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Short Communication

Relationship of Frequency of IVS 1-5 (G-C), Fr 8-9 (+G), Fr 41-42 and cd-5 Mutations of β-Thalassemia Traits with Red Cell Indices

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MW and HJ collects all samples, performed the experiments and wrote the article. HSM and UQ supervised the research work. NA and AH prepared the tables and the initial manuscript.

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ABSTRACT

A descriptive study was carried out to assess the mean ranges of red blood cell (RBCs) indices in β-thalassemia carrier patients with frequency of different common and uncommon/uncharacterized mutations that are IVS 1-5 (G-C), Fr8-9 (+G), Fr 41-42 and Cd 5 mutations of β-thalassemia traits. A total 293 β-thalassemia carrier patients were included in study. Fr 8-9 (+G) was found the most common mutation of β-thalassemia carrier patients in the population which was 112 (38.2%), followed by IVS1-5 (G-C) which was 82 (28%), Fr41-42 which was 24 (8.2%), Cd 5 which was 20 (6.8%) and others which were 55 (18.8%) patients. The IVS 1-5 (G-C) was the most common mutation in Punjabis while Fr 8-9 (+G) was the most common mutation in Pathan community. Lowest hemoglobin (Hb) level was found in IVS 1-5 mutations of β-thalassemia carrier patients, while the average Hb in β-thalassemia carrier patients was 11.54 but it also varied gender-wise, different ethnic origin and in different mutation. The mean MCV was 62.94fl, MCH 19.46pg, MCHC 30.74g/dl and RDW 39.06fl. The final findings showed that IVS 1-5 (G-C) was the most common mutation in Punjabis and Fr 8-9 (+G) is the most common mutation of Pathan community. Lowest Hb was found in IVS 1-5 Mutation of β-thalassemia carrier patients.

Pakistan has a Community of more than 160 million people with an overall carrier frequency of approximately 5.6% for β-thalassemia (Ahmed *et al*., 2002). Punjab is the largest province of the country with more than 50% of the population. The state of β-thalassemia is alarming as consanguinity rate is very high (>81%) and the literacy rate is low in South Punjab. It is observed that 58% of the siblings of β-thalassemia major children in Hazara division of Pakistan had β-thalassemia trait (Weatherall and Clegg, 2001).

More than 200 different mutations have been described in patients with β-thalassemia and related disorders such as the hyper unstable structural mutations with thalassemic phenotype in the world (Thein, 2004). Although most β-thalassemia mutations (substitutions, deletions or insertions) involve either a single or a few nucleotides, large deletions taking away part, the entire or multiple genes (Li *et al*., 2008). About 20 mutations account for 90% of β-globin genes in the world, and it is noted that each ethnic population has its own unique set of most most frequent mutations (Ansari *et al*., 2012). Assessment of red cell parameters an important laboratory investigation in the diagnosis process of thalassemia (Rund *et al*., 1992; Shen *et al*., 2010). The present investigation attempts to establish relationship between frequency of β-thalassemia mutations with red cell indices.

Materials and methods

Cross sectional descriptive design was used in this study. Inclusion criteria were known carriers of β-thalassemia whose mutations had been characterized by polymerase chain reaction (PCR) using Multiplex ARMS. Patients with beta thalassemia major or homozygous patients and iron deficient patient were excluded during study. Total 293 patients (149 males and 144 females) were included during this study period, of these 145 (49.5%) were Punjabis, 129 (44%) pathans, 7 (2.4%) Saraiki and 12 (4.1%) belonged to others ethnic groups. Out of 145 Punjabis 74 were males and 71 were females, out of 129 Pathans 65 were males and 64 were females, out of 7

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Saraiki 4 were males and 3 were females.

Blood samples (3ml) were collected in a tube containing EDTA from the patients received at Armed Forces Institute of Pathology, Rawalpindi. Complete blood counts of all the samples were determined by automated cell counter (Sysmex KX21) (Ansari *et al*., 2012).

PCR method known as amplification refractory mutation system (ARMS) was used for the detection of different mutations. Chelex 100 method was used for DNA extraction. Polar resin beads bind polar cellular components after breaking open cells. Non-polar nuclear DNA and RNA remain in water solution above chelex.

The target DNA was amplified using the primer complementary to the β-chain mutations to establish the homozygous and heterozygous state of the patient. Samples giving positive reaction with the normal primer were then tested for other mutations. Another set of primers was used to amplify an 861bp fragment of the distal part of the β-globin gene, to serve as an internal control for PCR (Table I).

PCR for the β-chain mutations was carried out using 2µl of DNA. The amplification on the thermal cycler was carried out consisting of 25 cycles, each of denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min and DNA extension at 72°C for 2 min. In the final cycle the extension was prolonged for another 3 min. The amplified product was visible on 6% polyacrylamide gel. The sequence of the primers used for the detection of mutations and the normal primers are given in Table I.

