European Journal of Medical Genetics 59 (2016) 355–362

Contents lists available at ScienceDirect

European Journal of Medical Genetics

journal homepage: http://www.elsevier.com/locate/ejmg

The molecular characterization of Beta globin gene in thalassemia patients reveals rare and a novel mutations in Pakistani population

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ARTICLE INFO

Article history:
Received 23 September 2015
Received in revised form
21 May 2016
Accepted 27 May 2016
Available online 1 June 2016

Keywords:
Pakistan
Beta globin gene
Hb Monroe
Multiplex GAP-PCR
XmnI polymorphism

ABSTRACT

Introduction: A multicentre study (including four cities in Pakistan) aimed to investigate the frequency and spectrum of alpha and beta thalassemia genetic mutations and XmnI polymorphism of the Gamma Globin gene.

Methods: One hundred and sixty one beta thalassemia patients, identified on the ground of haematological parameters, were screened for mutations of the Alpha (*HBA2* and *HBA1*) and Beta (*HBB*) Globin genes as well as Gamma (*HBG2*) Globin gene, -158 Gγ XmnI polymorphism, using a combination of multiplex GAP polymerase chain reaction (PCR), Sanger sequencing and restriction fragment length polymerase (RFLP) based PCR.

Results: Mutations of at least one *HBB* gene was identified in 157 of 161 patients screened. Among 16 identified mutations in the beta gene, *HBB*:c.27_28insG (p. Ser10Valfs*14) was the most prevalent. $\alpha^{-3.7}$ and $\alpha^{-4.2}$ deletions were co-inherited with beta thalassemia mutations. Rare mutations such as *HBB*:c.−138C > T and *HBB*:c.315 + 1G > A were also identified. One novel variant (*HBB*:c.-148T > A), two rare mutations [*HBB*:c.332T > C (p.Leu111Pro); *HBB*:c.92G > C (p.Arg31Thr] and a novel association, *HBB*:c.[92G > C (p.Arg31Thr)] and [-92C > G], were reported for the first time in our study. *HBG2*:c.-211C > T base-pair substitution (historically described as -158 GγXmnI polymorphism) was present in 36% of the patients.

Conclusion: Heterogeneity in clinical and haematological parameters in TM, show that monogenic disorders can present with a wide spectrum of disease severity. Our studies identified rare and novel mutations that will be useful in the prevention of highly prevalent disease of thalassemia in Pakistan following nationwide awareness campaign.

1. Introduction

Thalassemia is a quantitative deficiency of functional α- and β-like globin chains synthesis that leads to ineffective erythropoiesis and reduced hemoglobin synthesis. Increased gastrointestinal iron absorption, following frequent blood transfusions in the absence of proper chelation therapy, builds iron overload leading to increased morbidity and mortality and shorten life expectancy (Teh et al., 2014). More than 200 mutations have been identified that impair transcription, processing or translation of α- or β-globin mRNA. Twenty percent of all haemoglobin disorders are thalassemia, of which 90% occur in low or middle income countries (Jouini et al. 2013; Sharma et al., 2010). Despite being classified as a monogenic disease, phenotypic variations in thalassemia are linked to other factors. For instance, the amount of beta chain production defines the phenotypic response and is referred to as β^0, β^+, β^{++}. However, coinheritance of an alpha mutation and presence of certain polymorphisms also contribute to its phenotypic diversity. One third of genetic variance is associated with the *HBG2*:c.-211C > T base-pair substitution (historically described as -158 GγXmnI polymorphism) while, more than 50% genetic variance is caused by non-linked Beta globin gene genetic factors (Sharma et al., 2010).

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http://dx.doi.org/10.1016/j.ejmg.2016.05.016
1769-7212/

Thalassemia is considered as a major public health problem in Pakistan, with a carrier rate of approximately 5%. Over 5,000 new cases of beta thalassemia major (TM) are diagnosed annually. Among approximately 182 million people, there are six major ethnic groups in Pakistan: Punjabi (53.5%), Pashtun (Pathan) (15%), Sindhi (14%), Muhajirs (7.5%), Balochi (3.5%) and other (6.5%). The samples included in this study were collected from three provinces and four cities: Punjab (Lahore and Multan), Sindh (Karachi) and Khyber Pakhtunkhwa (KPK) (Peshawar). In Pakistan, previous studies have shown that six mutations [HBB:c.27_28insG (p.Ser10Valfs*14), HBB:c.92+5G > C, HBB:c.126_129delCTTT (p.Phe42Leufs*19), NG_000007.3:g.71609_72227del619, HBB:c.47G > A (p.Trp16*) and HBB:c.92+1G > T] account for more than 90% of all thalassemia genotypes (Moatter et al., 2012). In the absence of an effective treatment, (except for expensive bone marrow transplantation), thalassemia has a substantial impact on quality of life and health care in Pakistan.

