

Haematological and molecular characterization of sickle cell- β thalassemia in Dera Ismail Khan Division of Pakistan

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Abstract: Sickle cell- β thalassemia (HbS- β thalassemia) is an inherited erythrocyte disorder affecting multiple organs. It results from compound heterozygosity for sickle cell trait and β thalassemia trait. As individuals are living well into middle age due to advances in diagnosis and treatment, further disease-related complications are being recognized. The present study was undertaken to determine the genetic factors responsible for hematological and molecular variability of HbS- β thalassemia patients in Dera Ismail Khan (D.I.Khan), Pakistan. The current study was observed to analyse both molecular and haematological characterization of HbS- β thalassemia patients using Hb electrophoresis and allele specific primers through polymerase chain reaction to determine both α and β -thalassemia, and restriction enzymes for characterization of γ -globin gene arrangement. A total of fifteen HbS- β thalassemia cases with variable clinical manifestations were investigated. Ten patients showed milder clinical presentation against five patients who had severe clinical manifestations. Six β thalassemia mutations were identified: IVS 1-5 (G>C), codon 8/9 (+G), codon 30 (G>C), Cap+1 (A>G), -88 (C>T) and Cd 41/42 (-TCTT). Codon 30 (G>C) and -88 (C>T) mutations were found only in Pashtoon ethnic group while Cap+1 (A>G) mutation was observed only in Balochi ethnic group. HbS homozygous in Pashtoon ethnic group is being observed for the first time. α thalassemia and XmnI polymorphism in homozygous condition (+/+) were found to be common among the milder cases. The β S chromosomes were linked to the typical Arab-Indian subcontinent haplotype. Saudi haplotype is mostly associated with HbS and haplotype III is associated only with Pashtoon ethnic group. The phenotypic expression of HbS- β thalassemia is not uniformly mild and α thalassemia and XmnI polymorphism in homozygous condition (+/+) are additional genetic factors modulating the severity of the disease in the Pakistan. [Jabbar KHAN, Nafees AHMED, Sami SIRAJ, Shahid Niaz KHAN, Hamid SHAFIQ. **Haematological and molecular characterization of sickle cell- β thalassemia in Dera Ismail Khan Division of Pakistan.** *J Am Sci* 2018;14(9):41-46]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). <http://www.jofamericanscience.org>. 7. doi:[10.7537/marsjas140918.07](https://doi.org/10.7537/marsjas140918.07).

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1. Introduction

Hemoglobin S [HbS] is an inherited variant of normal adult hemoglobin A (HbA). It results from a substitution of valine for glutamic acid in the sixth position of the β globin chain (Serjeant, 2001).

From molecular point of view, HbS can be in one of the following three combinations.

Hemoglobin S trait that results when the gene for HbS is inherited from one parent and the second allele is normal from the other. This carrier state does not result in health problems.

Sickle cell anemia, homozygous sickle cell disease occurs when the gene for HbS is inherited from both parents. A moderate to severe hemolytic anemia develops in the first few months of life as a result of decrease in fetal hemoglobin (HbF) and increase in HbS (Brawley et al., 2008).

Sickle/beta [HbS- β] **thalassemia**, which results from co-inheritance of the gene for HbS and β

thalassemia, has mild to severe clinical manifestations, depending upon mutation affecting the HbA gene (Barbara & Barbara, 2006; Chirico & Pialoux, 2010).

Geographically, the disease is widely distributed, including Indian subcontinent, Africa, the Middle East, and parts of Mediterranean (Gladwin et al., 2002; Sampietro et al., 1992). Alterations in the shape of hemoglobin thus result in sickle-shaped erythrocytes with damaged cell membranes. The weakened cell membranes predispose cells to hemolysis, damage endothelial cells, and establish a favourable milieu for vascular obstruction (Sampietro et al., 1992).

The clinical features of HbS- β thalassemia are extremely variable, ranging from a completely asymptomatic state to a severe disorder similar to homozygous sickle cell disease. (Barbara & Barbara, 2006; Sampietro et al., 1992) This heterogeneity is

likely to be due to the presence of different β thalassemia alleles or interaction with modulating genetic factors like associated α thalassemia and/or a gene for raised HbF production (XmnI polymorphism). The disease leads to anemia of variable severity that becomes manifest in early childhood (Higgs et al., 1990; Wang et al., 2003; Yi et al., 2008).

