



Role of XmnI restriction site polymorphism and JAK2 gene mutation in β -Thalassemia

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Abstract

β -Thalassemia is one of the most prevalent hereditary disorder in the world. It is the most common hemoglobin disorder found in the Indian sub continent, caused by point mutations or more rarely deletions in the β -globin gene on chromosome 11, leading to reduced (β^+) or absent (β^0) synthesis of the β chains of hemoglobin (Hb). The objective of this study is to understand the role of modifier genes and their polymorphic forms in amelioration or deterioration of the disease and severity of β -thalassemias. The XmnI polymorphic site was determined by PCR-RFLP procedure. The XmnI restriction site at -158 position of the γ -gene is associated with increased expression of the γ -globin gene and higher production of HbF. The levels of γ and α chains were detected by HPLC. JAK2 V617F mutation indicate the phenotypic variations and for predicting the risk of major clinical events. In allele specific polymerase chain reaction (PCR) V617F mutation independently predicted the evolution toward large splenomegaly.

Keywords: β -Thalassemia, XmnI Polymorphism, JAK2 V617F mutation, DNA amplification, RFLP.

Introduction

β -Thalassemia is more common among people from Mediterranean countries, the Middle East, Central Asia, the Indian Subcontinent (South Asia) and South east Asia¹. β -thalassemia represents a great heterogeneity as more than 190 mutations have been identified for the β -globin gene responsible for this disease². β -thalassemia is characterized by absent to decreased synthesis of β -globin chains resulting in imbalance between α and β -chains and consequent ineffective erythropoiesis and hemolysis^{3,4}. It is one of the most common single gene disorder with >400,000 new borns affected per year worldwide⁵. The incidence of β -thalassemia trait in India ranges from 3.5 to 15%. Every year more than 10,000 children with thalassemia major are born in India⁶. Affected individuals also have a shortage of red blood cells (anemia), which can cause pale skin, weakness, fatigue, and more serious complications.

β -thalassemia patients who are unable to produce any HbA showed a mild disease with a reasonable level of HbF⁷. β -thalassemia is broadly classified into three groups, based on clinical severity β -thalassemia major, β -thalassemia minor and β -thalassemia intermedia. The two clinical forms of the disease requiring treatment are thalassemia major and intermedia. In β -thalassemia intermedia and major, the serum ferritin is raised or may be normal in the presence of iron chelation therapy⁸. HPLC is a sensitive and precise method for detecting thalassemia and abnormal Hemoglobins⁹⁻¹⁰.

β -thalassemia was first described in 1925 by Thomas Cooley and Lee¹¹. β -thalassemia major is a more serious blood disorder that affects some babies who inherit the defective gene from

both parents. In thalassemia major, the excess unpaired α -globin chains aggregate to form inclusion bodies¹². These chain inclusion bodies damage RBC membranes, leading to intravascular hemolysis. Transfusional hemosiderosis is the major cause of late morbidity and mortality in patients with thalassemia major¹³. Three of the general allele combinations are responsible for this thalassemia phenotype β^0/β^0 , β^0/β^+ , and β^+/β^{14} . Patients with β -thalassemia intermedia have mild to moderate anemia and in most cases do not require blood transfusions¹⁵. The clinical phenotype of thalassemia intermedia is roughly intermediate between thalassemia major and minor¹⁶. People with thalassemia minor are carriers of the disease. They are usually clinically asymptomatic but sometimes have a mild anemia¹⁷.

β -Thalassemia can be diagnosed by using blood tests, including a complete blood count (CBC), and special hemoglobin studies. Treatment for patients with β -thalassemia major includes chronic transfusion therapy, iron overload and chelation therapy, splenectomy, frequent blood transfusions, and stem cell transplantation. Thalassemia syndromes are prevalent in certain parts of the world¹⁸. There are about 240 million carriers of β -thalassemia in worldwide, and in India alone, the number is approximately 30 million with a mean prevalence of 3.3 %¹⁹.