As more than one mutation may be screened at the same time in a single PCR reaction (Multiplex PCR), provided the ARMS primers were coupled with the same common primer. The multiplex method of ARMS was accomplished by mixing several primers in the same primer mixer. The mixing was organized according to the product size.

The three multiplex primers AD-I, AD-2 and AD-3 were prepared as stock solutions containing 5pmol/µl each of all the primers for the mutant alleles. In addition, the two control primers and respective common primer were also added to the stock mixture. In each ARMS reaction 1µl of the stock primer was used. Mutations can be easily identified by resolution on PAGE because the sizes of the amplified products are sufficiently different from each other. There is no difference between IVSI-5 and Cd-30 (G-C and G-A). IVSI-I primer was added to AD-1 and AD-2 groups, whereas IVSI-5 was added in AD-1. Codon 30 was added in AD-2 and therefore amplification with AD-2 but not AD-1 indicated Cd-30. Same primers were used for the detection of the homozygote and heterozygote for Cd30 and IVSI-I. AD-3 includes the primers combination of Cd-15 and Cap +1. A control allelic ladder was also prepared. Each fragment was amplified in 50µl reaction mixture under standard ARMS condition. The individually amplified product were pooled and was used as reference ladder. Documentation of the amplified product was carried out on PAGE.

Results and discussion

Tables II, III and IV show the mean RBCs count and other hematological parameters in different ethnic groups and mutations.

Results of the current study showed that Fr 8-9 (+G) is the most common mutation of β-thalassemia carriers patients of Pakistani population which is 112 (38.2%) followed by IVS1-5 (G-C) which is 82 (28%), Fr41-42 which is 24 (8.2%), Cd 5 which 20 (6.8%) and other mutations which is 55 (18.8%).

About 4.5% of the population in Malaysia have been identified as heterozygous carriers for β-thalassemia, particularly among 53.5% of the population who are Malays (George, 2001; Tan *et al*., 2004).

Table I. The sequences of primers used for the identification of mutations by ARMS method and normal alleles of respective mutations.

Mutations	Primers	Fragment sizes
Identification of mutations		
IVS I-5 (G-C)	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	285
Fr 8-9 (+G)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	215
Fr 41-42	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	439
cd-5	ACA GGG CAG TAA CGG CAG ACT TCT CCG CGA	205
Identification of normal alleles of mutations		
IVS I-5	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC	285
Fr 8-9	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT	214
IVSI-I	GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG	450
Fr 41-42	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	443

Table II. Mean RBCs and Hemoglobin in male, female.

Hematological parameters	Male (n=149)	Female (n=144)
RBCs (RBCs×10^{12})	6.52	5.23
Hemoglobin (g/dl)	12.73	10.31

Table III. Hematological parameters in patients with different mutations. These all values lie with in 2SD.

Hematological parameters	IVS1-5 (G-C) (n=82)	Fr8-9 (+G) (n=112)	Fr41-42 (n=24)	Cd5 (n=20)	Others (n=55)
RBCs (RBCs×10^{12})	5.67	6.04	5.88	6.00	5.85
Hemoglobin (g/dl)	11.40	11.55	11.46	11.72	11.69
MCV (fl)	63.95	61.92	62.96	61.95	63.86
MCH (pg)	19.97	19.01	19.33	19.20	19.75
MCHC (g/dl)	30.95	30.60	30.47	30.58	30.88
RDW (fl)	39.38	38.83	40.66	37.78	38.80

MCV, mean corpuscular colume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution.

Table IV. Hematological parameters in different ethnic groups.

Hematological parameters	Punjabi (n=145)	Pathan (n=129)	Saraiki (n=07)	Others (n=12)
RBCs (RBCs×10^{12})	5.82	5.97	5.88	5.64
Hemoglobin (g/dl)	11.42	11.66	12.17	11.35
MCV (fl)	62.99	62.85	65.04	62.19
MCH (pg)	19.41	19.44	20.51	19.55
MCHC (g/dl)	30.66	30.77	31.12	31.15
RDW (fl)	39.35	38.68	41.17	38.35

For abbreviations, see Table III.

Serum ferritin estimation in carriers of β-thalassemia trait is important (Saleem *et al*., 1995). Two forms of hypochromic microcytic anaemia i.e. iron deficiency and β-thalassemia trait is common in Pakistan (Ansari *et al*., 2012). Iron deficiency was found in 9% while β-thalassemia was seen in 3% in the study carried out by Afroz *et al*. (1998). Ratio between the percentages of microcytic hypochromic cells as a screening test is a sensitive index which can be used for mass screening of β-thalassemia trait particularly in a population where iron deficiency is also prevalent (Saleem *et al*., 1995). Thalassemia trait is found in 11% of the Pakistani population (Molla *et al*., 1992). Khattak and Saleem (1992) reported the incidence of 5.4%. In Pathans it was 7.96% while in Punjabis it was 3.26%. Most frequent mutations were IVS-1-5 (G-C), Fr 8/9 (+G), IVS-1-1 (G-T), Fr 41/42 (-CTTT) and the 619bp deletion at the 3' end of the gene. Mutations at IVS-2-1 (G-A) and codon 30 (G-C), previously were described in Asian Indians in 1991 (Varawalla *et al*., 1991b). Five most common mutations identified in Pakistan are IVS1-5 (G-C), IVS1-1 (G-T), Fr 41-42 (-TTCT) Fr 8-9 (+G) and deletion 619bp (Khateeb *et al*., 2000).