In Pakistan, β -thalassemia is one of the most common Hb disorders (Khattak & Saleem, 1992; Ahmed et al., 1996). More than one third of Pakistani population is below the age of 15 years (Khattak & Saleem, 1992; Khan et al., 2015; Hashmi and F. Farzana, 1976). The carrier frequency is 5.4% [5]. The incidence of β thalassemia varies from 1.4 to 8% (Ahmed et al., 1996; Hashmi and F. Farzana, 1976; Khan & Riazuddin, 1998; Rehman et al., 1991). The disease highly prevails in areas along the Arabian Sea coast, in South and the Khyber-Pakhtunkhwa [KPK] province, situated near the Afghanistan border. Here, people from central Asia, the Mediterranean and Middle East settled as they invaded during various periods in history. A number of factors like, preference to marry within their own ethnic groups and cousin marriages have valuably contributed to the increased incidence of this disease in Pakistani population (Khan et al., 2015; Khan & Riazuddin, 1998; Rehman et al., 1991). Here, sickle cell disease is not well characterized but is grouped with the patients of thalassemia intermedia. Quality of life is very low for β -thalassemia patients in Pakistan, because of lack of public awareness about the disease and, also the provision of good quality care. Furthermore, the facilities for bone marrow transplantation are either very expensive or are rarely available in the country and thus adding to the miserable life of thalassemic patients of Pakistan (Khan et al., 2015; Khan & Riazuddin, 1998; Rehman et al., 1991; Khan et al., 2003).

Here the main focus was to characterize HbS- β -thalassemia patients in D.I.Khan division of KPK. The population survey revealed a high prevalence of the β s gene. During this study, 15 sickle cell- β thalassemia cases (3 homozygous and 12 compound heterozygous), in the age group of 3-32 years were identified using solubility test, hemoglobin electrophoresis and measurement of HbA2 and HbF levels by elution after electrophoresis and through amplification refractory mutation system (ARMS) technique by PCR. Further, It was the first report to observe the clinical, hematological and molecular pathological analysis of these HbS- β thalassemia cases from D.I.Khan division of Pakistan.

2. Materials and Methods

Informations on age at presentation and

requirements of blood transfusions were recorded. Ten millilitres of blood was collected in ethylenediaminetetraacetic acid (EDTA) after informed consent was obtained from all subjects. Blood samples for hematologic evaluation were collected at least 30 days after the last transfusion. Hemoglobin electrophoresis and quantitation of HbA2 was done using alkaline cellulose acetate electrophoresis at pH 8.9 and elution. The HbF level was quantified by the alkali denaturation method of (Singer et al., 2003). Molecular analysis was carried out in 15 HbS- β thalassemia cases. DNA was isolated from peripheral blood leukocytes by standard non-organic method (Miller et al., 1988) and stored at -20°C.

2.1. Detection of HbS β -thalassemia by allele specific PCR

All samples were screened out through tetra primer based allele specific PCR (Newton et al., 1989). For each sample, two PCR reactions were performed containing either of the allele specific primer and control primers. Each reaction of 25 μ l volume was performed by using 0.2 μ g genomic DNA. The reaction was carried out through 27 cycles that consisted of 1 minute denaturation at 94 °C, 1 minute annealing at 65 °C and 1 minute and 30 seconds extension at 72 °C. During the first cycle, denaturation was done at 95 °C for 3 minutes and the final extension was done at 72 °C for 5 minutes in the last cycle. Amplified products were electrophoresed on 1.5% agarose gel containing ethidium bromide and visualized under UV transilluminator. β -globin genotypes were assigned on the basis of presence or absence of allele specific bands. The positive controls used were previously characterized sample and dH₂O was used as a negative control.

2.2. Detection of α - globin region deletions by Gap-PCR

For the detection of $-\alpha^{3.7}$ kb deletions in α -globin genes, the forward primer used was C10; 5'-GATGCACCCACTGGACTTCCT-3' located in the homologous Y regions of both α 1 and α 2 genes and reverse primers used were C2; 5'-CCATGCTGGCAGTTTCTGA-3' and C3; 5'-CCATTGTTGGCACATTCCGG-3' located in the non homologous 3' non-coding regions of α 1 and α 2 genes in separate reactions. The reaction consisted of 30 cycles with 1-minute denaturation at 94 °C, 1 minute annealing at 52 °C and 1 minute and 30 seconds extension at 72 °C. During the first cycle, denaturation was done at 94 °C for 10 minutes, while in the last cycle extension was done for 10 minutes at 72°C. Electrophoresis of PCR products was done on 1.5% agarose gel containing ethidium bromide. A normal α 1 gene was detected as a 2.1 kb fragment with the

primers C10 and C2 primers while the $-\alpha^{3.7}$ mutation gave rise to 1.9 kb product with the same primers. A normal $\alpha 2$ gene and reciprocal event [$\alpha\alpha^{\text{anti } 3.7}$] were detected as 1.9 kb fragment and 2.1 kb respectively with the same C10 and C3 primers.