Genetics: β -thalassemia is a genetic disease in which an abnormal β -globin gene results in decreased (β^+ thalassemia) or completely absent (β^0 thalassemia) production of the normal β -globin chain. In particular the (C \rightarrow T) variation at position -158 upstream of the γ globin gene is detectable by the restriction enzyme XmnI. The sequence variation has been shown to increase HbF levels in β -thalassemia. HbF is reported to be

lower in transfused patients due to suppression of HbF synthesis²⁰. In Jak2 gene a G→T transversion causes phenylalanine to be substituted for valine at position 617 of Jak2 (V617F). Mutations in the HBB gene prevent the production of β -globin. HBB gene is located on the short (p) arm of chromosome 11 at position 15.5, base pairs 5,246,695 to 5,248,300. More than 250 mutations in the HBB gene have been found to cause β -thalassemia. Most of the mutations involve a change in a single DNA building block with in or near the HBB gene.

Role of XmnI (xanthomonas manihotis-I) restriction site polymorphism: XmnI restriction site at the 158 (C→T) position of the G_γ gene is associated with the increased production of HbF and can strongly influence the heterogeneity of β -thalassemia²¹⁻²². The presence of XmnI polymorphic site has affected on the clinical response to hydroxyurea therapy. Hydroxyurea enhances fetal hemoglobin production. The presence of XmnI site decreases the need of blood transfusion and also the rate of splenectomy and facial bone deformity. The screening of the XmnI G_γ polymorphism and HbF level in early childhood may help on the management of β -thalassemia major patients and possibly prevent severe complications.

Role of JAK2 (Janus kinase 2) gene mutation in β -Thalassemia: The JAK2 is especially important for controlling the production of blood cells from hematopoietic stem cells. These stem cells are located within the bone marrow and have the potential to develop into red blood cells, white blood cells, and platelets. Activation of JAK2 in β -thalassemia is associated with cell differentiation. Jak2 may have both direct and indirect effects on iron metabolism and regulate ferroportin degradation²³ and important mediator of ineffective erythropoiesis (IE). JAK2 contributes to a massive increase in the erythropoietic activity and a huge expansion of the erythron with related splenomegaly, a phenotype that is interestingly similar to observed in β -thalassemia²⁴.

Objectives: The present study is aimed to correlate the clinical features of β -thalassemia with variant HPLC. The study also focuses on serum ceruloplasmin levels and serum iron levels in β -thalassemia patients and polymorphism in XmnI and JAK2 gene mutation.

Material and Methods

A total of 25 specimens of EDTA blood samples were obtained from randomly selected clinically diagnosed thalassemia patients. 25 Control blood samples were collected from different communities with informed consent. The disease was diagnosed by clinical and hematological data.

HPLC which can be a relatively fast and reproducible method has been used for the determination of various hemoglobins, including hemoglobin A2 and F and to estimate the fetal hemoglobin by alkali denaturation method. Naked eye single tube red osmotic fragility test (NESTROFT) has been performed

as a simple objective preliminary screening test to detection of thalassemia²⁵. To estimate serum ceruloplasmin and serum iron levels in β -thalassemia patients and control samples. Genomic DNA was isolated from whole blood in anticoagulant (EDTA) by using sodium dodecyl sulphate and proteinase K, and digested overnight at 37°C. DNA was purified using phenol chloroform isoamyl alcohol method²⁶ and precipitated in ethanol. After DNA extraction, PCR reactions were set up for each sample.

PCR Conditions for XmnI: A total of 50 μ l of reaction volume was used for this purpose. The reaction volume was composed of 5 μ l of the genomic DNA template, 1 μ l of each of the two primers (forward primer 5'-AACTGTTGCTTTATAGGATTTT-3', reverse primer 5'-AGGAGCTTATTGATAACTCAGAC-3'), 5 μ l of taq buffer, 2 μ l of dntp's, 2 μ l of 25mM mgcl₂, 1 μ l of taq polymerase, 33 μ l of distilled water. The program was run in thermal cycler at 94°C for 6 minutes for an initial denaturation, followed by 34 cycles of DNA denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 2 minutes. After 34 cycles were over, the thermal cycler was set at 72°C for 8 minutes for final extension.