The results confirm and extend earlier findings for Thailand, Pakistan, India, Mauritius and Syria. Two novel mutations were identified, codon 55 (-A) and IVS-I-129 (A-C), both found in Sri Lankan patients. Two beta-thalassemia mutations were found to coexist in one beta-globin gene: Sri Lankan patients homozygous for the beta codon 16 (-C) frameshift were also homozygous for the beta+ codon 10 (C -A) mutations.

Table V. Correlation of the frequency of different mutations in different ethnic groups.

Mutation	Punjabi	Pathan	Saraiki	Others
IVS1-5	51 (35.2%)	26 (20.2%)	3 (42.9%)	2 (16.7%)
Fr8-9(+G)	46 (31.7%)	56 (43.4%)	3 (42.9%)	7 (58.3%)
Fr41-42	14 (9.7%)	9 (7.0%)	0	1 (8.3%)
Cd5	4 (2.8%)	14 (10.9%)	0	2 (16.7%)
Others	30 (20.7%)	24 (18.6%)	1 (14.3%)	0
Total	145	129	7	12

Studies of Sri Lankan, Pakistani, and Indian carriers suggested that codon 10 (C -A) mutations was a rare polymorphism on an ancestral allele in which β-codon 16 (-C) mutation has arisen. Each country was found to have only a few common mutations accounting for 70% or more of the β-thalassemia alleles (Primrose *et al*., 2001). For β-thalassemia a gene over 4000 homozygotes are born each year in Pakistan. β-thalassemia alleles from five major ethnic groups of Pakistan have been characterized. The complete spectrum comprises of 19 mutations. There are important ethnic and regional differences in the prevalence of mutations. The five most common mutations, IVSI-5 (G-C) (37.3%), Fr 8-9 (+G) (25.9%), del 619 (7.0%), Fr 41-42 (- TTCT) (6.7%) and IVSI-1 (G-T) (5.4%), constitute 82.3% of the total thalassemia population in Pakistan. Fr 8-9 (+G) is the most common mutation in Northern Pakistan (41.3%), whereas IVSI-5 (G-C) is the most frequent mutation in Southern Pakistan (52.2%). A novel 17bp deletion involving Cd126-13 was also identified (Qurat-ul-Ain *et al*., 2011). The IVS-I-5 (G-C) Asian Indian mutation was the most frequent mutation reported from United Arab Emirates (Rund *et al*., 1992) Determination of β-globin gene haplotypes in North-west Pakistan, Gujarat, Punjab and Sindh suggest that high frequency alleles i.e. intervening sequence 1 (IVS-1) nucleotide 5 (G-C) and Fr 41-42 (-CTTT) are older mutations as determined by multiple haplotype associations and widespread geographical distribution. Micro epidemiology of beta-thalassemia in this region

reflects considerable ethnic diversity, gene flow from population migration and natural selection by malaria infection (Varawalla *et al*., 1991a).

β-globin gene is present on the short arm of chromosome 11 in a cluster with the other Beta like genes. There are more than 200 mutations causing β-thalassemia. With exception of a few deletions, vast majority of β-thalassemia are caused by point mutations within the gene or in its immediate flanking sequences (Thein, 2005). The degree of globin chain imbalance is responsible for the pathophysiologic features of thalassemia syndromes. Globin chain imbalance is maximum in case of β-thalassemia major and minimal in case of silent β-thalassemia (Thein, 2004). The heterozygous state of β-thalassemia shows a tremendous phenotypic diversity. In typical Beta0 or severe Beta+ thalassemia, the alleles demonstrate lower Hb, MCV and MCH values while in some carriers the β-thalassemia is so mild that it is phenotypically silent with no anemia or hematological abnormality (Bianco *et al*., 1997).

Conclusion

Assessment of red cell parameters on complete blood count is the first and an important laboratory investigation in the diagnosis of thalassemia. Our results for the frequency of β-thalassemia mutations are in agreement with other studies across the country. The final findings show that IVS 1-5(G-C) was the most common mutation in Punjabis and Fr 8-9(+G) is the most common mutation in Pathan community. Lowest Hb is found in IVS 1-5 mutation of β-thalassemia carrier patients.

Conflict of interest statement

The authors have declared no conflict of interest.

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