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Journal of
Blood Disorders & Transfusion

Research Article

# Molecular Characterization and Frequencies of Different Genetic Ameliorating Factors in Transfusion Dependent Thalassemia Patients from District Peshawar

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## ABSTRACT

**Objectives:** 1) To re-assess diagnoses among local multi-transfused thalassemia syndrome patients. 2) To determine frequency of various genetic determinants of milder phenotypes of thalassemia among study patients.

**Methods:** Transfusion dependent thalassemia patients, upto 15 years age, were enrolled from Fatimid Foundation, Peshawar Pakistan. A comprehensive questionnaire encompassing demographic and clinical data was filled out for each patient. Genetic analyses for 2 alpha (α) and 13 prevalent beta (β) gene mutations and for polymorphisms at Xmn1-HBG2 and BCL11A were carried out on blood samples of the patients at National Institute of Blood Diseases, Karachi Pakistan. The data collected was analysed at Khyber Medical University (KMU) Peshawar Pakistan.

**Results:** A total of 54 transfusion dependent thalassemia patients were enrolled into the study. Homozygous or compound heterozygous combinations of β-globin gene mutations were identified in all the study patients. Eleven patients were found to have a co-existing heterozygous α (3.7kb) deletion, two patients had Xmn1-HBG2 polymorphism and 38 had BCL11A polymorphism. Homozygous Fr8-9 was the most frequent mutation, found in 19 (35.2%) patients. Only 13 patients were found to have isolated β-globin gene mutations. In total, 46 (85.2%) study patients were identified to have an ameliorating genetic factor (a co-existing α–globin gene mutation, an Xmn1-HBG2 polymorphism or a BCL11A polymorphism) besides the main β-globin gene mutation.

**Conclusion:** It was concluded that co-existing genetic ameliorating factors are frequently found in transfusion dependent β-thalassemia patients of Peshawar District. This factor imparts a milder phenotype to an otherwise severe disease. It is hence suggested that β-thalassemia patients from District Peshawar be screened for these factors and due consideration be paid by the physician in devising management plans.

**Keywords:** Ameliorating factors; α mutations; β mutations; BCL11A; Thalassemia; Xmn1

## INTRODUCTION

Thalassemia is one of the most common genetic blood disorders worldwide [1]. Beta (β) thalassemia represents a heterogeneous group of haemoglobin disorders characterized by quantitative reduction of β globin chains [2]. It is estimated that 80 million people, globally, have beta thalassemia trait [3]. The carrier rate of β thalassemia in Pakistan is found to be around 5%-7%, rendering it a major health concern [4]. Approximately 5000 children are diagnosed with thalassemia major every year in Pakistan [5]. Due to high numbers of consanguineous marriages in native population, defective genes tend to accumulate within the affected families, culminating in higher incidence rate of the disease [6]. Molecular lesions in β-thalassemia are usually caused by point mutations in the β-globin gene [7]. More than 200 causative molecular defects have been identified in β-globin genes [8]. About 20 mutations account for 90% of the abnormal β-genes [9]. Clinical severity of β-thalassemia is modified by different factors. The important ones include the type of disease causing mutation and the ability to produce α and γ globin chains [10]. A better understanding of these ameliorating factors may have a significant impact on disease management [11].

Conventional Hb studies can only provide information about β-globin chain abnormalities; these are mostly insensitive to α-globin chain disorders. Genetic confirmation for the disease

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**Received:** November 24, 2018; **Accepted:** April 23, 2019; **Published:** April 30, 2019

**Citation:** Sohail G (2019) Molecular Characterization and Frequencies of Different Genetic Ameliorating Factors in Transfusion Dependent Thalassemia Patients from District Peshawar. J Blood Disord Transfus 10:418.

and exclusion of other ameliorating genetic factors is rarely carried out. The current study elucidates frequency of these ameliorating factors in local thalassemia patients and explores their correlation with other clinical and haematological parameters.

## METHODS

Transfusion dependent thalassemia patients, upto 15 years age, were enrolled from Fatimid Foundation, Peshawar Pakistan. Purpose and benefits of the study were explained to the guardians and informed written consent was obtained. A comprehensive questionnaire encompassing demographic and clinical data was filled out for each patient. Venous blood samples were collected and stored till further analyses at National Institute of Blood Diseases, Karachi Pakistan. DNA was extracted from whole blood, manually, using PureLink™ Genomic DNA® extraction kit (Invitrogen, Carlsbad, CA, USA).