Epidemiological data about prevalence and distribution of mutations plays a vital role in the establishment of any effective management approach. Although, prior studies have played major part in establishing the occurrence and incidence of the six most common mutation panel (Ahmed et al., 1996; Ansari et al., 2012; Moatter et al., 2012) an update of their incidence is of operational importance. To this aim, we used Sanger sequencing to investigate mutations following PCR amplification of the *HBB* gene, we analysed the seven most common Alpha Globin deletions by GAP PCR and used restriction fragment polymorphism for the analysis of the *HBG2*:c.-211C > T base-pair substitution (XmnI polymorphism). The latter has not been previously reported as a part of any investigation of TM in the Pakistani population.

2. Material and methods

2.1. Patients' data

A total of 350 transfusion dependent beta thalassemia index samples were collected from four centres in different regions of Pakistan (e.g. 162 from Lahore, 64 from Multan, 71 from Karachi and 53 from Peshawar) between December 2011 and December 2013. All individuals had classical beta thalassemia blood features including hypochromic microcytic anemia [mean haemoglobin (Hb) 8 g/dl, mean corpuscular volume (MCV) 65 fL, mean corpuscular haemoglobin (MCH) 36 pg]. This study was carried according to the code of ethics of World Medical Association (Declaration of Helsinki). Approval for this study was obtained from Ethics committee of School of Biological Sciences, the University of the Punjab, Lahore, Pakistan. Informed written consents were obtained from the participants of this study before sample collection.

2.2. Haematological data and other findings

Peripheral blood counts and red blood indices were measured using Sysmex KX-21N™ automated blood cell analyser. Detailed clinical history including age of first blood transfusion (FBT), blood transfusion frequency, degree of organomegaly, levels of HbF, HBA, HBA_2 were obtained retrospectively in addition to clinical proforma. Participants were categorized into three socioeconomic status depending upon their monthly income: poor socioeconomic status with monthly income <10,000 PKR, average socioeconomic status with monthly income 10,000–20,000 PKR and higher socioeconomic status with monthly income >20,000 PKR.

2.3. Primary screening

All subjects were screened for the presence of Hepatitis B virus surface antigen (HBsAg), antibodies to Hepatitis C virus (HCV) and Human Immunodeficiency virus (HIV) using one step device (Accu-check®) following the manufacturer's guidelines. Participants positive for any of these were excluded on ground of safety concerns imposed by departmental and institutional ethical committees.

2.4. DNA isolation

Genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instructions.

2.5. Sanger sequencing for HBB gene

The *HBB* gene including 5′ and 3′ untranslated region was analysed for beta thalassemia mutations using Sanger sequencing following the amplification of three fragments, using previously published primers (Old, 2003). Samples were checked for the correct amplification by QIAGEN Qiaxcel and then subjected to sequencing following the manufacturers' instruction for the 3500×L genetic analyser. Sequencing data was checked using the Applied Biosystems SeqScape Software v2.7. GAP PCR was used to screen NG_000007.3:g.71609_72227del619 as it remained undetected by Sanger sequencing (Varawalla et al., 1991).

2.6. GAP-PCR for alpha thalassemia deletion mutation

Multiplex GAP PCR was applied to investigate seven common alpha thalassemia deletion ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $-\alpha^{20.5}$, $-^{SEA}$, $-^{FIL}$, $-^{MED}$ and $-^{Thai}$ deletions). They were run in parallel with positive controls for each of the mutations (Chong et al., 2000; Liu et al., 2000). For the purpose of achieving a correct diagnosis of a thalassemia and because in some instances it is impractical to carry out DNA analysis for all the TM samples, only samples with MCH of <25 pg were selected based on previously established selection criteria as proposed by White et al. (1993). In addition, we also considered that alpha deletions are not common in South Asian countries except the $\alpha^{-3.7}$ and $\alpha^{-4.2}$ deletions.

2.7. Restriction fragment length polymorphism (RFLP) for HBG2:c.-211C > T (XmnI polymorphism)

XmnI polymorphism is an established ameliorating factor in beta thalassemia worldwide and has been widely linked with the use of hydroxyurea to trigger HbF production as part of its management. In our study the presence of XmnI polymorphism was analysed by RFLP based PCR (Nemati et al., 2010) as detailed below. After PCR, the product was digested with XmnI restriction endonuclease. The 650bp amplicon will remain intact in a normal individual. In the presence of the *HBG2*:c.-211C > T mutation, a XmnI site will be generated, resulting in the 650bp amplicon being cut into a 450bp and a 200bp bands, respectively. Depending upon the number of analysed bands, the genotypes were classified as homozygous mutation (+/+, 450bp and 200bp bands), heterozygous (+/−, 650bp, 450bp and 200bp bands) or wild type (−/−, only 650bp band).

2.8. Statistical analysis

Data were analysed by both descriptive and analytic statistics using the SPSS version 20.0. Demographic characteristics' means and frequencies were obtained by descriptive statistics. Significant determinants were determined by Chi-squared test by using an online tool ©2016 GraphPad Software, Inc (http://graphpad.com/quickcalcs/contingency1/) to compare the mutation rates