PCR Conditions for JAK2: A total of 50 μ l of reaction volume was used for this purpose. The reaction volume was composed of 5 μ l of the genomic DNA template, 1 μ l of each of the two primers (forward primer 5'-GGGTTTCCTCAGAACGTTGA-3', reverse primer 5'-TCATTGCTTTTCCTTTTTTACAA-3'), 5 μ l of taq buffer, 2 μ l of dntp's, 2 μ l of 25mM mgcl₂, 1 μ l of taq polymerase, 33 μ l of distilled water. The program was run in thermal cycler at 94°C for 6 minutes for an initial denaturation, followed by 34 cycles of DNA denaturation at 92°C for 1 minute, annealing at 57°C for 1 minute, elongation at 72°C for 1 minute. After 34 cycles were over, the thermal cycler was set at 72°C for 7 minutes for final extension.

PCR products were subjected to electrophoresis on a 1.5 % agarose gel, visualized under UV light and gel image was documented further in gel documentation system. DNA ladder ranges from 100 bp to 1000 bp were used as marker to indicate the size of amplicon. After completion of PCR amplification the PCR products are digested with restriction enzymes.

RFLP Conditions for XmnI and JAK2: RFLP is a widely used technique to detect known mutations and variations using specific restriction endonucleases. A total of 30 μ l of reaction volume was used for this purpose. The reaction volume was composed of 15 μ l of nuclease free water, 2 μ l of 10X Digest buffer, 12 μ l of PCR product and 1 μ l of Enzyme. Mix gently and incubate at 37°C in a heat block or water thermostat for 8minutes. Analyze the restriction digested products on 3% agarose gel electrophoresis.

The XmnI polymorphism at -158 position of the G_γ -gene was confirmed by XmnI restriction enzyme digestion of a 650bp amplified DNA sequence from the promoter region of the G_γ -

gene. JAK2 Samples positive for the mutation were subsequently analyzed via polymerase chain reaction and digestion with the restriction endonuclease BsaXI, (New England Biolabs, Hitchin, UK) which allows for estimation between mutated and wild type alleles²⁷.

Results and Discussion

The anticoagulated blood was used for performing Naked eye single tube red osmotic fragility test (Nestroft). Nestroft results shows in the table 1.

Table-1
Nestroft results

S.No	Nestroft results	Patients n (25)	Controls n(25)
1	Number of positive cases	18	-
2	Number of negative cases	5	25
3	Number of cases to be Confirmed	2	-

Nestroft status in patients indicated that out of 25 samples 18 cases were positive and 5 cases were negative, and thalassemia to be confirmed in 2 cases.

The anticoagulated blood was used for performing High Performance Liquid Chromatography (HPLC) test. HPLC test results shows in the table 2.

Table-2
Variant HPLC results

S.No	HPLC results	
1	Normal	4
2	β-thalassemia carrier	3
3	β-thalassemia major	6
4	β-thalassemia minor	5
5	Sicklecell carrier	4
6	Sicklecell anaemia	2
7	E-thalassemia	1

HPLC status in patients indicated that out of 25 samples normal (4), β-thalassemia carrier (3), β-thalassemia major (6), β-thalassemia minor (5), Sicklecell carrier (4), Sicklecell anaemia (2), and E-thalassemia (1).

The mean values of HbF levels, serum ceruloplasmin levels and serum iron levels in β-thalassemia patients and healthy control comparisons are shows in table 3.

Table-3
Mean values of HbF, serum ceruloplasmin, and iron levels in patients and control samples

S. No	Variable	Patients n(25)	Controls n(25)
1	Mean HbF%	6.053 mg/dl	1.109 mg/dl
2	Mean Serum ceruloplasmin levels%	28.14 mg/dl	23.74 mg/dl
3	Mean Iron levels%	169.45 µg/dl	60.19 µg/dl

Analysis of the XmnI polymorphism: XmnI PCR products were digested with PdmI enzyme and electrophoresed on a 3% agarose gel. In the presence of T allele, two fragments of 450 and 200 bp were produced. The presence of the normal allele site for XmnI and thus an intact 650 bp fragment was produced (see figure-1).