β-thalassemia mutations were analyzed by Amplification Refractory Mutation System (ARMS) technique [12]. In the first round of ARMS, the five most common mutations in native population were screened followed by a second and third round for the uncommon and rare mutations. Three multiplex primer combinations (Table 1) were used with a final concentration of 5pM/µl of each primer. The two control primers and their respective common primers were added to the primer mix.

**Table 1:** Combinations of β–thalassemia mutations screened by multiplex ARMS PCR.

| MIX-I | | MIX-II | | MIX-III | |
|---|---|---|---|---|---|
| **Mutations** | **Fragment size** | **Mutations** | **Fragment size** | **Mutations** | **Fragment size** |
| Fr8-9 (+G) | 215bp | Cd 5 (-CT) | 205 bp | Cd15 (G-A) | 500 bp |
| IVS1-5 (G-C) | 285 bp | Fr16 (-C) | 238 bp | Cap+1 (A-C) | 567 bp |
| Fr 41-42 (-TTCT) | 439bp | IVS1-1 (G-T) | 280bp | - | |
| IVS1-1 (G-T) | 280bp | Cd30 (G-C) | 280 bp | | |
| Del 619 bp | 242 bp | Cd30 (G-A) | 280 bp | | |
| - | | IVS II-I (G-A) | 634bp | | |

PCR mix was prepared using 10 µl of 1X PCR-buffer, 1 µl of primer mixes 1, 2 or 3, 0.2 µl Taq polymerase and 2.5 µl of genomic DNA. The mixture was pipetted into PCR tubes and placed in ABI® Veriti 96 well Thermocycler. The machine was programmed at 95˚C for 5 minutes then 94˚C for 1 minute (denature), 65˚C for 1 minute (annealing) and 72˚C for 1.5 minutes (extension). After 25 cycles, the amplified product was stored at 4˚C.

α-thalassemia deletion mutations were detected by Gap-PCR [13]. The –α3.7 kb deletions were detected by amplifying α-globin gene using the forward primer C10; 5- GATGCACCCACTGGACTCCT-3 located in the homologous Y regions of both α1 and α2 genes and reverse primers C2: 5-CCATGCTGGCACGTTTCTGA-3 and C3: 5-CCATTGTTGGCACATTCCGG-3 located in the non-homologous 3- non coding regions of α1 and α2 genes in separate reactions. The PCR was performed using 0.3 µg genomic DNA, 7.5 pM of each of the primers, 200 µM of each dNTP, 1.9 mM MgCl2, 10% DMSO, 0.75 units Taq polymerase and 1x Taq reaction buffer [67mM Tris HCL (Ph 8.8), 16.6 mM (NH4)2 SO4, 66 µM EDTA, 10 mM β-mercaptoethanol and 170 µg mL BSA] in 30 µl reaction volume. Thermal cycling was carried out in ABI Veriti® 96 well thermocycler. Each cycle comprised 1 min denaturation at 940C, 1 minute annealing at 520C and 90 seconds of extension at 72˚C. During the first cycle denaturation was done for 10 minutes at 940C, while in the last cycle extension was performed for 10 minutes at 72˚C. The PCR products were electrophoresed on 2% Agarose gel electrophoresis containing ethidium bromide with 1 kb DNA ladder as a marker.

The α4.2 deletion was detected by the use of GAP-PCR using the PCR cycling conditions as for the α3.7 kb deletion. The reaction employs two GAP primers i.e. a forward primer: 5-TTAAGCTAGAGCATTGGTGGTC-3 and a reverse primer 5-GGTTTCTACATGTTGATCAGGG-3 located in the cap site of α2 and a pair of control primers. The two primers are 5519 bp apart so in PCR they will amplify only when a deletion of 4.5 kb is present, resulting in the band of 1.319 kb, while the normal control primers i.e. forward primer 5 TACAGCAGAGTGAGTGCTGCAT-3 and reverse primer 5-GGAGAAGTAGGTCTTCGTGGC-3 would yield a normal band of 1 kb in the same reaction. The PCR cycling conditions were same as for the detection of α3.7 kb.

Xmn1 genotyping (-158 Gγ t-c Xmn1 polymorphism) was carried out RFLP-PCR) (14). Primers amplified by a 641-bp fragment of DNA flanking the C-T polymorphism at -158 to the Gγ-gene was amplified by forward primer 5-GAACTTAAGAGATAATGGCCTA-3 and reverse primer: 5-ATGACCCATGGCGTCTGGACTAG-3 (Integrated DNA technologies, USA). The thermal cycling was carried out in ABI Veriti® 96 well thermocycler. The conditions set for thermocycling were: 95˚C for 1 minute (denaturation), 600C for 1 minute (annealing) and 720C for 1.5 minutes (extension). In the last cycle, final extension was performed at 370C with 10 units of pdmI (Xmn1) restriction enzyme (Fermentas Life Sciences, Vilnius, Lithuania). The products were electrophoresed on 2% agarose gel stained with ethidium bromide and the findings were recorded.

