Original Article

The rs4808793 Polymorphism of GDF-15 Associates with Significantly Elevated Ferritin in Both Thalassemia Major and Thalassemia Intermedia

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Abstract

Background: Previous studies have proposed the rs4808793 polymorphism of the *GDF-15* as a potent inducer of hypertension, cardiovascular disease, and renal failure. The current study was performed to investigate the role of rs4808793 polymorphism in the pathogenesis of iron overload in patients suffering from major or intermedia β-thalassemia. **Materials and Methods:** The study included 69 major thalassemia patients and 25 intermedia thalassemia patients as the control group. The study was conducted on 69 major thalassemia and 25 intermedia thalassemia patients who referred to Baqa'i Hospital 2 in Ahvaz, Iran. Five milliliter blood was collected and DNA was extracted. After DNA amplification by the use of polymerase chain reaction, the rs4808793 polymorphism was detected by AlwNI restriction enzyme application and restriction fragment length polymorphism. **Results:** Mean serum ferritin in patients with β-thalassemia major (3490.41 ± 169.22 ng/ml) was significantly higher than those with thalassemia intermedia (677.16 ± 388.80) ($P < 0.05$). The frequency of mutation showed no statistically significant difference between cases and controls (41% vs. 32%) ($P > 0.05$). Both cases and controls with rs4808793 polymorphism showed significantly elevated serum ferritin concentrations compared to patients without mutations ($P < 0.05$). **Conclusion:** Incidence of rs4808793 *GDF-15* polymorphism can be considered as an effective factor in iron overload, exposing people to thalassemia, both in thalassemia major and intermediate groups.

Keywords: *Ferritin, GDF-15, rs4808793 polymorphism, β-thalassemia intermedia, β-thalassemia major*

Introduction

Iron overload can be considered as one of the major problems in patients with β-thalassemia. Iron accumulation in the cell induces reactive oxygen species production, apoptosis, necrosis, and inflammation.[1] The other complications include heart damage, liver damage, liver cirrhosis, pancreatic islet cell damage, diabetes, hypothyroidism, and hypogonadism.[2,3] Currently, much research is being done to reduce the problem of iron overload in β-thalassemic patients. Some research studies have suggested *GDF-15* overexpression as a trigger for iron overload.[4] GDF-15 is an inflammatory cytokine that belongs to the transforming growth factor-β superfamily. The *GDF-15* is expressed in most tissues.[5] However, mature hemoglobin-containing erythroblasts, prostate, and placenta show the maximum values of expression.[6] Previous studies have shown significant elevations of *GDF-15* expression in patients suffering from prostate cancer, cardiovascular disease, type 2 diabetes mellitus, and β-thalassemia.[6,7] The elevated levels of *GDF-15* in β-thalassemia can be justified by an unusual proliferation of erythroid cells and ineffective erythropoiesis. Recent studies have demonstrated that GDF-15 serum concentrations above 10,000 pg/ml inhibit hepcidin expression in β-thalassemia patients.[8] *GDF-15* overexpression causes decreased expression of hepcidin that ultimately results in iron overload. Some surveys have proposed a probable role of GDF-15 polymorphisms in altering iron homeostasis. The rs4808793 polymorphism of *GDF-15* results from the replacement of G allele instead of C allele in the upstream region of *GDF-15*. The study conducted by Athiyarath *et al*. revealed that among eight different polymorphisms of GDF-15, the rs4808793 polymorphism was associated with significant elevation in *GDF-15* expression.[9] The results of this study showed a significant reverse association between *GDF-15* expression and hepcidin levels. However, no significant

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change in Ferritin concentration was noticed.[9] In another study, a significant positive correlation between GDF-15 concentrations and ferritin levels in transfusion-dependent β-thalassemia patients with Iron overload was reported. Due to the remarkable increase of *GDF-15* expression in β-thalassemia patients and its inhibitory effect on hepcidin expression, the genetic variations in *GDF-15* may influence the Iron overload condition in β-thalassemia patients.[10] In this regard, the study of iron deposition statue in β-thalassemia patients with genetic variations in *GDF-15* may be a useful approach to ameliorate iron overloading. Here, we investigated the prevalence of the rs4808793 polymorphism of *GDF-15* and the association of this polymorphism with iron overload in patients with β-thalassemia major compared to patients with intermediate thalassemia.

