original research report

Frequency of Gγ-globin promoter −158 (C>T) XmnI polymorphism in patients with homozygous/compound heterozygous beta thalassaemia

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BACKGROUND: Response to hydroxyurea therapy in homozygous or compound heterozygous beta thalassaemia (BT) has been reported as more favourable in the presence of XmnI polymorphism. The prevalence of XmnI polymorphism may vary with BT phenotypes and genotypes, and differs geographically in distribution. Prevalence of XmnI polymorphism is not known in northern Pakistan.

OBJECTIVE: To determine the frequency of Gγ-globin promoter −158 (C>T) XmnI polymorphism (XmnI polymorphism) in patients with homozygous or compound heterozygous beta thalassaemia.

MATERIALS: Polymerase chain reaction (PCR) for common beta thalassaemia mutations and Gγ-globin promoter −158 (C>T) XmnI polymorphism was performed on 107 blood samples of transfusion dependent beta thalassaemia (BT) patients in Pakistan. One hundred samples of unrelated BT traits and 94 samples of healthy subjects as controls were also analysed for BT mutations and XmnI polymorphism.

RESULTS: Out of 301 DNA samples, XmnI polymorphism was detected in 71(24%); in normal controls, XmnI polymorphism was detected in 34/94 (36%) subjects; while in homozygous/compound heterozygous BT, it was detected in 14/107(13%) patients (Fisher's exact test, $p = .0002$). In heterozygous BT group, XmnI polymorphism was detected in 23/100 subjects (Fisher's exact test, $p = .03$ with normal controls, and $p = .049$ with homozygous/compound heterozygous BT). The most common BT genotype was Frame Shift (Fr) 8–9/Fr 8–9, and none of the patients with this genotype had XmnI polymorphism. The second most common genotype was IVSI-5/IVSI-5; 4/26 (15%). Cases with this genotype had XmnI polymorphism.

CONCLUSION: XmnI polymorphism in homozygous/compound heterozygous BT group is 13%. The most common genotype associated with XmnI polymorphism was IVSI-5/IVSI-5.

KEYWORDS: Beta thalassemia; Hbf; Hydroxyurea; Xmn 1Gγ polymorphism

Beta thalassaemia is one of the major health problems in Pakistan. About 5% of the country's population are carriers of this genetic disorder.[1] Patients with beta thalassaemia major (BTM) require lifelong blood transfusions unless cured with stem cell transplant or gene therapy. The prevalence of beta thalassaemia (BT) mutations varies among different ethnic groups in Pakistan. The five common genetic defects comprising IVS-1–5 (G→C), Fr 8/9 (+G), Fr 41/42 (-TTCT), IVS-1-1 (G→T) and Del 619 cover 90% of BT in southern Pakistan, while Cd 15 (G-A), Cd 5 (-CT), and IVSI-1 (G-T) are also frequently detected among Punjabis and Pathans in northern Pakistan.[2,3] Genetic defects covering >90% of BT in Pakistan cause transfusion dependent anaemia.[4]

It is known that coinheritance of genetic factors like C>T polymorphism at −158 base pair upstream Gγ gene (XmnI polymorphism) may affect the haemoglobin F (HbF) production in patients with BTM or thalassaemia intermedia (TI).[5,6] In conditions of erythropoietic stress, as occurs in BTM, the presence of XmnI polymorphism may activate production of HbF, leading to amelioration of the phenotype.[7] Hydroxyurea (HU) promotes HbF production via reactivation of γ-genes. It also suppresses the β globin expression through its cytotoxic effects, which may ameliorate the effects of the alpha/non alpha chain imbalance.[8] The response to hydroxyurea therapy has been reported as more favourable in the presence of XmnI polymorphism, though this association has not been well established.[7,9] The prevalence of XmnI polymorphism may vary with BT phenotypes and with geographical distribution.[10–12] The aim of our study is to determine the frequency of XmnI polymorphism in patients with homozygous/compound heterozygous beta thalassaemia in our patient population.