Single nucleotide polymorphism (SNP) of rs 4671393 (A) and rs 4671393 (G) in the BCL11A gene was detected by ARMS PCR [14,15]. A 548bp fragment was amplified using the following primers:

rs 4671393 (A) (F) 5′-CTG TGG ACA GCA AAG CTG CA-3′

rs 4671393 (A) (R) 5′-TCT CCC CCT TGC ATT GTT GTC-3′

rs 4671393 (G) (F) 5′- CCC CCA CTA GCT CAG AAA TGGA-3′

rs 4671393 (G) (R) 5′- GGG AAT CTT AAT TTC CTG CCC C-3′

Conditions set for thermocycling were: 95˚C initial denaturation followed by 94˚C 40 seconds (denaturation), 62˚C 30 seconds (annealing) and 72˚C 40 seconds (extension). Final extension was set at 72˚C for 5 minutes followed by a hold at 4˚C. Electrophoresis was carried out on 2% agarose gel.

## RESULTS

In total, 54 regularly transfused thalassemia patients, 26 (48.1%) female and 28 (51.8%) male, were screened for mutations in the α and β globin loci and for polymorphisms of BCL11A and Xmn1. All the patients belonged from district Peshawar of Khyber Pakhtunkhwa (KP) province of Pakistan. The mean age of study patients was 11 ± 3.7 years (range 3 to 15 years). Twenty three (42.6%) patients were in the normal Body Mass Index (BMI) range while 31 (57.4%) were underweight. Among the underweight

patients 27 (50%) were of age >10 years old. Age-at-diagnosis ranged from neonatal diagnosis to two years.

The mean total Hb level at diagnosis was 6.202 ± 1.5g/dl. The initial diagnostic Hb studies were performed on a post-transfusion sample among eight study patients. Diagnoses were established in these patients on the basis of circumstantial evidence obtained from family history of thalassemia and/or evidence of thalassemia trait in both the parents. HbF levels recorded in this set of patients ranged from 5.1% to 60%. In rest of the patients, HbF levels ranged from 70.0% to 98.5%

Gross enlargement of liver and/or spleen was observed in 23 (42.6%) patients; three (5.6%) had isolated hepatomegaly whereas four patients (7.4%) had isolated splenomegaly. Rest of the 16 (69.5%) patients had co-existing hepato-splenomegaly.

All the study patients were regularly transfused with packed red blood cells (pRBCs). Twenty-two (40.7%) patients were transfused every 10 days. A majority of the patients (n=28, 51.9%) were transfused every 11 to 20 days. Only four (7.4%) patients were transfused every 21 to 30 days. The total number of blood transfusions was calculated in the year of enrolment into the study. It was found that on the average 27 ± 11.1 transfusions were received by each of the study patients.

The mean serum ferritin level found in the study cohort was 7231.13 ± 5358.5 ng/ml. The minimum ferritin level found was 609.48 ng/ml while the maximum level was 29793.28 ng/ml. Among the study patients, eight (14.8%) never received any type of iron chelation therapy. Adequate chelation therapy was observed in only two (3.7%) patients whereas most (n=52, 96.3%) had inadequate iron chelation.

All 54 patients comprising were screened by allele specific PCR for mutations previously reported in native population. Of the 54 patients, 37 were homozygous $\beta^{\circ}/\beta^{\circ}$ and 17 were compound heterozygotes, i.e. β+/β+ or $\beta^{\circ}$/ β+, for the β-globin gene allele. Fr 8-9 was found to have been the most prevalent mutation (Table 2).

**Table 2:** Frequency of β-mutations in study patients.

| β-mutations | No of patients | Percentage (%) |
|---|---|---|
| Fr 8-9/Fr 8-9 | 19 | 35.2 |
| IVS1-5/IVS1-5 | 9 | 16.7 |
| IVS1-5/Fr 8-9 | 6 | 11.1 |
| Fr 41-42/Fr 41-42 | 5 | 9.3 |
| Cd5/Cd5 | 4 | 7.4 |
| Fr16/Fr16 | 2 | 3.7 |
| Fr 8-9/Cd5 | 2 | 3.7 |
| Fr 8-9/Fr 41-42 | 2 | 3.7 |
| IVS1-5/Fr16 | 1 | 1.9 |
| Fr 8-9/Del619 | 1 | 1.9 |
| Fr 41-42/Del619 | 1 | 1.9 |
| Cd15/Cd15 | 1 | 1.9 |
| Fr 41-42/Cap+1 | 1 | 1.9 |
| Total | 54 | 100 |

The study patients were further analysed for locally prevalent α-thalassemia mutations. A total of nine (16.66%) patients were found to have heterozygous αα/-$\alpha^{3.7}$ deletion, one patient carried homozygous -$\alpha^{3.7}$/-$\alpha^{3.7}$ while another patient had the αα/αααanti$^{3.7}$ mutation (Table 3).