Materials and Methods

Study design

This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1397.940). A case–control study was conducted. The study was conducted in accordance with ethical procedures and policies approved by the Ethical Committee of Ahvaz Jundishapour University of Medical Sciences, Ahvaz, Iran, and Helsinki Declaration of 1975. The study group was selected from the patients suffering from major or intermedia thalassemia who were referred to Baqa'i Hospital 2 in Ahvaz, Iran. The patients' incidence of thalassemia was previously confirmed by specialists with regard to medical examinations and blood experiments. Patients with similar hemoglobinopathies such as sickle cell anemia were excluded from the study. The study was conducted on 69 patients with major thalassemia as a case group and 25 patients with intermedia thalassemia as a control group.

Blood samples preparation method

Informed consent was obtained from all patients and controls. Five milliliter of peripheral blood was collected in the tubes that contained ethylenediaminetetra acetic acid.

DNA extraction

DNA was extracted using the Yekta Tajhiz Azma (IRAN) kit according to the manufacturer's instructions. The DNA concentration was determined at the wavelength of 260 nm using a NanoDrop spectrophotometer (NanoDrop 2000™; Thermo Fisher Scientific, Waltham, MA, USA). The unimpaired DNA was confirmed by OD 260/280 nm between 1.8 and 2. The extracted DNAs were labeled and stored at −20°C until use.

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out in the total volume of 25 μL using Taq DNA Polymerase × 2 Master Mix RED (Ampliqon, Odense, Denmark). The specific primers (Bioneer Corporation) used for the amplification of full-length GDF-15 gene included 5'-GCAACAGAGCGAGACTCCA-3' and 5'-CCACGCCGGTCGGATTAAAACT-3'. The primers were designed using primer premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). The accuracy of primers was confirmed using Bioinformatics org/sms[2]/ primer stats online software. The PCR was carried out using a thermal cycler (Eppendorf Mastercycler International, Hamburg, Germany). The thermal program given consisted of 40 cycles as follows: initial denaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were transferred on 1% agarose gel stained by Safe stain (SinaClon BioSciences) and subjected to electrophoresis at 100V for 40 min then visualized using a Vilber Lourmat (France) transilluminator.

Enzymatic digestion

The proper restriction enzyme (AlwNI) (Thermo, USA) was chosen by the use of NEB Cutter online software. The intact PCR products were subjected to enzymatic restriction as follows: 5 μl of PCR product was mixed with 1 μl AlwNI enzyme, 1 μl of enzymatic buffer, and 8 μl of deionized water and incubated at 37°C for 2 h. The products were quantified versus a 100 bp DNA ladder on 1% agarose gel.

DNA sequencing

For final confirmation of enzymatic digestion results, a number of 10 AlwNI digested DNAs (5 mutated DNAs and 5 wild DNAs) were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA).

Statistical analysis

The quantitative data normality was assessed using the Kolmogorov–Smirnov test. Evaluation of the relationship between quantitative variables was carried out using an independent *t*-test. The relationship between two qualitative variables was examined by the Chi-square test. The significance level was considered <0.05. Data analysis was performed using USA, Microsoft company. 22 software.

Results

Demographic information

The present study was performed on 69 individuals with thalassemia major and 25 individuals with intermediate thalassemia. In total, 43 subjects were female (45.7%) and 51 were male (54.3%). In the group with thalassemia major, the mean age was calculated as 26 ± 9.11 years and the mean age of control group was 24.36 ± 9.64 years. The mean age showed no statistically significant difference between cases and controls ($P > 0.05$).

Polymerase chain reaction results

In accordance with *GDF-15* expression, all samples showed a single clear band on 665 bp region [Figure 1].

