ORIGINAL ARTICLE

*Xmn*I POLYMORPHISM AND DISEASE SEVERITY IN PATIENTS WITH BETA THALASSEMIA FROM NORTHERN PAKISTAN

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Background: Thalassemia is a heterogeneous disorder and several genetic factors influence the severity of thalassemia. An accurate and early diagnosis of a mild thalassemia genotype helps to avoid unnecessary transfusion and its complications. The aim of this study is to identify the association between *Xmn*I polymorphism and disease severity in patients with β-thalassemia from northern Pakistan. **Methods:** The cross sectional study was conducted at the Department of Haematology, Armed Forces Institute of Pathology (AFIP) Rawalpindi, from September 2006 to June 2009. A total of 90 subjects including 30 with thalassemia major, 30 with thalassemia intermedia and 30 normal individuals were studied. DNA from each subject was tested for 15 β-thalassemia mutations and the presence of *Xmn*I polymorphism using Amplification Refractory Mutation System and Restriction Fragment Length Polymorphism respectively. **Results:** One normal and one thalassemia major subject were found to be positive for homozygous and heterozygous *Xmn*I polymorphism respectively. Among the thalassemia intermedia group, *Xmn*I polymorphism was found in 12/30 patients, of whom 10 were homozygous and 2 were heterozygous for it. **Conclusion:** *Xmn*I polymorphism is an important genotypic factor in Pakistani population for making a prospective diagnosis of thalassemia intermedia and predicting the severity of the disease.

Keywords: Thalassemia intermedia, *Xmn*I endonuclease, Restriction Fragment Length Polymorphism, Northern Pakistan

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INTRODUCTION

Distinguishing thalassemia major from thalassemia intermedia at the time of presentation is vital to designing the appropriate treatment of the patients.[1] Accurate prediction of a mild phenotype may avoid needless blood transfusion and its complication, while an early diagnosis of thalassemia major will allow timely start of the transfusion program, thus preventing/delaying hypersplenism, thalassemia related bone changes and growth retardation.[2,3]

Unfortunately, the accurate identification of these two phenotypes at the onset may prove extremely difficult owing to the wide variety of β-thalassemia mutations and the factors that modify the severity of the disease.[4,5] A multidimensional approach involving careful analysis of clinical, haematological, and molecular data is therefore needed to reach the correct diagnosis.[6–7]

Presence of *Xmn*I polymorphism is one of the many genetic modifiers that can reduce the severity of homozygous β-thalassemia.[9] This polymorphism is associated with increased levels of HbF in thalassemic patients and is found in variable frequency in various populations.[10–12] A recent study of *Xmn*I polymorphism in Pakistani patients with delta beta thalassemia demonstrated *Xmn*I positivity in all the subjects.[13] This prompted us to determine an association between *Xmn*I polymorphism and disease severity in patients with β-thalassemia in northern Pakistani population.

MATERIAL AND METHODS

The cross-sectional study was carried out at the Department of Haematology, AFIP, Rawalpindi over a period of two years and ten months, from September 2006 to June 2009. A total of 90 unrelated subjects including 30 with thalassemia major, 30 with thalassemia intermedia and 30 normal individuals were studied. Relatives (first and second cousins) of the already enrolled subjects were excluded. Study was approved by the ethical review board of Armed Forces Institute of Pathology. An informed consent was obtained from each subject and a brief history was taken. Severity of the disease was determined by three factors, i.e., age at the start of transfusion requirement, interval between transfusion and total number of transfusions received. 5 ml of venous blood was drawn from the antecubital vein. Ethylenediamine tetra-acetic acid at concentrations of 1.5±0.5 mg/ml was used as the anticoagulant. Deoxyribonucleic acid (DNA) was extracted using genomic DNA purification kit by gentra systems (USA).

All the samples were first tested by Amplification Refractory Mutation System (ARMS) polymerase chain reaction (PCR) technique for the 15 known mutations [IVSI-5 (G-C), Fr 8-9 (+G), IVSI-1 (G-T), Fr 41-42 (–TTCT), Del 619 bp, Cd 15 (G-A), Cd 5 (–CT), Cd30 (G-C), Cd 30 (G-A), Fr 16 (–C), IVSII-1 (G-A), Cd 26 (G-T) (Hb-E), Cap +1 (A-C),

Fr 47-48 (+ATCT) and IVSI-25 (25b del)] previously reported in Pakistani subjects.[14]