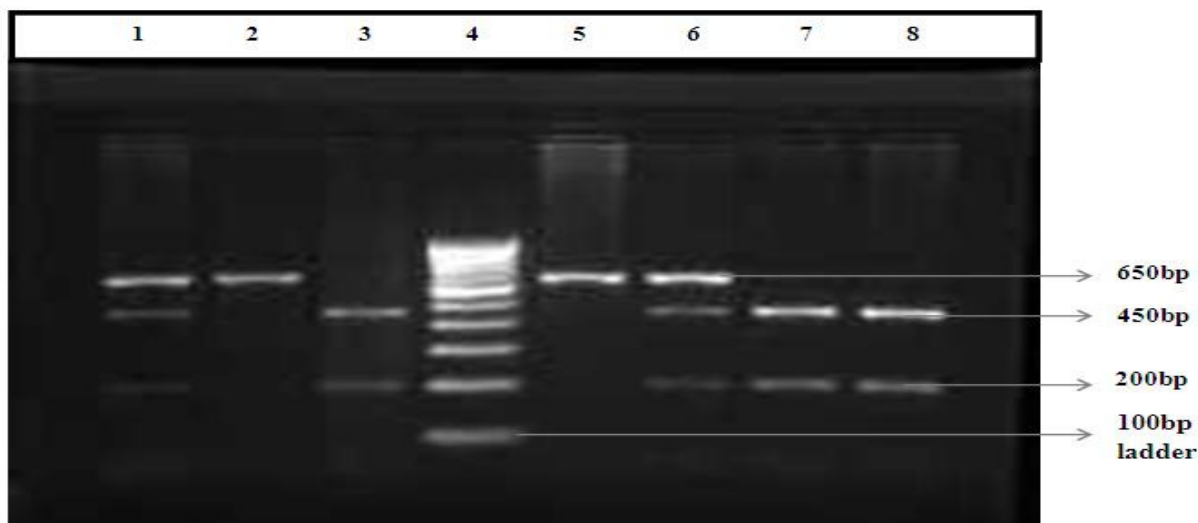


Figure-1
RFLP of the XmnI PCR product

In figure-1 RFLP of the XmnI PCR products were digested with PdmI restriction enzyme produced bands of 450bp and 200bp when both chromosomes possessed the XmnI polymorphic site Homozygous mutant (+/+) (lanes 3, 7 and 8), bands of 650bp, 450bp and 200bp were observed when only one chromosome possessed the site Heterozygous (+/-) (lanes 1 and 6) and lane 4 represents 100bp ladder, absence of the XmnI site in both chromosomes Homozygous (-/-) (lane 2 and 5).

Analysis of XmnI status in patients indicated that out of 25 samples CC-Homozygous 12 (48%), TT-Homozygous mutant 4 (16%), CT-Heterozygous 9 (36%). XmnI status in controls indicated that out of 19 samples CC-Homozygous 19(100%).

Analysis of the JAK2 V617F mutation: JAK2 PCR products were digested with BsaXI enzyme and electrophoresed on a 3% agarose gel. This approach allows both normal and mutant alleles to be visualized and can distinguish between homozygous and heterozygous mutations (see figure-2).

In figure-2 RFLP of the JAK2 PCR products digested with BsaXI, Lanes 2, 3 and 5 represent GG homozygotes. Lanes 1 and 6 represent GT heterozygotes. Lane 7 and 8 represents TT homozygotes mutant and lane 4 represents 100 bp ladder.

Analysis of JAK2 status in patients indicated that out of 25 samples GG-Homozygous 5(20%), GT-Heterozygous 18 (72%) and TT-Homozygous mutant 2(8%), JAK2 status in controls indicated that out of 17 samples CC-Homozygous 17(100%).

