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Short Communication

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*.

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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) *Hind*II 5' of ε; (2) *Hind*III 5' of $^{G}\gamma$; (3) *Hind*III in the IVSII 5' of $^{A}\gamma$; (4) *Hinc*II in the $^{\Psi}$b; (5) *Hind*II 3' of $^{\Psi}$b; (6) *Ava*II in the β and (7) *Hinf*I 3' β (Orkin *et al.*, 1982; Rahimi *et al.*, 2003; Falchi *et al.*, 2005). Additionally, polymorphism at *Xmn*I in the 5' of $^{G}\gamma$ 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,

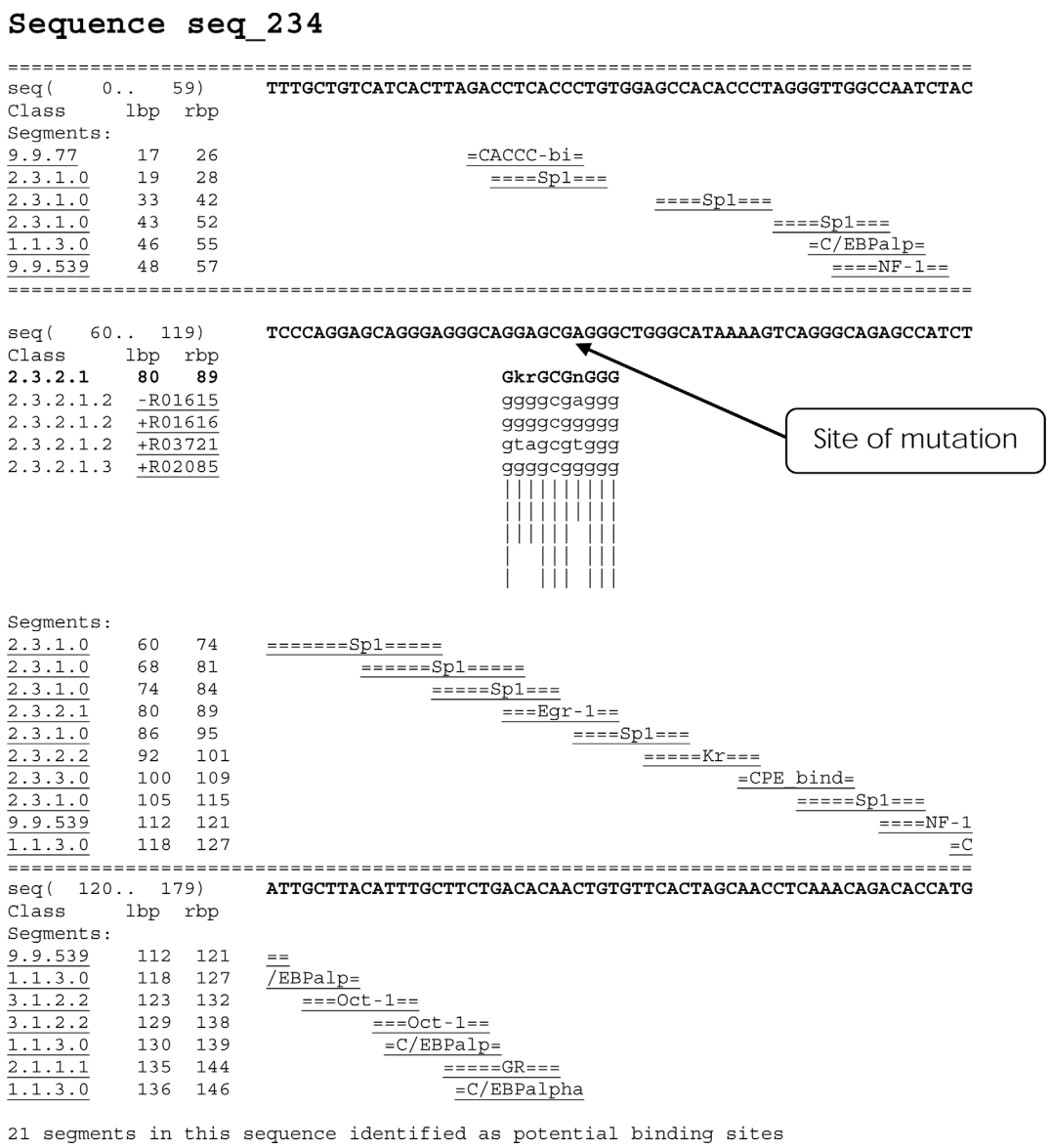

Figure 1 - Putative *Egr1* binding site present at the mutation site. Transcription factor binding site predicted by AliBaba2.1 program (Grabe, 2002), using the TRANSFAC (Wingender *et al.*, 1996) database.

respectively. The propositus had the *Xmn*I 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 erythropoesis. *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-κB (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 *Xmn*I site at the 5' end of $^{G}\gamma$. Although a positive correlation between *Xmn*I polymorphism and HbF level has been reported (Bandyopadhyay *et al.*, 2001), no association between *Xmn*I polymorphism and incidence of the mild form of β-thalassaemia, namely thalassaemia intermedia was found (Neishabury *et al.*, 2010).

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References

Amer J and Fibach E (2004) Oxidative status of platelets in normal and thalassemic blood. Thromb Haemost 92:1052-1059.

Atweh GF and Forget BG (1986) Identification of a beta-thalassemia mutation associated with a novel haplotype. Am J Hum Genet 38:855-859.

Ayub MI, Moosa MM, Sarwardi G, Khan W, Khan H and Yeasmin S (2010) Mutation analysis of HBB gene in selected Bangladeshi β thalassemic individuals: Presence of rare mutations. Genet Test Mol Biomarkers 14:299-302.