2.3. β -globin haplotype and XmnI polymorphism in γ -globin gene

Haplotype analysis for β -globin gene region and a $G\gamma$ -158T>C polymorphism in the γ -globin gene was done by PCR with specific primers (Table 3). A 25 μ l standard PCR reaction PCR reactions were carried out through 30 cycles that consisted of 30 seconds denaturation at 94 °C, 30 seconds annealing at 55 °C or 62 °C (Table 1), 1 minute and 30 seconds extension at 72 °C. During the first cycle denaturation was done at 95 °C for 5 minutes and the final extension at 72 °C for 3 minutes after the last cycle. Amplified product was digested with appropriate restriction enzyme under the conditions recommended by the manufacturer (New England Bio Labs, Inc, USA). Electrophoresis of digested product was carried out on 3% agarose gel containing ethidium bromide and visualized under UV light. β -globin gene haplotypes were assigned by the presence or absence of specific restriction site. The restriction enzymes with their restriction sites used to construct haplotypes were

Hinc II 5' to ϵ , XmnI 5' to $G\gamma$, Hind III within $G\gamma$ and $A\gamma$ Hind II 3' to $\psi\beta$, AvaII within β and Hinf-1 3' to β .

3. Statistical Analysis:

The data was analyzed using Korea Statistics 9. Results are presented as mean \pm wherever necessary.

4. Results

The age at presentation ranged from 3 to 32 years. Ten (66.6%) HbS- β thalassemia patients had a milder clinical presentation, whereas, five cases (33.4%) had severe clinical manifestations in the form of acute pain in the joints, abdomen, bones and chest and were dependent on regular blood transfusions. Although hepatosplenomegaly was observed in both mild and severe cases, however, splenomegaly was more common in severe cases (80.0%) as compared to milder cases (40.0%).

The mean Hb levels among the severe cases were slightly lower (7.7 ± 0.60) than the patients with milder clinical manifestations (9.2 ± 2.04). HbF levels varied from 4 to 32.6%. HbF levels among the patients with milder clinical presentations were higher (18.0 ± 3.17) than the severe cases (13.1 ± 6.20). XmnI polymorphism was studied in all 15 patients, of whom 9 (60.0%) were homozygous (+/+), 5 (33.3%) were heterozygous (+/-) and only patient was lacking this mutation (-/-).

Table 1: Sequences of the RFLP Primers for the β Globin Gene Haplotype and -158 $G\gamma$ HPFH Gene (X mnI RFLP)

5'>3' Primer	Product Size (bp)	Absence Of Site (bp)	Presence Of Site	Annealing Temp
Hinc II/ ϵ TCTCTGTTTGGATGACAAATTC AGTCATTGGTCAAGGCTGACC	760	760	446 314	55°C
X mn-1/ $G\gamma$ ACTGTTGCTTTATAGGAT CTGGTCGACTAGGAGCTTATTGAT	657	657	465 188	55°C
Hind III/ $G\gamma$ TGCTGCTAATGCTTCATACAA AAGTGTGGAGTGCACATGA	326	326	235 91	62°C
Hind III/ $A\gamma$ TGCTGCTAATGCTTCATTACAA TAAATGAGGAGCATGCACACAC	635	635	327 308	62°C
Hind II 3' $\Psi\beta$ GTACTCATACTTTAAGTCCTAACT TAAGCAAGATTATTTCTGGTCTCT	913	913	479 434	55°C
Ava II 5'/ β GTGGCTCACCCCTGGACCCAGAGG TTCGICTGTTTCCCATTTCTAAACT	328	328	228 100	55°C
Hinf-1 3'/ β AGTAGAGGCTTGATTTGGAGG GTTAAGGTGGTTGATGGTAAC	474	320 plus constant fragment of 154	213 107 and 154	55°C

HbF levels were found to be significantly higher in patients homozygous for XmnI polymorphism as compared to those who were heterozygous for the said mutation ($p < 0.001$). Interestingly, the patient lacking XmnI polymorphism possessed the least level of HbF. Almost all the severe cases (Table 2, S.No. 11-15) showed the presence of the XmnI polymorphism in heterozygous state (XmnI +/-), with only one case having no XmnI polymorphism. In contrast, with only one exceptional case, which is heterozygous for this mutation, all the milder cases (Table 2, S.No. 1-10) were homozygous (+/+).