MATERIALS AND METHODS

Polymerase chain reaction (PCR) for common beta thalassaemia mutations and Gγ-globin promoter −158 (C>T) XmnI polymorphism was performed on 107 blood samples of transfusion-dependent BT patients in Pakistan. One hundred samples of unrelated BT trait, and 94 samples of healthy subjects as controls were also analysed for BT mutations and XmnI polymorphism. The study was approved by the ethical committee of the institute. Blood samples were collected in ethylene-diamine-tetra-acetic-acid (EDTA) vacutainers, and DNA was extracted from peripheral blood using phenol–chloroform DNA extraction method.

Beta thalassaemia mutations/deletions

PCR for BT mutations/deletions was performed by multiplex amplification refractory mutation system (ARMS) in three separate reaction mixtures. The first multiplex contained primers (Invitrogen life technologies USA) for IVSI-5 (G-C), Fr 8–9 (+G), Fr 41-42 (-TTCT), IVSI-1 (G-T) and Del 619 bp. The second multiplex contained primers for Cd 5 (-CT), Fr 16(-C), IVSI-1 (G-T), Cd30 (G-C), Cd 30 (G-A), and IVSII-1 (G-A). The third multiplex contained primers for Cd 15 (G-A) and Cap +1 (A-C). These genotypes cover almost 98% of beta thalassaemia mutations/deletions in Pakistan. PCR was performed in a 25 µl reaction mixture containing 5 pmol of previously described set of primers, 0.3 units of Taq polymerase (Fermentas Life Sciences, Vilnius, Lithuania), 30 µM of each dNTP, (Fermentas Life Sciences, Vilnius, Lithuania), 10 mmol Tris HCl (pH 8.3), 50 mmol KCl, 1.5 mmol MgCl2, 100 µg/ml gelatin and 0.3–0.5 µg of genomic DNA. The PCR process consisted of 25 cycles of denaturation at 94 °C for one minute, primer annealing at 65 °C for one minute, and DNA extension reaction at 72 °C for 1.5 min. In the final cycle, the extension reaction was prolonged to three minutes. The results were read after 5% mini-polyacrylamide gel electrophoresis (PAGE) and staining by silver nitrate. Where single mutation was detected, a second PCR was run with a set of primers containing the normal allele of the respective mutation to detect the presence of normal (wild type) sequence. Where the normal allele was also amplified, the DNA in question was assigned as heterozygous. Where the normal counterpart was not detected, DNA was assigned as homozygous for the respective mutation.

XmnI polymorphism

A restricted fragment length polymorphism (RFLP) PCR was performed using the previously described set of primers F5′-GAACTTAAGAGATAATGGC CTAA and R5′-ATGACCCATGGCGTCTGGA CTAG (Fermentas Life Sciences, Vilnius, Lithuania) that amplified 641-bp fragment of DNA flanking the C–T polymorphism at −158 to the Gγ-gene. PCR was carried out in a 25 µl reaction mixture containing 5 pmol of primers, 0.3 units of Taq polymerase (Fermentas Life Sciences, Vilnius, Lithuania), 30 µM of each dNTP, (Fermentas Life Sciences, Vilnius, Lithuania), 10 mmol Tris HCl (pH 8.3), 50 mmol KCl, 1.5 mmol MgCl2, 100 µg/ml gelatin and 0.3–0.5 µg of genomic DNA. Thermal cycling consisted of 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for one minute and DNA extension reaction at 72 °C for 1.5 min. In the final cycle, the extension reaction was prolonged to three minutes. The amplified fragments were digested overnight at 37 °C with 10 units of PdmI (XmnI) restriction enzyme (Fermentas Life Sciences, Vilnius, Lithuania). The results were read after running 5% PAGE and staining by silver nitrate. Where the digested product contained only 641 bp fragment, the −/− genotype was assigned; where 641 bp, 418 bp and 223 bp were detected, the +/− genotype was assigned. Where the digested product contained 418 bp and 223 bp fragments, the +/+ genotype was assigned (Figure 1).