PCR was carried out in a 25 µl reaction mixture containing 5 pmol of each primer, 0.3 units of Taq polymerase (Advanced Biotechnologies. U. K), 30 µM of each Deoxynucleotide Triphosphate (dNTP), (Boehringer Manheim), 10 mmol Tris HCl (pH 8.3), 50 mmol KCl, 1.5 mmol $MgCl_2$, 100 µg/ml gelatin and 0.3–0.5 µg of genomic DNA. The thermal cycling consisted of 25 cycles of denaturation at 94°C for 1 minute, primer annealing at 65°C for 1 minute and DNA extension reaction at 72°C for 1.5 minute. In the final cycle the extension reaction was prolonged to 3 minutes.[14]

A 641-bp fragment of DNA flanking the C–T polymorphism at–158 to the $^{G}\gamma$-gene was amplified with primers 5'-GAACTTAAGAGATAATGGCCTAA and 5'-ATGACCCATGGCGTCTGGACTAG (Invitrogen, Carlsbad, CA). The amplified fragments were digested overnight at 37°C with 10 units of *Xmn*I restriction enzyme (Fermentas Life sciences, Vilnius, Lithuania).[13]

The PCR of amplified products were run by 6% mini polyacrylamide gel electrophoresis at 150 V for 40 minutes. The gels were stained by 0.1% silver nitrate for 20 minutes. The reaction was developed by adding freshly prepared solution of 1.5% NaOH and 0.15% formaldehyde. Within 10–15 minutes the bands of DNA could be seen clearly. The gel was washed with tap water and transferred to a clean sheet of filter paper. It was covered by cling film and was dried on a gel dryer.[15]

Presence of *Xmn*I polymorphism in thalassemia intermedia patients was compared with thalassemia major patients and normal controls. The frequency of occurrence of *Xmn*I polymorphism, i.e., –/–, –/+ and +/+ genotype in each group was calculated along with 95% Confidence Interval. The frequencies in each group were compared using Chi Square test and p-values below 0.05 were considered statistically significant. Data was analysed in Statistical Package for Social Sciences version12.0.

RESULTS

A total of 90 subjects including 30 with thalassemia major, 30 with thalassemia intermedia and 30 normal individuals were enrolled in the study.

One case of *Xmn*I homozygosity and one of heterozygosity was found in the normal and thalassemia major group respectively. All other subjects in these two groups were *Xmn*I –/–. Among the thalassemia intermedia group, *Xmn*I polymorphism was found in 12/30 patients out of which 10 were homozygous and two were heterozygous for it (Figure 1). Among the *Xmn*I +/+ group, one patient had homozygous Inv/Del $^{G}\gamma(^{A}\gamma\delta\beta)$ mutation as an additional ameliorating factor. Similarly, one of the *Xmn*I +/– patient also had HbE/β-thalassemia. CAP+1 mutation was found in 10/30 subjects to be the cause of reduced severity. An uncharacterized mutation was present in two patients, one of whom was homozygous, while the other was compound heterozygous with CAP+1 mutation. Among the rest in thalassemia intermedia group, six were homozygous and one was compound heterozygous for β^0-thalassemia. The frequency of occurrence of *Xmn*I polymorphism, i.e., –/–, –/+ and +/+ calculated with 95% confidence interval is mentioned in table 1.

The frequencies in each group are compared by using Chi Square test in table 3. Causes of reduced disease severity in thalassemia intermedia group are summarized in Table-4. *Xmn*I polymorphism was found to be closely associated with IVS I-5, Cd 30, IVS II-1 and Inv/Del $^{G}\gamma(^{A}\gamma\delta\beta)$ mutations. No such association was seen with any other β-thalassemia mutations that were found in the thalassemia intermedia group. Frequency of *Xmn*I polymorphism in various mutations is mentioned in table 2.

Most of the patients of thalassemia major group presented and became transfusion dependent during first year of life. On the other hand, age at the need for transfusion was higher in thalassemia intermedia group, ranging between 2–6 years in majority of cases. However, a few patients presented in the third decade of life. The median age at presentation for thalassemia major and thalassemia intermedia group was six months and six years respectively.

Table-1: The frequency of occurance of *Xmn*I +/+, +/– and –/– in thalassemia major, thalassemia intermedia and normal groups

Group	The frequency of occurrence along with its 95% CI					
	Xmn I +/+		*Xmn* I +/–		*Xmn* I –/–	
	Freq n (%)	95% Cl	Freq n (%)	95% CI	Freq n (%)	95% CI
TM	0	-	1 (3.3)	0–9	29 (96.6)	89–100
TI	10 (33.3)	16–50	2 (6.6)	0–15	18 (60)	42–77
N	1 (3.3)	0–9	0	-	29 (96.6)	89–100

***CI** = confidence interval, **TM**=thalassemia major, **TI**= thalassemia intermedia, **N**=normal, **Freq**=frequency.*

Table-2.Comparison of the presence of *Xmn*I polymorphism between thalassemia intermedia, major and normal group of individuals

Group	Chi square value	*p*-value
Thalassemia intermedia and thalassemia major	11.88	0.0005
Thalassemia intermedia and normal	11.88	0.0005