All the patients were screened out for β thalassemia mutations. Six different β thalassemia mutations were found. Three patients were found homozygous for HbS (Table 2). IVS 1-5 (G>C) was the commonest mutation (20.0%), followed by codon 30 (G>C) (13.4%), codon 8/9(+G) (13.4%), -88 (C>T) (6.7%), Cap+1 (6.7%) and codon 41/42 (-TCTT) (6.7%). Interestingly, HbS homozygous cases were found only in Pashtoon ethnic group and Cap+1 (A>G) mutation has been observed only in Balochi

ethnic group. Similarly, IVSI-5 (G>C) was observed only in Punjabi ethnic group and codon 30 G>C was found only in Pashtoon ethnic group (Table 2).

All the patients were further characterized for α -thalassemia rearrangements. Five patients were found with $\alpha\alpha/\alpha\alpha$ genotype, four patients with $-\alpha/\alpha\alpha$ genotype and two cases were found with $-\alpha/-\alpha$ genotype. Moreover, two patients possessed $-\alpha^{3.7}/-\alpha^{3.7}$ genotype and one patient each with $\alpha\alpha/-\alpha^{3.7}$ and $\alpha\alpha/\alpha\alpha^{3.7}$ genotypes (Table 2). Hence, all the milder patients had abnormal α genotype except the one case possessing a normal $\alpha\alpha/\alpha\alpha$ genotype, and, all the severe cases had a normal α genotype ($\alpha\alpha/\alpha\alpha$) except one case possessing inherited $\alpha\alpha/\alpha\alpha^{\text{anti}3.7}$ genotype (Table 2).

Haplotype analysis revealed six different haplotypes where Saudi haplotype was mostly associated with HbS and haplotype III was found only in Pashtoon ethnic group (Table 2).

Table 2: Haematological and molecular analysis of HbS- β thalassemia patients from D.I.Khan Division of KPK, Pakistan

S.No	Age/sex	Transf/Y	Hb (g/dl)	HbA2 (%)	HbS (%)	HbF (%)	XmnI	β Thal. mut	α -Genotype	Haplotype
Pashtoon	11/F	0	9.1	6.2	99.2	22.1	+/+	HbS homo	$\alpha\alpha/\alpha\alpha$	VII/Saudi
Pashtoon	32/M	0	9.7	4.4	100.0	18.7	+/+	HbS homo	$-\alpha/\alpha\alpha$	Saudi/Saudi
Pashtoon	16/F	0	9.1	5.0	99.1	22.8	+/+	HbS homo	$-\alpha/\alpha\alpha$	IX/Saudi
Balochi	20/M	0	8.6	4.7	75.7	19.0	+/+	Cap+1 (A>G)	$-\alpha/\alpha\alpha$	VII/VII
Pashtoon	16/F	0	8.4	5.0	78.1	19.4	+/+	-88 (C>T)	$-\alpha/-\alpha$	IX/VII
Balochi	9/F	1	9.0	4.9	78.4	12.1	+/+	Cap+1 (A>G)	$-\alpha^{3.7}/-\alpha^{3.7}$	Saudi/VII
Balochi	15/M	0	9.2	4.8	72.3	18.8	+/+	Cap+1 (A>G)	$-\alpha/-\alpha$	IX/I
Punjabi	6/F	0	10.5	4.9	64.7	32.6	+/+	IVS 1-5 (G>C)	$-\alpha/\alpha\alpha$	Saudi/I
Punjabi	23/M	0	10.4	4.3	67.1	23.6	+/+	IVS 1-5 (G>C)	$\alpha\alpha/-\alpha^{3.7}$	Saudi/I
Punjabi	25/F	1	8.6	4.2	34.6	12.7	+/-	IVS 1-5 (G>C)	$-\alpha^{3.7}/-\alpha^{3.7}$	IX/I
Pashtoon	12/F	3	8.3	5.6	60.5	13.5	+/-	Cd 30 (G>C)	$\alpha\alpha/\alpha\alpha$	VII/I
Pashtoon	16/F	2	8.7	6.6	61.3	12.6	+/-	Cd 30 (G>C)	$\alpha\alpha/\alpha\alpha^{3.7}$	Saudi/I
Pashtoon	19/M	4	7.2	4.4	67.1	14.6	+/-	Cd 8/9 (+G)	$\alpha\alpha/\alpha\alpha$	IX/III
Pashtoon	7/F	10	6.9	3.9	50.0	10.0	+/-	Cd 41/42 (-TCTT)	$\alpha\alpha/\alpha\alpha$	Saudi/III
Pashtoon	8/M	10	7.1	3.9	60.0	4.0	-/-	Cd 8/9 (+G)	$\alpha\alpha/\alpha\alpha$	IX/I