Data was collected for statistical analysis using SPSS 19 version. Fisher's exact test was applied to

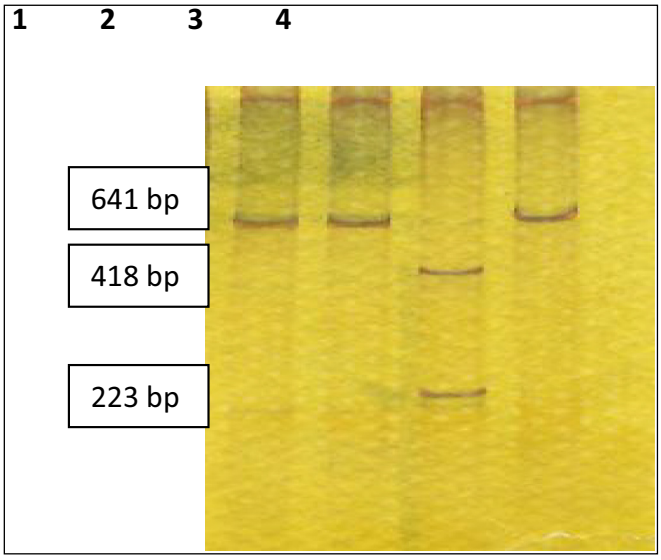

Figure 1. PCR for XmnI polymorphism, polyacrylamide gel electrophoresis and staining by silver nitrate showing patterns of normal, enzyme digested and undigested amplified products. 1, Normal control; 2, XmnI -/-; 3, XmnI +/+; 4, Un-cut.

compute the statistical significance of association between the groups. A p value <0.05 was taken as significant.

RESULTS

Out of 301 DNA samples, XmnI polymorphism was detected in 71(24%); in normal controls, XmnI polymorphism was detected in 34/94 (36%); while in homozygous/compound heterozygous, BT XmnI polymorphism was detected in 14/107(13%) patients (Fisher's exact test, $p = .0002$). In heterozygous BT group XmnI polymorphism was detected in 23/100 subjects (Fisher's exact test, $p = .03$ with normal controls, and $p = .049$ with homozygous/compound heterozygous BT). Frequency of XmnI polymorphism in all three groups is summarised in Table 1.

All patients with homozygous/compound heterozygous BT ($n = 107$) were transfusion-dependent, between six months to 31 years of age, mean 6 + 5 years). Sixty-three (59%) were male and 44(41%) were females. XmnI polymorphism was detected in 14 (13%) patients; 5/14 were heterozygous, and 9/14 were homozygous for XmnI polymorphism. Mean age of those with XmnI polymorphism was 8 years +7.9, while mean age of those without XmnI polymorphism was 5.8 + 4.5, (Un-paired t-test, $p = .15$). XmnI polymorphism was equally distributed in βo/βo (5/14, 35.5%) phenotype, and in β+/β+(5/14, 35.5%) phenotype, while in βo/β+ it was less common than the above two groups (4/14, 29.0%). The most common BT genotype was Fr 8-9/Fr 8-9, and none of the patients with this genotype had XmnI polymorphism. The second most common

Table 1. Frequency of XmnI polymorphism in controls with normal BT genotype, heterozygous BT, and cases with homozygous/compound heterozygous BT.

XmnI status	Normal genotype	Heterozygous BT	Homozygous/compound heterozygous BT
−/−	60 (64%)	77 (77%)	93 (87%)
−/+	32 (34%)	15 (15%)	5 (5%)
+/+	2 (2%)	8 (8%)	9 (8%)
	94	100	107

BT, Beta thalassaemia; XmnI, Gγ-globin promoter −158 (C>T) XmnI polymorphism.

Table 2. Frequency of XmnI polymorphism in homozygous/compound heterozygous BT.

*Genotype	XmnI −/−	XmnI −/+	XmnI +/+	Total
Fr 8-9	44 (40%)	0	0	44 (40%)
IVS 1–5	30 (28%)	5 (5%)	4 (4%)	39 (36%)
Fr 41-42	3 (3%)	0	1 (1%)	4 (4%)
Fr 16	2 (2%)	0	0	2 (2%)
Cd 5	7 (7%)	0	1 (1%)	8 (8%)
Cd 15	7 (7%)	0	1 (1%)	8 (8%)
Cd 30	0	0	1 (1%)	1 (1%)
IVSII-1	0	0	1 (1%)	1 (1%)
Total	93 (86%)	5 (5%)	9 (9%)	107 (100%)

BT: Beta thalassaemia.

*Genotype, Homozygous or compound heterozygous; XmnI, Gγ-globin promoter −158 (C>T) XmnI polymorphism.

