAGCTCTACAGAC</td>
<td>55.8</td>
</tr>
<tr>
<td>Outer reverse</td>
<td>5′TGGAGCTACAGACAAGAAGG</td>
<td>55.9</td>
</tr>
<tr>
<td>Inner reverse</td>
<td>5′TGGGTTGGAGTTAGCCATGAC</td>
<td>53.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR fragments</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (CC) (bp)</td>
<td>317, 122</td>
<td></td>
</tr>
<tr>
<td>Heterozygote (CT) (bp)</td>
<td>317, 239, 122</td>
<td></td>
</tr>
<tr>
<td>Variant homozygote (TT) (bp)</td>
<td>317, 239</td>
<td></td>
</tr>
</tbody>
</table>

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RESULTS

Among the 51 patients studied, 28 (54.9%) subjects were male, and 23 (45.1%) subjects were female. Patients were aged between 15 and 55 years. Among the total patients, 22 (43.1%) subjects were splenectomized. Mean (SD) Hb levels in male and female patients were 9.21 ± 1.48 g/dl and 8.77 ± 1.49 g/dl, respectively. In addition, mean (SD) HbF levels in male patients were 83.37 ± 28.74 g/dl and in female patients were 81.85 ± 33.76 g/dl. Chi-square test showed that these differences between male and female were not statistically significant (P = 0.46 and P = 0.41, respectively).

The XmnI\(^{G}\) polymorphism genotype was determined in patients using the Tetra-Primer ARMS-PCR technique (Fig. 2). Patients 1 and 2 had three bands, indicating the heterozygous genotype (C/T). Patients 3-7 had 122-bp and 317-bp bands; therefore, its genotype was homozygous (CC).

Among the 51 patients, 35 (68.6%) subjects were heterozygous (CT) and 16 (31.4%) subjects were homozygous (CC). The frequency of allele T in males and females was 34.8% and 33.9%, respectively. Chi-square test indicated that there was no significant correlation between the presence of allele T at XmnI polymorphic site and sex (P = 0.89).

As shown in Table 2, the Hb and HbF levels in the patients with heterozygous (CT) genotype were significantly higher (9.58 ± 1.25 gm/dl and 89.30 ± 21.87, respectively) than the homozygous (CC) genotype (7.94 ± 1.34 gm/dl and 70.32 ± 40.56, respectively). Based on independent t-test, the differences were statistically significant (P = 0.001 and P = 0.04, Table 2).

The prevalence of splenectomy in heterozygous (CT) genotype of XmnI polymorphism was 15 (42.9%) and in homozygous (CC) state was 7 (43.8%). Chi-square test indicated no significant association between the presence of T allele at XmnI polymorphic site and splenectomy (P = 0.59, Table 2).

Among the 51 patients studied, 30 (58.8%) subjects were under treatment with hydroxyurea (17 [56.7%] males and 13 [43.3%] females). Mean Hb levels before and after treatment were 8.47 ± 1.36 gm/dl and 9.01 ± 1.52 gm/dl, respectively. Using the paired sample t-test, the observed differences were statistically significant (P = 0.03, Table 3). Mean HbF levels before and after treatment were 81.87 ± 26.95 and 86.11 ± 28.00, respectively. Paired sample t-test showed that the differences were not statistically significant (P = 0.32, Table 3).
Among the patients under treatment by hydroxyurea, 20 (66.7%) subjects were heterozygous (CT) and 10 (33.3%) subjects were homoyzogous (CC). In this study, we observed that Hb and HbF levels in the patients with heterozygous (CT) genotype (0.7 ± 1.26 and 5.95 ± 14.8, respectively) were increased more than the patients with homoyzogous (CC) genotype (0.26 ± 1.43 and 0.8 ± 1.31, respectively). Paired sample t-test showed that more elevation in total Hb level was statistically significant (P = 0.03, Table 4), but differences in HbF level were not statistically significant (P = 0.37, Table 4).

### DISCUSSION

β-thalassemia is the most common monogenic disease in humans. Genetic and non-genetic factors such as (C→T) polymorphism administration of hydroxyurea have been reported to influence γ-globin gene expression and the severity of clinical symptoms of β-thalassemia [10]. Xmn1γ affects Hb and HbF levels only in erythropoietic stress conditions [11]. Some studies have reported that there is no association between the presence of T allele at this site and the reduction of clinical symptoms in β-thalassemia intermediate patients [12, 13]. In the present study, association between Xmn1γ polymorphism and Hb/HbF levels and the effects of hydroxyurea on β-thalassemia intermediate patients in Isfahani population were studied by the Tetra-Primer ARMS-PCR technique.