5. Discussion

HbS- β -thalassemia not only leads to hematopoietic problems but also causes the involvement of other organs as clinical phenotypes develop. This very generalization leaves the researchers with a lot of unexplored domains to look into. The clinical and hematologic features in HbS- β thalassemia are quite variable. The clinical severity largely depends upon the nature of the β thalassemia mutations, the α -globin gene rearrangement, polymorphism in the promoter region of γ -globin gene, and some unknown factors, which need to be explored (Sampietro et al., 1992; Alsultan et al., 2011; Thein et al., 2009; Guida et al., 2006; Uda et al., 2008).

Due to strong cultural preference for consanguineous marriages, there is high prevalence of recessively inherited disorders in Pakistan. The five most common mutations are IVI-5 (G>C) (37.7%), codon 8/9 (+G) (21.1%), 619bp del. (12.4%), IVS1-1 (G>T) (9.5%) and codon 5 (-CT) (9.1%) (Hashmi & Farzana, 1976; Khan et al., 2003). The same ethnic specificity has been found with respect to mutations in β globin gene, as has been previously reported (Khan & Riazuddin, 1998; Khan et al., 2003). The only exceptional case with this study is that of HbS homozygous condition that is found only in Pashtoon ethnic group. Previously this mutation was reported in Sindh ethnic group only in Sindh region of Pakistan (Hashmi & Farzana, 1976; Khan & Riazuddin).

Similarly, codon 30 G>C has been found in Pashtoon ethnic group, which has not been reported in previous studies. Hence, HbS is quite heterogeneous, found in all the three major ethnic groups of this region, which is in contrast to what has previously been reported (Khattak et al., 1996; Khan & Riazuddin, 1998; Khan et al., 2003).

As reported earlier, the coinheritance of α -thalassemia with β -thalassemia reduces the severity of disease (Khan & Riazuddin, 1998; Uda et al., 2008; Papachatzopoulou et al., 2007; Dode et al., 1993; Higgs et al., 1990; Adams et al., 1990). In this study, the coinheritance of α -thalassemia with β -thalassemia has also been found. Interestingly, except in one case, possessing normal α globin gene genotype, all the milder patients possessed deleted α globin gene, either in homozygous or heterozygous condition. Similarly, with only one exceptional case, possessing triplicated α globin gene genotype, all the other severe patients had normal $\alpha\alpha/\alpha\alpha$ genotype.

All the HbS- β thalassemia patients were examined for Haplotype analysis. It is reported that there is a close but not completely consistent association of each haplotype with a specific β globin gene mutation (Higgs et al., 1983; Labie et al., 1989; Makani et al., 2010; Mukherjee et al., 1997), such as codon 8/9 (+G) mutation was found with two different haplotypes. Similarly IVSI-6, HbE, codon 41/42 (-TCTT) and IVSI-1 (G>A) were also reported with different haplotypes, but haplotype VII was found only with 619 bp deletions (Khan & Riazuddin, 1998; Khan et al., 2003; Guida et al., 2006; Schmugge et al., 2008). The present study of haplotype analysis showed that Saudi haplotype was the most frequent haplotype (30.0%), followed by haplotype I (23.3%), haplotype VII and IX each with 20.0% and haplotype III (6.6%). This Haplotype analysis showed that Saudi haplotype was mostly associated with HbS and this is what has previously been reported. Interestingly, haplotype III was only associated with Pashtoon ethnic group, which has not mentioned in previous reports. This study, hence, shows that origin of the β thalassemia mutation in this area is unicentric and that the haplotype association is typical Arab-Indian-subcontinent haplotype.

6. Conclusion:

The clinical and haematological manifestations of Pakistani HbS- β thalassemia patients are not only influenced by β thalassemia mutations but also by associated α thalassemia and XmnI polymorphism.

7. Author contributions

JK designed and performed the experimental work, analyzed results and wrote the manuscript. NA and SS conceived the study and reviewed the

manuscript. HS participated in selection of patients and reviewed the manuscript. All authors have read and approved the final manuscript.

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