



Combination of two rare mutations causes β -thalassaemia in a Bangladeshi patient

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Abstract

Screening of mutations that cause β -thalassaemia in the Bangladeshi population led to the identification of a patient with a combination of two rare mutations, Hb Monroe and HBB: -92 C > G. The β -thalassaemia major male individual was transfusion-dependent and had an atypical β -globin gene cluster haplotype. Of the two mutations, Hb Monroe has been characterized in detail. Clinical effects of the other mutation, HBB: -92 C > G, are unknown so far. Bioinformatics analyses were carried out to predict the possible effect of this mutation. These analyses revealed the presence of a putative binding site for *Egr1*, a transcription factor, within the HBB:-92 region. Our literature survey suggests a close relationship between different phenotypic manifestations of β -thalassaemia and *Egr1* expression.

Key words: Hb Monroe, HBB: -92 C > G, transcription factor, *Egr1*.

Received: March 18, 2011; Accepted: March 21, 2011.

Thalassaemia is one of the major monogenic disorders worldwide (Weatherall, 2001). Approximately 400 mutations have been reported to cause this disease in the world population. These mutations show notable variability in their prevalence and effects in disease prognosis (Winichagoon *et al.*, 2000; Thein, 2005). Thus, the presence of rare mutations with debilitating phenotypic manifestations imposes a significant challenge to the successful implementation of prenatal diagnosis-based disease management strategies.

A preliminary study reported that the number of thalassaemic births in Bangladesh is 7483 per year (Khan *et al.*, 2005). This study also revealed that 7% of the population are carriers of this trait. As part of a screening program for mutations in β -thalassaemic individuals, a combination of two rare mutations, Hb Monroe (HBB: c. 92G > C) and HBB: -92C > G, was found in the same male subject, who presents both mutations in homozygous form (Patient 1 of

Ayub *et al.*, 2010). This study aimed to characterize the physiological effects caused by the mutations in the patient.

Based on haematological findings, the patient was diagnosed as having β -thalassaemia major, requiring regular transfusion. Further confirmation of β -thalassaemia was obtained by haemoglobin electrophoresis, using the Sebia Hydragel Haemoglobin (E) K 20 system. The patient also presented mild facial bone deformity.

DNA was isolated using the modified DNAzol method (Chomczynski *et al.*, 1997; Ayub *et al.*, 2010). A 587 bp segment of the *HBB* gene was amplified using methods described elsewhere (Ayub *et al.*, 2010). The selection was based on mutation data from other populations (Old, 2001; Vrettou *et al.*, 2003). The region includes a section of the upstream sequence, 5' -UTR, exon 1, intron 1 and part of exon 2, covering most of the part of *HBB* gene which contains the most prevalent five mutations in the Southeast Asian population (Panigrahi and Marwaha, 2007). The PCR product was purified using QIAGEN QIAquick PCR Purification Kit. Then the purified product was sequenced commercially by Macrogen Inc. (Korea). PCR and subsequent sequencing step were repeated.

The propositus's sequence was compared with the NCBI RefSeq entry for the *HBB* gene (NG_000007.3). The HbVar database was used for the identification of the presence of mutations reported in other populations (Patrinos *et al.*, 2004). The transcription factor binding site was predicted by AliBaba 2.1 (Grabe, 2002), using the