**Table 3:** Frequency of α-mutations in study patients.

| α-mutations | No of patients | Percentage (%) |
|---|---|---|
| αα/-$\alpha^{3.7}$ | 9 | 16.7 |
| -$\alpha^{3.7}$/-$\alpha^{3.7}$ | 1 | 1.9 |
| αα/αααanti$^{3.7}$ | 1 | 1.9 |
| αα/αα | 43 | 79.6 |
| Total | 54 | 100 |

All the study patients were screened for BCL11A polymorphism. Thirty-eight patients (70.4%) had detectable G>A polymorphism; the rest (n=16, 29.6%) lacked BCL11A polymorphism. On the other hand, only two patients (3.7%) had heterozygous Xmn1 polymorphism; 52 patients (96.3%) lacked the polymorphism.

Among the 37 patients with $\beta^{\circ}/\beta^{\circ}$ genotype, eight carried a co-existing -α3.7 deletion, 25 had BCL11A polymorphism and only one carried Gγ- 158 XmnI polymorphism [+/-] (Table 4).

None of the patients with β+/β+ genotype (n=9) carried the -α3.7 deletion and XmnI polymorphism. Of the nine patients with the β+/β+ genotype, seven carried BCL11A polymorphism (Table 4). Of the eight patients with $\beta^{\circ}$/β+ genotype, three were heterozygous for -α3.7 deletion, six carried BCL11A polymorphism and only one had Gγ- 158 XmnI polymorphism [+/-] (Table 4).

**Table 4:** Coinheritance of α -thalassemia in multi transfused β-thalassemia patients.

| α -genotype | XmnI polymorphism | | | BCL11A polymorphism | | Total |
|---|---|---|---|---|---|---|
| | [-/-] | [+/-] | [+/+] | Detected | ND | |
| $\alpha^{\circ}$/ $\alpha^{\circ}$ genotype | | | | | | |
| αα / αα | 28 | 1 | - | 19 | 8 | 56 |
| αα /-$\alpha^{3.7}$ | 6 | - | - | 3 | 3 | 12 |
| α3.7/-$\alpha^{3.7}$ | 1 | - | - | 1 | - | 2 |
| αα / ααα anti$^{3.7}$ | 1 | - | - | 1 | - | 2 |
| Total | 36 | 1 | - | 24 | 11 | 72 |
| $\alpha^{\circ}$/ α+ genotype | | | | | | |
| αα /αα | 5 | - | - | 3 | 2 | 10 |
| αα /-$\alpha^{3.7}$ | 2 | 1 | - | 3 | - | 6 |
| Total | 7 | 1 | - | 6 | 2 | 16 |
| α+/ α+ genotype | | | | | | |
| αα /αα | 9 | - | - | 7 | 2 | 18 |
| Total | 52 | 2 | - | 37 | 15 | 106 |

Most of the patients (n=46, 85.2%), in the current study, had ameliorating factors. This advocates a higher prevalence of milder thalassemia phenotypes in local population. Different study parameters were compared with the ameliorating genetic factors in individual patients. Among those with an ameliorating factor, majority (n=26; 56.5%) were diagnosed after the age of five months (Table 5). In contrast, all the patients without an ameliorating factor were diagnosed before five months age.

Majority of the patients with ameliorating factors (n=28; 60.9%) had moderate anaemia at the time of diagnosis while only 18 (39.1%) had severe anaemia at time of diagnosis. In contrast, seven patients out of eight with no ameliorating factors had severe anaemia at time of diagnosis. Statistically significant interconnection was found

between haemoglobin at diagnosis and presence of ameliorating factors (p-value 0.019).

All eight patients lacking ameliorating factors required very frequently transfusions, i.e. at 10 days interval. In contrast, all of the patients with relatively better transfusion frequency (every 11-20 days and 21-30 days) had ameliorating factors. A statistically significant association was found between the ameliorating factors and frequency of transfusion (p-value 0.001).

Among the 46 patients with an ameliorating factor, equal number of patients (n=23) were found with and without visceromegaly. In contrast, seven out of eight patients lacking ameliorating factors had visceromegaly (Table 5).