The frequency of T allele at the Xmn1 polymorphic site has been reported differently in various populations, varying from 10-76.9% [13-22]. However, in the present study, the frequency of T allele at Xmn1 polymorphic site in 51 patients with β-thalassemia intermediate was found 34%.

Different studies have proved that the existence of T allele at Xmn1 polymorphic site is associated with an increased amount of total Hb and HbF in intermediate β-thalassemia patients [22-25]. The presence of T allele in Xmn1 polymorphic site reduces the binding of transcription silencers to the γ-globin gene promoter. Therefore, the γ-globin gene is reactivated in adult life in erythropoietic stress conditions [2, 7]. Numerous studies have revealed that there is a significant correlation between the occurrence of T allele at Xmn1 polymorphic site and increased amount of HbF and even reduction of severity of clinical symptoms in patients [6, 16, 20, 23-29]. However, some other studies have indicated that there is no association between the presence of T allele at this site and increased HbF level [15]. It has been also reported that there is no association between the presence of T allele at this site and the reduction of clinical symptoms in β-thalassemia intermediate patients [12]. In line with the majority of the first group, we have found that the levels of Hb and HbF are significantly increased in the presence of T allele at the Xmn1 polymorphic site. These different results in various studies could be caused by the complexity of gene regulation pathways for γ-globin gene expression and also HbF levels [12]. Hydroxyurea is a chemical agent that may increase Hb

### Table 2. Parameters associated with Xmn1 polymorphism in 51 patients according to independent t-test and Chi-square test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Xmn1 (CT)</th>
<th>Xmn1 (CC)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (gm/dl)</td>
<td>9.58 ± 1.25</td>
<td>7.94 ± 1.34</td>
<td>0.001</td>
</tr>
<tr>
<td>HbF</td>
<td>89.30 ± 21.87</td>
<td>70.32 ± 40.56</td>
<td>0.04</td>
</tr>
<tr>
<td>Splenectomy (%)</td>
<td>15 (42.9)</td>
<td>7 (43.8)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

### Table 3. Mean Hb and HbF levels in 30 patients under treatment with hydroxyurea according to paired sample t-test

<table>
<thead>
<tr>
<th>Mean level</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb* (g/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CT)</td>
<td>n = 20</td>
<td>n = 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.47 ± 1.36</td>
<td>9.01 ± 1.52</td>
<td>0.03</td>
</tr>
<tr>
<td>(CC)</td>
<td>n = 10</td>
<td>n = 10</td>
<td></td>
</tr>
<tr>
<td>HbF**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CT)</td>
<td>n = 15</td>
<td>n = 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>81.87 ± 26.95</td>
<td>86.11 ± 28.00</td>
<td>0.32</td>
</tr>
<tr>
<td>(CC)</td>
<td>n = 10</td>
<td>n = 6</td>
<td></td>
</tr>
</tbody>
</table>

*Of 30 patients, 20 and 10 patients were heterozygote (CT) and homoyzogote (CC), respectively; **HbF information for 22 out of 30 patients (before receiving hydroxyurea) and 16 out of 30 patients (after treatment with hydroxyurea) was prepared according to hospital files.

### Table 4. Parameters associated with Xmn1 polymorphism in 30 patients under treatment with hydroxyurea using paired sample t-test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Xmn1 (CT)</th>
<th>Xmn1 (CC)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevation of Hb levels with hydroxyurea</td>
<td>0.70 ± 1.26</td>
<td>0.26 ± 1.43</td>
<td>0.03</td>
</tr>
<tr>
<td>Elevation of HbF levels with hydroxyurea</td>
<td>5.95 ± 14.8</td>
<td>0.80 ± 1.31</td>
<td>0.37</td>
</tr>
</tbody>
</table>
and HbF levels. This effect can be exerted through γ-globin expression, and it seems to be associated with allele at the XmnI polymorphic site [10, 17, 26, 30-32].

In the present study, the frequency of XmnI polymorphic site in 51 patients with β-thalassemia intermediate was determined, and its correlation with levels of Hb and HbF was analyzed. The results indicated that in the presence of T allele at XmnI polymorphic site, the Hb and HbF levels were increased. In addition, the association between XmnIγ variation and the effect of hydroxyurea was studied. In the current investigation, it has been demonstrated that in the patients carrying T allele, Hb and HbF levels are increased statistically, and they also respond to hydroxyurea treatment better than patients without the T allele.

ACKNOWLEDGMENTS

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REFERENCES

22. Arab A, Karimipoor M, Rajabi A, Hamid M, Arjmandi S, Zeinali S. Molecular characterization of β-


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