Bandyopadhyay S, Roychowdhury K, Chandra S, Das M and Dasgupta UB (2001) Variable severity of β-thalassemia patients of Eastern India: Effect of α-thalassemia and *Xmn*I polymorphism. Clin Exp Med 1:155-159.

Chomczynski P, Mackey K, Drews R and Wilfinger W (1997) DNAzol: A reagent for the rapid isolation of genomic DNA. Biotechniques 22:550-553.

Ciccoli L, Signorini C, Scarano C, Rossi V, Bambagioni S, Ferrali M and Comporti M (1999) Iron release in erythrocytes from patients with -thalassemia. Free Radic Res 30:407-413.

Ciccoli L, Rossi V, Leoncini S, Signorini C, Paffetti P, Bracci R, Buonocore G and Comporti M (2003) Iron release in erythrocytes and plasma non protein-bound iron in hypoxic and non hypoxic newborns. Free Radic Res 37:51-58.

Falchi A, Giovannoni L, Vacca L, Latini V, Vona G and Varesi L (2005) Beta-globin gene cluster haplotypes associated with beta-thalassemia on Corsica island. Am J Hematol 78:27-32.

Fattoum S, Guemira F, Öner C, Öner R, Li H, Kutlar F and Huisman T (1991) β-thalassemia, Hb S-β-thalassemia and sickle cell anemia among Tunisians. Hemoglobin 15:11-21.

Fedorov A, Nasyrov F, Smirnova E, Bocharova T and Limborska S (1993) IVS-I-1 (G > C) in combination with-42 (C > G) in the promoter region of the beta-globin gene in patients from Tajikistan. Hemoglobin 17:275-278.

Gonzalez-Redondo J, Stoming T, Lanclos K, Gu Y, Kutlar A, Kutlar F, Nakatsuji T, Deng B, Han I and McKie V (1988) Clinical and genetic heterogeneity in black patients with homozygous beta-thalassemia from the southeastern United States. Blood 72:1007-1014.

Gonzalez-Redondo J, Stoming T, Kutlar F, Kutlar A, Hu H, Wilson J and Huisman T (1989) HB Monroe or $_{\alpha}2\alpha230$ (B12) Arg > Thr, a variant associated with β-thalassemia due to a G > C substitution adjacent to the donor splice site of the first intron. Hemoglobin 13:67-74.