Enzyme digestion results

After enzymatic cleavage, the samples were visible on agarose gel in two ways: (1) nonmutant samples that lacked enzymatic cleavage and had a single band in the 665 bp region and (2) mutant samples that had an enzymatic section and showed two bands on the gel, one in the 243 bp region and the other in the 422 bp region [Figure 2].

Prevalence of rs4808793 polymorphism

In total, 38% of the total population showed G/C mutations. The frequency of rs4808793 polymorphism was 41% in patients with thalassemia major and 32% in patients with thalassemia intermedia. The frequency of mutations between the two groups did not show a statistically significant difference ($P > 0.05$) [Table 1].

Comparison of ferritin levels in the two groups

Mean serum ferritin in patients with β-thalassemia major was 3490.41 ± 169.22 ng/ml, which was significantly higher than those with thalassemia intermedia (677.16 ± 388.80) ($P < 0.05$).

Relationship between the prevalence of polymorphism and serum ferritin

Data analysis showed that in both the group of patients with β-thalassemia major and in the control group, the level of serum ferritin in people with polymorphism was significantly higher than those without mutations ($P < 0.05$).

Discussion

Today, the iron overload is the most important risk factor for patients with thalassemia, which puts patients at high risk for chronic liver failure, heart disease, thyroid hormone disorders, sex hormones disturbances, infections, osteoporosis, and spleen damages.[11,12] Therefore, researchers are trying to identify the factors affecting iron overload and prevent the accumulation of harmful iron accumulation in tissues. Blood transfusions are the most important cause of iron overload.[13,14] Due to the fact that patients' red blood cells lack the proper function to deliver oxygen to the body's tissues, patients need regular blood transfusions. Although blood transfusions have largely prevented the progression of the disease and improved the clinical condition of patients, regular transfusions have a number of side effects for patients.[15] In 2020, Advani *et al*. conducted a study to evaluate iron overload on cardiac function in patients with thalassemia major. Their results showed that iron overload leads to increased QT time and impaired echocardiogram.[16] Therefore, they concluded that assessing heart function using echocardiograms could be a diagnostic tool in people at high risk for iron overload. Another study by Çetinçakmak *et al*. to evaluate iron overload on endocrine organs showed that spleen resection in thalassemia patients resulted in iron accumulation in the heart and liver and reduced survival.[17] The GDF-15 is known to play an important role in the regulation of iron metabolism in the body. GDF-15 plays an important role in regulating hepcidin expression by preventing its expression.[18] Hepcidin in normal condition prevents the absorption of iron by cells and thus prevents the accumulation of iron in the heart and liver.[19] Thus, GDF-15 overexpression results in iron overload through hepcidin downregulation. According to previous studies, mutations in the gene encoding *GDF-15* in patients with thalassemia major increase its expression in patients, so mutations in the *GDF-15* gene may possibly increase iron overload and intensify the thalassemia complications.[20,21] In this study, we performed a study on patients with thalassemia major and intermedia to evaluate the incidence of *GDF-15* polymorphism. Intermediate thalassemia has less clinical severity than thalassemia major. Patients with thalassemia

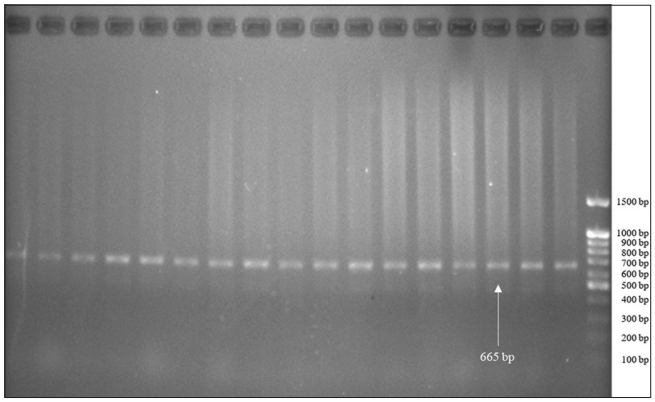

Figure 1: Polymerase chain reaction products on agarose gel. A clear single band on 665 bp region was yielded

