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Short Communication

Molecular Heterogeniety of β-Thalassemia in Karak District, Khyber Pakhtunkhwa, Pakistan

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SR designed project, performed experimental work. RMK and MA did sample collection and clinical diagnosis. JK did manuscript writing and helped in experimental work. ZM did statistical analysis and experimental work.

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ABSTRACT

β-Thalassemia (β-thal) is a monogenic disease characterized by mutations on the HBB gene, affecting the production of globin that results in hypochromic and microcytic anemia. The objective of this study was to determine the prevalence of six common β-thal mutations, their frequency, consanguinity in parents and inheritance pattern in patients of Karak region, Khyber Pakhunkhwa (KP) province, Pakistan. During the study, 200 peripheral blood samples were collected both from families having at least one transfusion dependent child and sporadic patients from different areas of karak region. Using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique, all samples were analyzed for the most common β-thalassemia mutations reported in Pakistani population. The most common mutations detected in karak region were frameshift codons (FSC) 8/9 (þG) (HBB: c.27_28insG), followed by IVS-I-5(G>C), FSC 5 (–CT) and Codon 15 (G>A). The present study hence showed differences with previous results from other regions of the Pashtun ethnic group, which demarcates the heterogeneity in mutations found in the Pashtun ethnicity. These observations may help in arranging counseling about disease recurrence in future, large scale mutation screening and PND not only for Kohat region but also the whole community of KP province. Such study could provide valuable information regarding thalassemia prevention like prenatal diagnosis (PND), genetic counseling and carrier screening for controlling the affected births in the population. Consanguinity, lack of awareness and non-availability of health facilities have thus contributed to the high occurrence of β-thal and miserable life of the patients.

Beta-thalassemia (β-thal) is an autosomal recessive genetic disorders, characterized by mutation in β-globulin gene clusters positioned at the short arm (p) of chromosome number 11 band 11p15.4 - 1p15.5. The disease mainly results from a variety of molecular defects including point mutations, insertion of stop codons in mRNA encoding the β-thal genes (Chen *et al*., 2010). The remarkable clinical features associated with heterozygous β-thal carriers include hypochromia, microcytosis, increased Hb A_2 and an unbalanced α/β-globin chain synthesis ratio (Laiska *et al*., 2016). More than 400 different mutations have recently been detected disturbing the diverse levels of β-globin gene expression and causing β-thal in which 35 mutations are predominant in Pakistan (Jalilian *et al*., 2017). Despite the fact that these mutations are not equally distributed, but have a geographical specificity and racial origin, each is identified by the presence of some common mutations and variable numbers of rare ones (Hussain *et al*., 2017). Globally, β-thal is one of the most important autosomal single gene disorders and in around more than 60 countries it can be found with a carrier population of up to 150 million (Kountouris *et al*., 2014). In Pakistan, the carrier rate in different ethnic groups has been estimated to be around 5.0-7.0%. Over 9000 children inheriting homozygous β-thal are born every year (Khan *et al*., 2018). The present study was aimed to assess the prevalence of the six common β-thal mutations in the district Karak of KP Province, their inheritance pattern and its relation to consanguinity. Moreover, this study can support public health efforts such as carrier screening, genetic counseling and prenatal diagnosis to control affected births by screening the population for

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the presence of common selected mutations through the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique.