Table 3. Frequency of XmnI polymorphism in heterozygous BT.

Genotype	XmnI −/−	XmnI +/−	XmnI +/+	Total
Fr 8-9	29 (29%)	5 (5%)	2 (2%)	36 (36%)
IVS 1–5	23 (23%)	5 (5%)	2 (2%)	30 (30%)
Fr 41-42	8 (8%)	0	0	8 (8%)
Cap +1	5 (5%)	0	0	5 (5%)
Fr 16	2 (2%)	0	0	2 (2%)
Cd 5	5 (5%)	1 (1%)	3 (3%)	9 (9%)
Cd 15	4 (4%)	4 (4%)	0	8 (8%)
Cd 30	1 (1%)	0	1 (1%)	2 (2%)
Total	77 (77%)	15 (15%)	8 (8%)	100

BT, Beta thalassaemia; XmnI: Gγ-globin promoter −158 (C>T) XmnI polymorphism.

genotype was IVSI-5/IVSI-5; 4/26 (15%) cases with this genotype had XmnI polymorphism. The genotypes of homozygous/compound heterozygous beta thalassaemia and XmnI polymorphism detected with each type are summarised in Table 2. In the heterozygous BT group, Fr 8-9(36%), followed by IVSI-5(30%), were the most common mutations. XmnI polymorphism was detected in 23/100(23%) cases of this group; 70% of XmnI polymorphism (16/23) was associated with βo phenotype. IVSI-5, followed by Fr 8-9, were the most common genotypes associated with XmnI polymorphism (23% and 19% respectively). The genotypes of heterozygous BT and XmnI polymorphism detected with each type are summarised in Table 3.

DISCUSSION

Beta thalassaemia is a major health burden in Pakistan. Despite preventive measures, around 5000 children with transfusion dependent TI or TM are annually added to the registry. No permanent cure is available, except stem cell transplantation, which is unaffordable due to high cost. The majority of

patients with transfusion-dependent thalassaemia have no choice but to have regular blood transfusions and iron chelation therapy. Haemoglobin F augmentation through HU therapy is another ray of hope to avoid blood transfusions and associated complications.[13–15] The response to HU therapy has been correlated with certain BT genotypes and XmnI polymorphism. However, this association has not been well established.[9,16]

XmnI polymorphism is heterogeneously distributed in different parts of the world. Our study was performed in northern Pakistan where Punjabi and Pathan populations dominate. We found XmnI polymorphism in 13% of patients with TM and TI. In a study by Ansari et al. performed in southern Pakistan among a dominant Sindhi and Indian migrant population, XmnI polymorphism was detected in 45% of patients.[17] XmnI polymorphism was detected in 18–33% of patients with BT in India, in 39–71% of patients in Iran, 7% in Turkey, and 4% of BT patients in Egypt.[11,18–22] Such a wide variation can only be explained on the basis of the genetic distribution of polymorphism, unrelated to the type of BT mutations, though frequently related to BT genotypes. The molecular mechanisms describing BT mutation effects on the distribution of XmnI polymorphism are not well outlined. XmnI polymorphism may also be associated with a completely deleted β-gene as in the case of δβ thalassaemia where it can be assumed that the loss of regulatory regions for the γ-genes, the rearrangement of the β-gene complex that brings enhancer sequences close to the Gγ-globin gene promoter, and the loss of competition for a common locus control region (LCR) between the γ-, δ-, and the β-gene promoters may be involved.[23] No such association of specific BT mutations with XmnI polymorphism has been described.

XmnI polymorphism was equally distributed in βo/βo (5/14, 35.5%), and in β+/β+ (5/14, 35.5%) phenotypes. In the heterozygous BT group, 70% of XmnI polymorphism was associated with βo phenotype, which agrees with studies carried out in India and Turkey.[10,21] It can be inferred that there is a selective effect of genotypes with the distribution of XmnI polymorphism, which varies from region to region. It was also observed that IVSI-5/IVSI-5 could possibly be a genotype that is associated with XmnI polymorphism, and hence can predict better response to HU therapy, if XmnI polymorphism is taken as the major determinant. In the Beta thallasaemia trait group, 23% of IVSI-5 genotype had XmnI polymorphism, which further supports the importance of this genotype. Internationally, other studies also show an association of XmnI polymorphism with specific genotypes like IVS 1-1 (G → T) in India, IVS II-1(G > A) in Turkey, and frame shift codon 8 (-AA) in Morocco.[11,21,24]