β -thalassemia is an autosomal recessive genetic disorder with many mutations in a single genes involved. Homozygous β -thalassemia is accounting for a majority of β -thalassemic

syndromes in India. Despite seemingly genotypes, the patients, especially those with β -thalassemia, have a remarkable variability in anemia, growth development, and hepatosplenomegaly and transfusion requirements. In our study, along with XmnI polymorphism the other gene we studied is JAK2. XmnI allele frequency differences across populations. The association of some β -globin mutations with XmnI site with elevated HbF expression. The role of increased HbF response as an ameliorating factor has become evident in patients who were mildly affected despite being homozygotes or compound heterozygotes for β^0 or β^+ thalassaemia²⁸.

JAK2 V617F plays in the phenotypic progression of the disease. JAK2 V617F mutation screening can also be used for other indications such as unexplained erythrocytosis and thrombocytosis. The T allele frequency was found to be high in thalassemia patients with JAK2 V617F mutation than in patients with XmnI polymorphism. Mean values of fetal hemoglobin levels, serum ceruloplasmin levels, and iron levels were elevated in patients when compared to control samples.

Conclusion

β -thalassemia is highly prevalent and is a major public health problem in the Indian Subcontinent and Middle East. Only a few prevalent HBB mutations underlie the majority of patients with β -thalassemia. Such as blood transfusions and treatment with iron chelators, can have potentially serious complications. Thus, prenatal diagnosis or other preventative approaches may be the most important strategy to control the clinical problems arising from β -thalassemia.

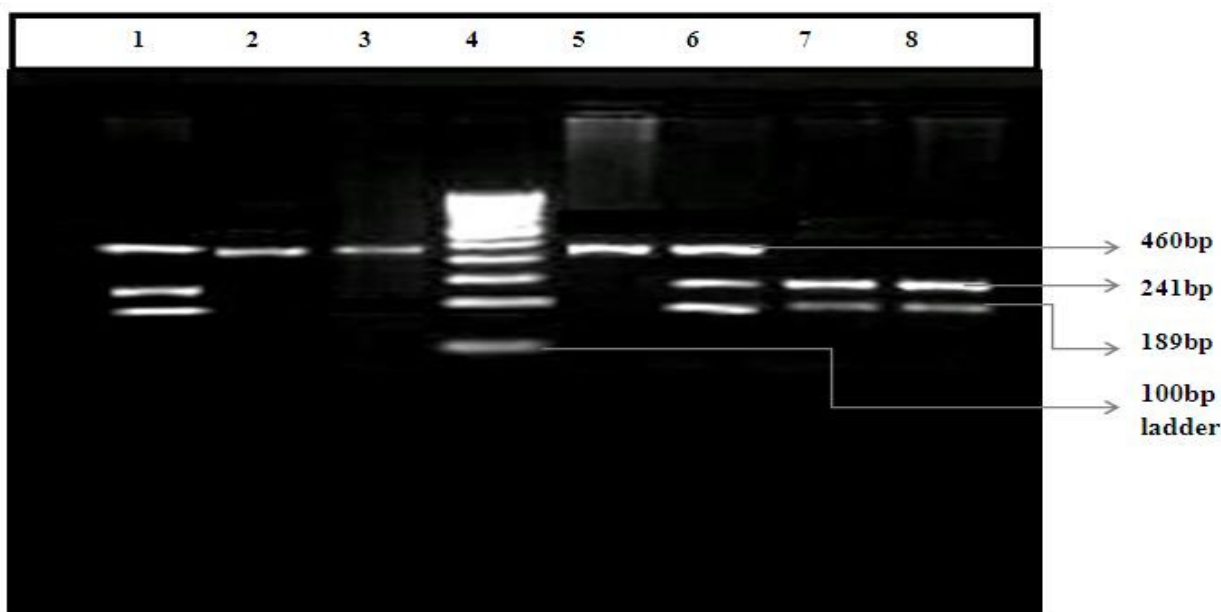


Figure-2
RFLP of the JAK2 PCR product

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