Materials and methods

The current study was undertaken in the Molecular Laboratory of the Department of Biotechnology, University of Science and Technology Bannu, KP, Pakistan. Different demographic parameters including sex, age, clinical symptoms and family history of β-thal with important pathological parameters were collected on a study-designed proforma (Arif *et al*., 2008). A written consent was obtained from patients and their parents. In total, 200 blood samples were collected from 110 unrelated families having at least one affected blood transfusion-dependent β-thal [Cooley's anemia or β-thal major (β-TM)] child (Ahmed, 2016). The study was permitted by the Ethnic Board of the Thalassemia Center and Bannu University review board. The participating patients in the study were blood transfusion-dependent and diagnosed as β-TM patients through peripheral blood morphology and Hb electrophoresis Laboratory tests (Shakeel *et al*., 2016; Ahmed *et al*., 2000). Most of the families were inbred and patients were born due to consanguineous marriages. Socioeconomic status of the non-consanguineous families was more satisfactory than consanguineous (Woods *et al*., 2006). The genomic DNA from the collected blood samples was isolated using standard protocol (Sambrook, 1987). Screening the samples was done using tetra primer based allele specific PCR (Newton *et al*., 1989). Two PCR reactions were done either with allele specific primer or control primers (Table I). To perform PCR reaction, 100 ng genomic DNA, 200 µM of each dNTP, 1 unit of Taq polymerase, 10 pmol of each primer i.e. two primers for control fragment and two primers for each of the mutant or control allele and 1x Taq reaction buffer were used in 20 µ reaction volume. The reaction was done through 27 cycles, consisting of denaturation at 94°C for one minute, annealing at 65°C for one minutes and extension at 72°C for 1 minute and 30 seconds. Denaturation during the first cycle was done at 95°C for 6 minutes while the final extension was done at 72°C for 10 minutes. Gel electrophoresis of PCR product was done on 3% agarose gel, containing ethidium bromide for visualization. Hind III digest was used as a marker. β-globin genotypes were assigned on the basis of presence or absence of allele specific bands. The previously characterized samples and dH_2O were used as positive and negative controls respectively.

Results

DNA from 200 individuals was screened for the presence of six β-thal mutations reported in Pakistani population. Only four point mutations; FSC 8/9 (HBB: c.27_28insG), IVS-1-5 (G>C) (HBB: c.92+5G>C), FSC 5 (-CT) (HBB: c.17_18delCT) and codon 15 (G>A) (HBB: c.47G>A) were found to be the most common in 181 patients (Table II). Nineteen patients remained uncharacterized for

Table I. The amplification refractory mutation system-polymerase chain reaction allele specific primers used in this study.

Primers	Sequences (5'>3')	Product size (bp)
Control A	CAA TGT ATC ATG CCT CTT TGC ACC	861
Control B	GAC TCA AGG CTG AGA GAT GCA GGA	861
Common C	TCA CTT AGA CCT CAC CCT GTG GAG CCA C	-
Codons 41/42 (Mt)	GAG TGG ACA GAT CCC CAA AGG CCT TGT TAG	439
Codons 41/42 (N)	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	-
IVS-1-5 (Mt)	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	285
IVS-1-5 (N)	CTC CTT AAA CCT GTC TTG TAA CCT GAT ACG AAA	-
IVS-1-1 (Mt)	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA	280
IVS-1-1 (N)	GAT GAA GTT GGT GAC GCC CRG GGT AGG	-
FSC 8/9 (Mt)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	215
FSC 8/9 (N)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT	-
Codon 15 (Mt)	CAC CAA CTT CAT CCA CG5 TCA CCT TGG CCT	500
Codon 15 (N)	CAC CAA CTT CAT CCA CGT TCA CCT TGG CCC	-
FSC (Mt)	ACA GGG CAG TAA CGG CAG ACT TCT ACT CG	170
FSC (N)	ACA GGG CAG TAA CGG CAG ACT TCT CAT CAG	-

IVS, Intervening sequence; FSC, frameshift codon; Mt, mutant; N, normal.

Table II. The frequency of mutant alleles in the studied individuals.

Serial number	HbVar nomenclature	Mutations	Homozygous	heterozygous	Total no. of mutant alleles (n)	Frequency (%)
1	HBB: c.27_28insG	FSC 8/9 (+G)	34	57	125	49
2	HBB: c.92+5G>C	IVS-I-5(G>C)	21	32	74	29
3	HBB: c.17_18delCT	FSC 5 (–CT)	13	11	37	14.5
4	HBB: c.47G>A	Codon 15 (G>A)	06	07	19	7.5
Total no (%)			74	107	255	100.00%