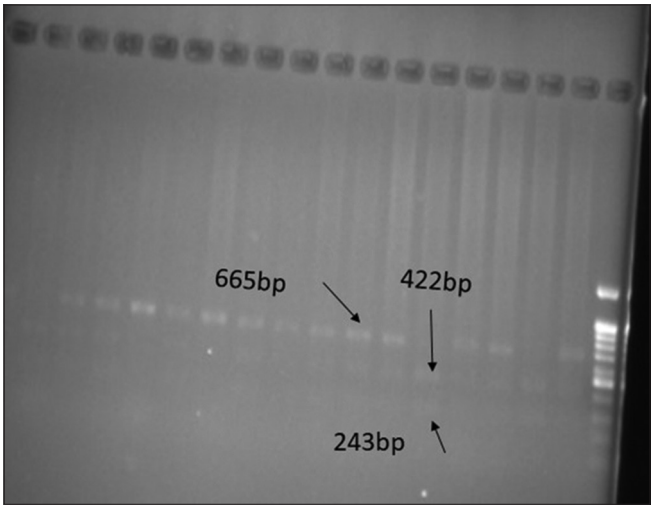

Figure 2: Bands created after enzymatic cleavage. The wild samples showed an intact band on 665 region, but the mutants showed two distinct bands on 243 bp and 422 bp regions

Table 1: Prevalence of rs4808793 polymorphism

Type of disease	*n*	Number of mutation	Percentage
Major	69	28	41
Intermedia	25	8	32

intermedia despite the reduced symptoms of the disease and less blood transfusion requirement show some symptoms of the iron overload. Therefore, in this study, the frequency of rs4808793 *GDF-15* gene polymorphism in patients with thalassemia major and intermedia was compared. The results showed that the prevalence of polymorphism in thalassemia major patients was 41% versus 32% in intermediate thalassemia patients. This difference was not statistically significant. Therefore, the incidence of rs4808793 polymorphism may be regarded as one of the predisposing factors for developing thalassemia. The present results showed that the amount of ferritin in patients with thalassemia major was higher than patients with thalassemia intermedia and the difference was statistically significant. This result confirmed the increased chance of developing iron overload in people with thalassemia major compared to people with intermediate thalassemia. In addition, the present study showed that in both the groups with thalassemia major and intermedia, the level of serum ferritin in people with polymorphism is significantly higher than those without mutations. Therefore, the incidence of rs4808793 polymorphism in both the groups can be considered as a predisposing factor for iron overload. Similarly, in a study conducted by Athiyarath *et al*. to evaluate the rs4808793 polymorphism association with ferritin levels in patients with thalassemia major, the results showed that the presence of G allele in this polymorphism was associated with a significant increase in GDF-15 levels in patients and significantly decreased hepcidin levels in major thalassemic patients compared with normal control subjects. However, no statistically significant difference in ferritin levels was noticed between the patients and controls.[9] The study conducted by Tantawy *et al*. showed that increased ferritin levels in thalassemia patients were associated with cardiovascular disorders (CVD).[22] In the study conducted by Zhou *et al*., patients with CVD showed significant elevated levels of GDF-15.[23] Another study by Sayani *et al*. showed that in patients with thalassemia major, increased *GDF-15* expression was associated with increased ferritin and iron overload in the liver and heart, which ultimately led to the dysfunction of both organs and endocrine disorders.[24] The study conducted by Li *et al*. in dialysis patients showed no significant association between *GDF-15* expression levels and ferritin.[25]

Conclusion

It is concluded that the incidence of rs4808793 polymorphism in the gene encoding *GDF-15* could be regarded as one of the predisposing factors to thalassemia. Considering that in this study, in both the groups with thalassemia major and intermedia, the incidence of rs4808793 polymorphism was significantly associated with serum ferritin concentration; it can be concluded that the presence of rs4808793 polymorphism has a chance of iron overload and increases incidence-related disorders in patients with thalassemia. However, further studies are needed in this issue.

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Conflicts of interest

There are no conflicts of interest.

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