Table-3: Frequency of *Xmn*I polymorphism in various β-thalassemia mutations in thalassemia intermedia group

β-thalassemia mutation	Number of alleles	*Xmn* I positive alleles	%
Cd 15	2	0	0
IVS I-5	18	13	72
Fr 8-9	12	0	0
CAP+1	10	0	0
Cd 30	4	4	100
IVS II-1	4	4	100
Fr 41–42	4	0	0
HbE	1	0	0
Inv/Del $^{G}\gamma(^{A}\gamma\delta\beta)$	2	2	100
Uncharacterized	3	0	0

Table-4: Causes of reduced severity in thalassemia intermedia group

XmnI Polymorphism	10 cases (33%)
CAP+1 mutation	10 cases (33%)
XmnI Polymorphism with other ameliorating factors	2 cases (7%)
Unknown	8 cases (27%)

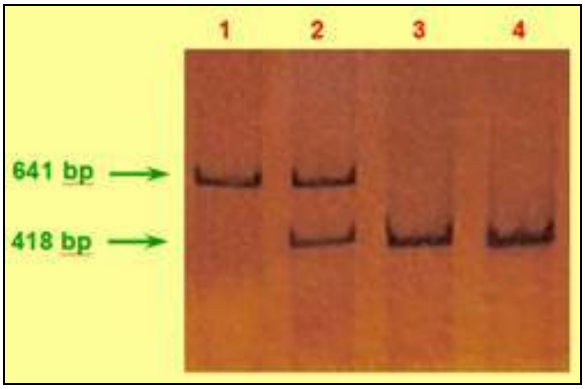

Figure-1: Silver stained polyacrylamide gel electrophoresis of the *Xmn*I restriction enzyme digested products from patients with thalassemia intermedia. The 641-bp fragments represent the uncut (–) site, while the 418-bp fragments represent the cut (+) site. Samples 1 is –/–, sample 2 is +/– whereas samples 3 and 4 are +/+ genotypes

DISCUSSION

Thalassemia intermedia is a clinically diverse syndrome which is influenced by a large number of genetic factors.[16] However, based on the underlying pathophysiology, three general mechanisms have been described that influence the clinical severity of the disease. These include inheritance of mild β-thalassemia mutations, co-inheritance of α-thalassemia and inheritance of genetic determinants that cause enhanced production of γ-globin chains.[17] Our study revealed that predominant cause of reduced severity in thalassemia intermedia group was the presence of *Xmn*I polymorphism. Coinheritance of the silent CAP+1 mutation with β^0 mutation was the next most common ameliorating factor. These results are in accord with past studies among Indian and Iranian populations where *Xmn*I polymorphism was found to be the leading cause of reduced severity among thalassemia intermedia patients.[10] However, studies from Europe and Mediterranean region show different results demonstrating the heterogeneity of the disease. In these regions, the commonest cause of reduced severity is β^+ mutations, though the spectrum of these mutations also changes from region to region.[11,18,19]

During this study, a large number of patients (33%) with compound heterozygosity for CAP+1 mutation and β^0 mutation were observed in thalassemia intermedia group. In fact, this was the only β^+ mutation that significantly reduced the disease severity among our patients. Surprisingly, this mutation, although of Indian origin, has been reported in very low frequency (approximately 3%) in Indian thalassemia intermedia patients.[18] A study on the Asian Indian population in UK has also showed the presence of this mutation among the subjects tested.[19] -88 C-T mutation is another mild mutation of Punjabi origin and has been known to be prevalent among the Sikh population of Punjab[20], however, it was not found in any of our subjects. Although this mutation wasn't screened with PCR in this study, its presence was ruled out by the fact that none of the three uncharacterized alleles produced any β-globin chain (demonstrable by the presence of HbA band on haemoglobin electrophoresis). Comparison with contemporary studies shows that the mutation pattern differs with the region and ethnic origin of the population, and each group has its own set of mild mutations that results in thalassemia intermedia.[11,18,19,21]

*Xmn*I polymorphism has been known to be associated with certain β-globin gene mutations.[10,11,13] In this study, *Xmn*I polymorphism was found to be associated with IVS I-5, Cd 30, IVS II-1 and Inv/Del $^{G}\gamma(^{A}\gamma\delta\beta)$ mutations. No association was found with Fr 41-42 and Fr 8-9 mutations. Further research is needed to establish genetic mechanism behind these associations.

CONCLUSION

Thalassemia intermedia is a heterogeneous group of genetic disorders that range in severity from mild to moderately transfusion dependent anaemia. *Xmn*I polymorphism is an important genotypic factor in northern Pakistani population for making a prospective diagnosis of thalassemia intermedia and predicting the severity of the disease.

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