known 22 mutations reported so far in Pakistani community (Table II). Of the 181 characterized samples, 74 (40.88%) were found to be homozygous for four mutations and 107 (59.12%) were heterozygous for the same mutations. The frame-shift mutation at codon 8/9 (+G) was found to as the most prevalent mutation (49%), followed by IVS-I-5(G>C) (29%), codon 5 (-CT) (14.5%) and codon 15 ((G>A) (7.5%) (Table II). A total of 255 mutant alleles observed for the above mentioned four mutations. Of the 74 homozygous patients 34 (45.9%) had codon 8/9 (+G) mutation, 21(28.3%) possessed IVS-I-5(G>C), and 13 (17.6%) and 6 (8.1%) were homozygous for FSC 5 (–CT) and codon 15 (G>A) respectively (Table II). Similarly, maximum number of patients (57=53.3%) were found with codon 8/9 (+G) mutation out of 107 compound heterozygous thalassemia patients, followed by IVS-I-5(G>C) (29.9%), codon 5 (–CT) (9.4%), and only 6.5% patients with Codon 15 (G>A) mutation.

Moreover, out of 200 patients, 40 (20.0%) were reported to be over 15 years old, 110 (55.0%) patients demonstrated early onset of disease, i.e. ≤5 years old, and 50 (25.0%) patients were >10 years old. Furthermore, the study also showed 109 (54.5%) patients with poor socioeconomic status, having a monthly income of <20,000 PKR. The parents of 89 (44.5%) patients were first cousins, 84 (42.0%) were related but not first cousins, and 27 (13.5%) were not related. The above mentioned data suggests consanguinity as the main driving force behind the inheritance of β-thalassemia in KP province.

Discussion

Approximately 270 million carriers of hemoglobinopathies including 80 million β-thal have been estimated worldwide. Reviewing the previous reports, it can be concluded that 300,000–400,000 children with abnormal HbS including 23,000 β-thal are born every year (Shakeel *et al*., 2016). In Pakistan, β-thal is very common with an estimated inheritance rate of 5.0–7.0%, and over 9000 homozygous β-thal cases are born every year (Ansari *et al*., 2011; Ahmed *et al*., 2000). About 35 β-thal mutations have been reported in the country (Woods *et al*., 2006). In every population or ethnic group, usually four to seven mutations are reported to be common, comprising nearly 90.0% of all β-thal cases (Woods *et al*., 2006). The frequency of homozygosity in Pakistan relates to certain aspects, the notable of which are frequent first-cousin marriages, increase in annual birth rates, contact with mutagens and migration of highly conserved and consanguineous Afghani population to Pakistan (Khan *et al*., 2015, 2019). Moreover, the increase in annual birth rate in Pakistan accelerates the number of homozygous thalassemic patients. From the Indian subcontinent, nearly 40 β-thal mutations have been identified, of which five, i.e. FSC 8/9 (+G), IVS-I-5 (G>C), codons 41/42 (-TCTT), IVS-I-1 (G>T) and FSC 5 (-CT), are predominant (Khan *et al*., 2018; Arif *et al*., 2008). It has been reported that the existing treatment strategies (i.e. chelation therapy, bone marrow transplantation, blood transfusion) may not eliminate the disease due to its high treatment cost and required conditions (Ahmed, 2016; Ahmed *et al*., 2000). The reasons for the numerous birth rate of β-thal patients and carriers in developing countries include lack of public awareness and lack of facilities in the form of thalassemia centers, molecular diagnosis, premarital screening and prenatal screening (Ahmed, 2016; Ahmed *et al*., 2000). This study reports four common mutations in the local population, which are in agreement with previous findings (Ansari *et al*., 2011; Khan *et al*., 2015, 2019). Most Pashtuns prefer inter-family marriages. Interestingly, IVS-I-5(G>C) was reported as the second most common mutation in the region, which is contrary with the what has previously been reported (Khan *et al*., 2015, 2019). The above contradiction can be correlated to regional distribution of the Pathan tribes containing a large number of small tribal groups, unequal sample size and sample sites. The other reason for contradiction could be that the area is comparatively closer to the Afghan border and a large population has migrated here during the Russian invasion of Afghanistan.

Conclusion

Consanguinity and lack of awareness and non-

availability of health facilities have contributed to the high occurrence of β-thal and miserable life of the patients. All the patients must be molecularly characterized before first transfusion.

Statement of conflict of interest

The authors have declared no conflict of interest.

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