In our study, the most common BT genotype was Fr 8-9/Fr 8-9, and none of the patients with this genotype had XmnI polymorphism. The second most common genotype was IVSI-5/IVSI-5, and 15% of patients with this genotype had XmnI polymorphism. Overall, the distribution of XmnI polymorphism varied with the genotype. It was more common when both of the alleles were normal, and was detected in 36/192 normal chromosomes, 31/200 chromosomes in the heterozygous BT group, and 23/214 chromosomes in the homozygous BT group. Our findings differ from the findings of the study performed in Turkey by Bahader and Atalay, who found XmnI polymorphism in 43/200 normal chromosomes, 37/210 with heterozygous BT, and 4/54 chromosomes of homozygous BT 21. It can be inferred that the distribution and frequency of XmnI polymorphism differs greatly among regions, as seen in Iran, India, and southern Pakistan. Northern Pakistan has a low frequency of this important determinant of response to HU therapy.[5,6,11,16–19] However, many studies did not find a significant correlation of response to HU with XmnI polymorphism or BT genotype; additional factors may affect the response to HU therapy or amelioration of the BT phenotype.[7,9,10,14,25] It should be noted that beta thalassaemia is heterogeneous in genetic defects, clinical presentation, and response to therapy. Further clinical studies based on response to HU therapy are strongly recommended in this part of the country.

Limitations of study

The study did not cover the genotype/phenotype correlation of BTM and TI patients, correlation of genotype with basal HbF levels, or age of first transfusion in BTM/TI patients.

CONCLUSION

XmnI polymorphism in homozygous/compound heterozygous BT group is 13%. The most common genotype associated with XmnI polymorphism is IVSI-5/IVSI-5.