Majority of the patients (n=35; 76.1%) with ameliorating factors had <90% HbF at presentation. Total 17 patients had HbF >90% at diagnosis. Of these 17 patients, 11 had ameliorating factors while six patients lacked an ameliorating factor. The association between HbF at presentation and ameliorating factors was statistically significant (p-value 0.009) favoring lesser HbF levels at diagnosis in patients with ameliorating factors.

**Table 5:** Ameliorating genetic factors in comparison to various clinical parameters in study patients.

| | | | | |
|---|---|---|---|---|
| **Age at diagnosis** | | | | |
| Upto 5 months | 20 | 8 | 28 | 0.015 |
| 5.1-11 months | 22 | 0 | 22 | |
| 11.1-24 months | 4 | 0 | 4 | |
| **Hb at diagnosis** | | | | |
| severe anaemia (<6 g/dl) | 18 | 7 | 25 | 0.019 |
| Moderate anaemia (6-10 g/dl) | 28 | 1 | 29 | |
| **Frequency of transfusion** | | | | |
| Upto 10 days | 16 | 8 | 24 | 0.001 |
| 11-20 days | 26 | 0 | 26 | |
| 21-30 days | 4 | 0 | 4 | |
| **Visceromegaly** | | | | |
| Yes | 23 | 7 | 30 | 0.063 |
| No | 23 | 1 | 24 | |
| **HbF at presentation** | | | | |
| < 90% | 35 | 2 | 37 | 0.018 |
| >90% | 11 | 6 | 17 | |
| Total | 46 | 8 | 54 | |

*p-value calculated by Fischer's exact test

## DISCUSSION

Thalassemia is a common monogenic disorder manifesting a wide spectrum of phenotypes [16]. Besides debilitating the haemopoietic system, thalassemia adversely affects other organs of the body as well and is associated with a variety of abnormalities and complications.

In this study, the most frequent contributor to transfusion dependent β-thalassemia was found to be homozygous or compound heterozygous inheritance of β° mutations (β°/β°). Accordingly, in our neighboring country Iran, β° mutations was found to be frequent in comparison to β+ in thalassemia intermedia patients [17]. In the current study, the most frequent β-mutation was homozygous Fr 8-9 (35.2%) followed by homozygous IVS1-5 (16.7%), compound heterozygous IVS1-5/Fr8-9 (11.1%), homozygous Fr 41-42 (9.3%) and homozygous Cd-5 (7.4%), in respective order. The most commonly found α-globin gene mutation in our study patients was –α3.7 deletion (11/54). It is well known that the co-inheritance of α-thalassemia with β-thalassemia minimizes the disease severity [18].

In the present study, only two patients (3.7%) had Xmn1 polymorphism (+/-) one with β°/β° genotype while the other had β°/β+ genotype. This is consistent with the findings of Verma and coworkers that demonstrated a similar pattern among 325 TI patients from different ethnic backgrounds in the Mediterranean region and Asia and concluded that this polymorphism is the commonest ameliorating factor in cases with β° mutations but not β+ [19].

Majority of the patients from current study (n=38, 70.3%) had BCL11A polymorphism. The result was in agreement with a study conducted on Sardinian population [20]. The same study also elucidated that variants at BCL11A locus influence HbF levels, modulating the clinical phenotype of β-thalassemia favourably [20].

Inheritance of β+ alleles in homozygous or compound heterozygous state in 31.4% of the patients in current study advocate milder phenotypes in these patients. Co-inheritance of α3.7kb deletion in 20.3% of the patients is another major ameliorating factor. About 3.7% of the patients have co-existing Xmn1-polymorphism in heterozygous state, which also ameliorates the disease phenotype. In addition, 70.4% of the patients have co-inherited BCL11A-polymorphism which is a known stand-alone ameliorating factor. All these findings suggest a milder phenotype of the disease. Transfusion dependency in the study patients, however, do not agree with the scenario. It rather advocates a severe phenotype. An explanation to this discrepancy may be the occurrence of intercurrent infections in settings of thalassemia intermedia mimicking picture of thalassemia major. This is more likely in patients from poor families and from regions with dearth of health care facilities. Pakistan, being a low-middle income country, is facing these issues at large. A careful clinical assessment of the disease is hence advised before categorically declaring a patient thalassemia major.

## CONCLUSION

The current study affirms a high frequency of genetic ameliorating factors among the transfusion dependent β-thalassemia patients from district Peshawar of Pakistan. Presence of these factors favours a milder disease phenotype and warrant accordingly management. Genetic screening for these ameliorating factors and their response to different treatment strategies in larger study groups is suggested for definitive recommendations.

## CONFLICT OF INTEREST

There are no conflicts of interest to disclose from author.

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