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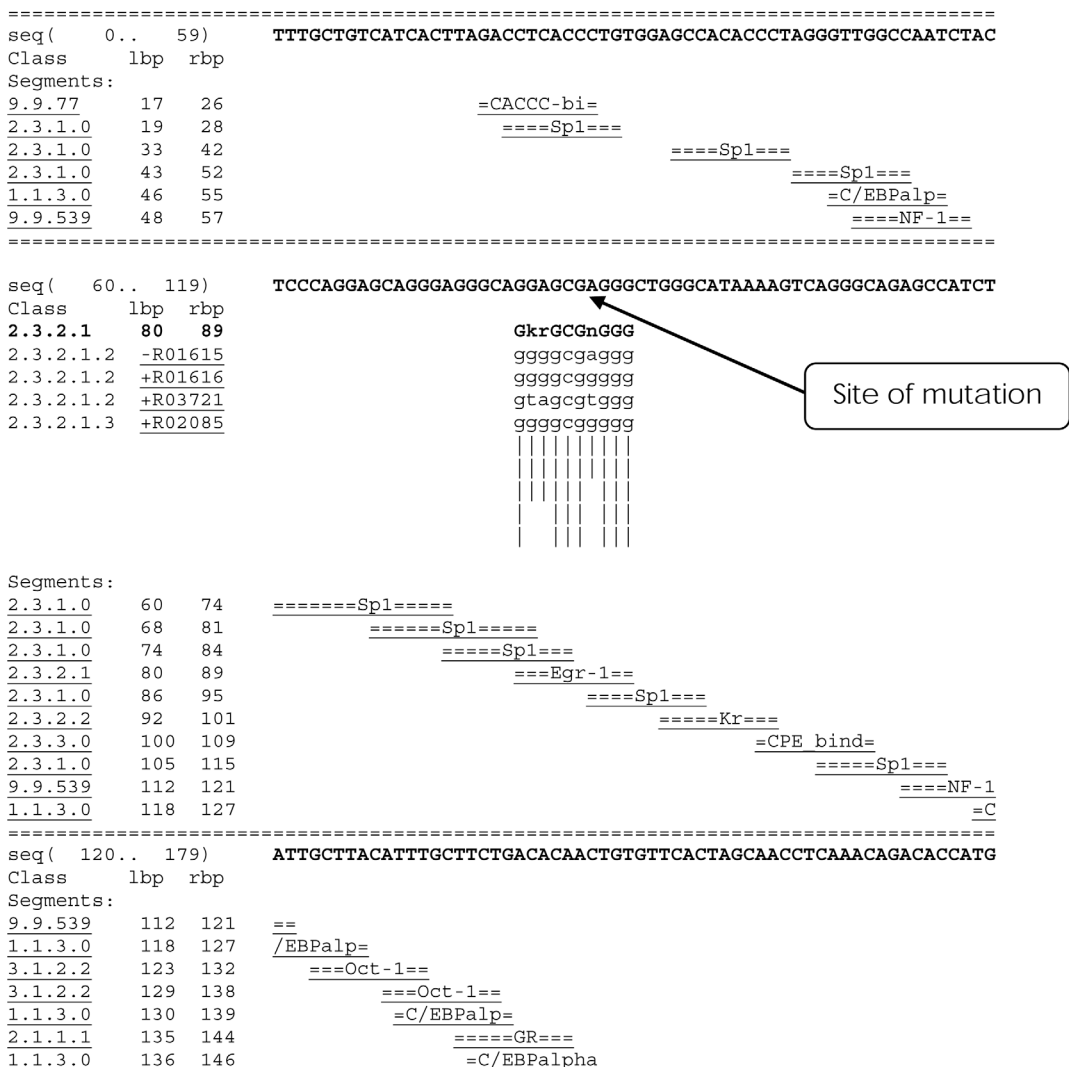
TRANSFAC database of transcription factors (Wingender et al., 1996).

Haplotypes were determined by analysis of the following polymorphic restriction sites in the β-globin gene cluster: (1) *HindII* 5' of ε; (2) *HindIII* 5' of Gγ; (3) *HindIII* in the IVSII 5' of Aγ; (4) *HincII* in the Ψb; (5) *HindII* 3' of Ψb; (6) *AvaII* in the β and (7) *HinfI* 3' β (Orkin et al., 1982; Rahimi et al., 2003; Falchi et al., 2005). Additionally, polymorphism at *XmnI* in the 5' of Gγ site was also checked, according to methods described by Rahimi et al. (2003).

This analysis revealed three single-nucleotide differences in the proband's sequence compared to the NCBI reference entry. The differences were: (1) HBB: -92C > G, (2) HBB: c. 9T > C, and (3) HBB: c. 92G > C. Mutation HBB: c. 92G > C, also known as Hb Monroe, alters the β-globin

codon 30 from Arg to Thr and is present within the donor splice site of exon-intron junction 1 of the *HBB* gene. It is likely that the mutation plays a role in pathogenesis by reducing the splicing efficiency (Gonzalez-Redondo et al., 1989). Mutation HBB: c. 9T > C is a silent mutation. This polymorphism was also found in Mediterranean populations (Atweh and Forget, 1986). The rare mutation HBB: -92C > G is located upstream of the transcription start site (TSS). Search for a putative transcription factor site in TRANSFAC using AliBaba 2.1 showed the putative *Egr1* binding site includes the point of mutation (Figure 1). Haplotype analysis revealed an atypical haplotype in the homozygous phase (- - + - + -). The presence and absence of a restriction enzyme site is represented by + and - signs,

Sequence seq_234



21 segments in this sequence identified as potential binding sites

Figure 1 - Putative *Egr1* binding site present at the mutation site. Transcription factor binding site predicted by AliBaba2.1 program (Grabe, 2002), using the TRANSFAC (Wingender et al., 1996) database.

respectively. The proband had the *XmnI* cutting site in the 5' region of $G\gamma$ of both chromosomes.