REFERENCES

1. Khattak MF, Saleem M. Prevalence of heterozygous beta-thalassemia in northern areas of Pakistan. J Pak Med Assoc 1992;42(2):32–4.
2. Ansari SH, Shamsi TS, Ashraf M, Farzana T, Bohray M, Perveen K, et al. Molecular epidemiology of β-thalassemia in Pakistan: far reaching implications. Indian J Hum Genet 2012;18(2):193–7.
3. Khattak SA, Ahmed S, Anwar J, Ali N, Shaikh KH. Prevalence of various mutations in beta thalassaemia and its association with haematological parameters. J Pak Med Assoc 2012;62(1):40–3.
4. Baig SM, Azhar A, Hassan H, Baig JM, Kiyani A, Hameed U, et al. Spectrum of beta-thalassemia mutations in various regions of Punjab and Islamabad, Pakistan: establishment of prenatal diagnosis. Haematologica 2006;91(3):ELT02.
5. Badens C, Joly P, Agouti I, Thuret I, Gonnet K, Fattoum S, et al. Variants in genetic modifiers of β-thalassemia can help to predict the major or intermedia type of the disease. Haematologica 2011;96(11):1712–4.
6. Nguyen TK, Joly P, Bardel C, Moulsma M, Bonello-Palot N, Francina A. The XmnI (G) gamma polymorphism influences hemoglobin F synthesis contrary to BCL11A and HBS1L-MYB SNPs in a cohort of 57 beta-thalassemia intermedia patients. Blood Cells Mol Dis 2010;45(2):124–7.
7. Bradai M, Abad MT, Pissard S, Lamraoui F, Skopinski L, de Montalembert M. Hydroxyurea can eliminate transfusion requirements in children with severe beta-thalassemia. Blood 2003;102(4):1529–30.
8. Mabaera R, West RJ, Conine SJ, Macari ER, Boyd CD, Engman CA, et al. A cell stress signaling model of fetal hemoglobin induction: what does not kill red blood cells may make them stronger. Exp Hematol 2008;36(9):1057–72.
9. Karimi M, Haghpanah S, Farhadi A, Yavarian M. Genotype-phenotype relationship of patients with β-thalassemia taking hydroxyurea: a 13-year experience in Iran. Int J Hematol 2012;95(1):51–6.
10. Verma IC, Kleanthous M, Saxena R, Fucharoen S, Winichagoon P, Raizuddin S, et al. Multicenter study of the molecular basis of thalassemia intermedia in different ethnic populations. Hemoglobin 2007;31(4):439–52.
11. Sharma N, Das R, Kaur J, Ahluwalia J, Trehan A, Bansal D, et al. Evaluation of the genetic basis of phenotypic heterogeneity in north Indian patients with thalassemia major. Eur J Hematol 2010;84(6):531–7.
12. Haj Khelil A, Morinière M, Laradi S, Khelif A, Perrin P, Ben Chibani J. Xmn I polymorphism associated with concomitant activation of Gγ and Aγ globin gene transcription on a β0-thalassemia chromosome. Blood Cells Mol Dis 2011;46(2):133–8.
13. Yavarian M, Karimi M, Bakker E, Harteveld CL, Giordano PC. Response to hydroxyurea treatment in Iranian transfusion-dependent beta-thalassemia patients. Haematologica 2004;89(10):1172–8.
14. Musallam KM, Taher AT, Cappellini MD, Sankaran VG. Clinical experience with fetal hemoglobin induction therapy in patients with β-thalassemia. Blood 2013;121(12):2199–212.
15. Danjou F, Anni F, Perseu L, Satta S, Dessì C, Lai ME, et al. Genetic modifiers of β-thalassemia and clinical severity as assessed by age at first transfusion. Haematologica 2012;97(7):989–93.
16. Ansari SH, Shamsi TS, Ashraf M, Perveen K, Farzana T, Bohray M, et al. Efficacy of hydroxyurea in providing transfusion independence in β-thalassemia. J Pediatr Hematol Oncol 2011;33(5):339–43.
17. Ansari SH, Shamsi TS, Munzir S, Khan MT, Erum S, Perveen K, et al. Gγ-Xmn I polymorphism: a significant determinant of β-thalassemia treatment without blood transfusion. J Pediatr Hematol Oncol 2013;35(4):e153–6.
18. Aditya R, Verma IC, Saxena R, Kaul D, Khanna VK. Relation of Xmn-1 polymorphism and five common Indian mutations of thalassaemia with phenotypic presentation in β-thalassaemia. JK Sci 2006;8(3):139–43.
19. Karimi M, Yarmohammadi H, Farjadian S, Zeinali S, Moghaddam Z, Capellini MD, et al. Beta-thalassemia intermedia from southern Iran: IVS-II-1 (G→A) is the prevalent thalassemia intermedia allele. Hemoglobin 2002;26(2):147–54.
20. Neishabury M, Azarkeivan A, Najmabadi H. Frequency of positive XmnIGgamma polymorphism and coinheritance of common alpha thalassemia mutations do not show statistically significant difference between thalassemia major and intermedia cases with homozygous IVSII-1 mutations. Blood Cells Mol Dis 2010;44(2):95–9.
21. Bahadir A, Atalay EO. Frequency of Gγ-globin promoter −158 (C>T) Xmn I polymorphism in Denizli, Turkey. Int J Phys Sci 2012;7(12):1927–31. http://dx.doi.org/10.5897/IJPS12.055.
22. Tantawy AA, Andrawes NG, Ismaeil A, Kamel SA, Emam W. Prevalence of XmnI Gγ polymorphism in Egyptian patients with β-thalassemia major. Ann Saudi Med 2012;32(5):487–91.
23. Ahmed S, Anwar M. XmnI Ggamma-polymorphism in six unrelated Pakistani families with Inv/Del Ggamma(Agammadeltabeta) degrees deltabeta-thalassemia. Am J Hematol 2005;80(4):303–5.
24. Agouti I, Badens C, Abouyoub A, Khattab M, Sayah F, Barakat A, et al. Genotypic correlation between six common beta-thalassemia mutations and the XmnI polymorphism in the Moroccan population. Hemoglobin 2007;31(2):141–9.
25. Kumar R, Kaur A, Agarwal S. Influence of Xmn 1(G)γ (HBG2 c.-211 C→T) Globin Gene Polymorphism on Phenotype of Thalassemia Patients of North India. Indian J Hematol Blood Transfus 2014;30(4):286–90. http://dx.doi.org/10.1007/s12288-013-0293-9.