Grabe N (2002) AliBaba2: Context specific identification of transcription factor binding sites. *In Silico* Biol 2:1-15.

Gupta R, Tiwary R, Pande P, Kutlar F, Öner C, Öner R and Huisman T (1991) Hemoglobinopathies among the Gond tribal groups of Central India; interaction of alpha-and beta-thalassemia with beta chain variants. Hemoglobin 15:441-458.

Hasan RN and Schafer AI (2008) Hemin upregulates Egr-1 expression in vascular smooth muscle cells via reactive oxygen species ERK-1/2 Elk-1 and NF-κB. Circ Res 102:42-50.

Khan WA, Banu B, Amin SK, Selimuzzaman M, Rahman M, Hossain B, Sarwardi G, Sadiya S, Iqbal A, Rahman Y, *et al.* (2005) Prevalence of beta thalassaemia trait and Hb E trait in Bangladeshi school children and health burden of thalassaemia in our population. DS (Child) H J 21:1-7.

Koren A, Levin C, Dgany O, Kransnov T, Elhasid R, Zalman L, Palmor H and Tamary H (2008) Response to hydroxyurea therapy in beta-thalassemia. Am J Hematol 83:366-370.

Neishabury M, Azarkeivan A and Najmabadi H (2010) Frequency of positive *Xmn*I Gγ polymorphism and coinheritance of common alpha thalassemia mutations do not show statistically significant difference between thalassemia major and intermedia cases with homozygous IVSII-1 mutation. Blood Cells Mol Dis 44:95-99.

Old JM (2001) DNA based diagnosis of the hemoglobin disorders. In: Steinberg MH, Forget BG, Higgs DR and Nagel RN (eds) Disorders of Hemoglobin. Cambridge University Press, Cambridge, pp 941-957.

Orkin S, Kazazian H, Antonarakis S, Goff S, Boehm C, Sexton J, Waber P and Giardina P (1982) Linkage of beta-thalassaemia mutations and beta-globin gene polymorphisms with DNA polymorphisms in human beta-globin gene cluster. Nature 296:627-631.

Panigrahi I and Marwaha R (2007) Mutational spectrum of thalassemias in India. Indian J Hum Genet 13:36.

Patrinos GP, Giardine B, Riemer C, Miller W, Chui DHK, Anagnou NP, Wajcman H and Hardison RC (2004) Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies. Nucleic Acids Res 32:D537-541.

Rahimi Z, Karimi M, Haghshenass M and Merat A (2003) Beta-globin gene cluster haplotypes in sickle cell patients from southwest Iran. Am J Hematol 74:156-160.

Thein S (2005) Genetic modifiers of beta-thalassemia. Haematologica 90:649-660.

Varawalla NY, Old JM, Sarkar R, Venkatesan R and Weatherall DJ (1991) The spectrum of beta-thalassaemia mutations on the Indian subcontinent: The basis for prenatal diagnosis. Br J Haematol 78:242-247.

Vrettou C, Traeger-Synodinos J, Tzetis M, Malamis G and Kanavakis E (2003) Rapid screening of multiple beta-globin gene mutations by Real-Time PCR on the LightCycler: Application to carrier screening and prenatal diagnosis of thalassemia syndromes. Clin Chem 49:769-776.

Wang M, Tang DC, Liu W, Chin K, Zhu JG, Fibach E and Rodgers GP (2002) Hydroxyurea exerts bi-modal dose-dependent effects on erythropoiesis in human cultured erythroid cells via distinct pathways. Br J Haematol 119:1098-1105.

Weatherall D (2001) Phenotype-genotype relationships in monogenic disease: Lessons from the thalassaemias. Nat Rev Genet 2:245-255.

Wingender E, Dietze P, Karas H and Knuppel R (1996) TRANSFAC: A database on transcription factors and their DNA binding sites. Nucleic Acids Res 24:238-24.

Winichagoon P, Fucharoen S, Chen P and Wasi P (2000) Genetic factors affecting clinical severity in β-thalassemia syndromes. J Pediatr Hematol Oncol 22:573-580.

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