Hb Monroe was first reported in a transfusion-dependent 15-year-old black female from USA (Gonzalez-Redondo *et al.*, 1988, 1989). Later on, this mutation was also found in a limited number of individuals from other populations, such as Indians (Gupta *et al.*, 1991; Varawalla *et al.*, 1991), Tunisians (Fattoum *et al.*, 1991) and Tajiks (Fedorov *et al.*, 1993). The second mutation (HBB: -92 C > G) was previously reported only in a Tajik patient who incidentally had the Hb Monroe mutation too (Fedorov *et al.*, 1993). Nevertheless, it is unlikely that the two individuals (patient of current study and the Tajik patient) shared common ancestry. To our knowledge, no analysis was done so far on the possible role of this mutation in disease progression.

HBB: -92 C > G is located 42 bp upstream of the TSS. In eukaryotic systems, upstream regions of TSS are associated with gene regulation. The experimentally determined transcription factor binding site (TFBS) from the TRANSFAC database (Wingender *et al.*, 1996) was searched for within this region, using the Ali Baba 2.1 program (Grabe, 2002). This search showed that the mutation was present within the predicted binding site of *Egr1*, a transcription factor gene. This transcription factor belongs to the EGR family of C2H2-type zinc-finger proteins. The products of the target genes it activates are required for differentiation, mitogenesis and regulation of erythropoiesis. *Egr1* is also known as zif268; Krox-24; NGFI-A; 225; ETR103. It is associated with the CREB signalling pathway (Wang *et al.*, 2002).

An interesting role of *Egr1* in the response to the drug hydroxyurea (HU) has been observed in cell culture experiments. Treatment with HU can increase the production of HbF as well as the haemoglobin content, thus ameliorating β -globin-deficient β -thalassaemia (Koren *et al.*, 2008). It was also found that low-level pulsed doses of HU change the expression level of a wide range of genes, including *Egr1*. The HU treatment increased its expression nearly 2-fold in cell culture experiments (Wang *et al.*, 2002). The relative role of *Egr1* in increasing the γ -chain: β -chain ratio is yet to be determined.

The imbalance in the α -chain: β -chain ratio and the instability of the β -chain in β -thalassaemia result in the increase of serum-free haem (Ciccoli *et al.*, 1999; Koren *et al.*, 2008). Free haem results in increased generation of reactive oxygen species (Ciccoli *et al.*, 2003; Amer and Fibach, 2004), which in turn results in increased *Egr1* expression via MAPK ERK-1/2, Elk-1 and NF- κ B (Hasan and Schafer, 2008). Thus, *Egr1* also plays a role in the ROS-mediated pathogenesis of β -thalassaemia.

Although the aforementioned studies coupled with the bioinformatics analyses of the current study suggest a possible role of *Egr1* in certain forms of β -thalassaemia,

our literature survey failed to find any experimental evidence of *Egr1* binding at the HBB: -92 position. Experimental studies on *Egr1* binding to the HBB: -92 position may confirm the prediction of our current study. The effect of *Egr1* inhibition in a β -thalassaemia model organism may also shed further light on the molecular mechanism of the role played by *Egr1* in the ROS-mediated pathogenesis of individuals affected by this disease.

Haplotype analysis showed that the patient described here had an atypical haplotype (- - - + -). Both chromosomes displayed the same form of polymorphism, which can be explained by the fact that his parents were second/third cousins. The patient was positive for the *XmnI* site at the 5' end of $G\gamma$. Although a positive correlation between *XmnI* polymorphism and HbF level has been reported (Bandyopadhyay *et al.*, 2001), no association between *XmnI* polymorphism and incidence of the mild form of β -thalassaemia, namely thalassaemia intermedia was found (Neishabury *et al.*, 2010).

Acknowledgments

This study was partly funded by the Biotechnology Research Centre, University of Dhaka. MIA was supported by an NSICT Fellowship, granted by the Ministry of Science and Information & Communication Technology, Government of Bangladesh.

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Associate Editor: Francisco Mauro Salzano

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