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Co-inheritance of the Hb Sun Prairie mutation with a point mutation at 5′-UTR in the eastern Indian population

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Summary

Haemoglobin (Hb) Sun Prairie (α2-globin cd130, GCT → CCT, Ala → Pro) is detected in three unrelated chromosomes, in association with a C → T transition in the 5′-untranslated region (UTR), two bases upstream from the translation start site. Reported inversion of α/β-mRNA ratio observed in Hb Sun Prairie mutants might stem from the second mutation and should be investigated. Molecular modelling studies indicate that the 130th residue of α-globin faces primarily the central cavity of the molecule and is not in contact with any β-chain residue; further, no significant disruption of the Hb structure because of the Sun Prairie mutation is discernible. Depression of translation because of the second mutation of a conserved base in the 5′-UTR might explain the observed clinical severity.

Keywords: α-thalassaemia, haemoglobin Sun Prairie, 5′-untranslated region, C → T transition, eastern India.

Haemoglobin (Hb) Sun Prairie (codon 130 of the α2-globin gene; GCT → CCT, Ala → Pro) was first detected in the USA, in a young adopted Indian patient with the clinical symptoms of severe haemolytic anaemia, microcytosis and hypochromia, in the homozygous state. The study was based on the detection and later preparation of the unstable α-chain, present in 3–5% of the total Hb, by reverse phase high-performance liquid chromatography (HPLC). Further confirmation of this unstable variant was performed with structural and dot-blot analyses by the same group (Harkness *et al*, 1990). Later, the variant was detected in two daughters, also of Asian Indian origin, where an autosomal recessive inheritance was clearly discernible (Ho *et al*, 1996a). This observation was noted to be unusual amongst variants in the region of helix-H. Inversion of the α/β-globin mRNA ratio was demonstrated, which makes it more difficult to identify it as an α-thalassaemia, rather than a β-thalassaemia (Ho *et al*, 1996a).

In this study we describe the coinheritance of Hb Sun Prairie with a C → T transition two bases upstream from the initiation AUG codon, in the homozygous and heterozygous states. Our molecular modelling studies indicate that the substitution in the 130th residue causing the Hb Sun Prairie mutant does not significantly alter the quaternary structure of the Hb molecule. The incidental coinheritance of a substitution in the 5′-untranslated region (UTR) of the same α2-globin gene containing the Sun Prairie substitution, in three unrelated chromosomes, thus altering the consensus Kozak sequence, arouses interest in the effect of 5′-UTR substitutions on the expression of the major α2-globin gene.

Methods and materials

Haematological studies and Hb analysis

Written informed consent was obtained from patient families before collection of blood samples. Hb profiling was performed by standard techniques. Cation-exchange HPLC was performed using the Bio-Rad Haemoglobin variant analyser using the beta-short programme.

Southern hybridization and polymerase chain reaction for detection of α^+/α°-deletions

Southern hybridization was performed for the detection of common deletional α-thalassaemia or novel deletions if present (Rienzo *et al*, 1985). Hybridization was performed after complete digestion of the genomic DNA by BglII. The α-P^{32} dCTP labelled ξ probe (cDNA, kindly provided by Dr A. Bhattacharya, University of Pennsylvania, USA) and α-probe (PstI fragment including the α2-globin gene, kindly provided by Dr G. Garewal, IPGMR, India) were used for hybridization.

Previously established GAP-polymerase chain reaction (PCR) protocols were slightly modified and used for the detection of the common α^+ deletions, namely the 3·7 and 4·2 kb deletions (Baysal & Huisman, 1994), where the normal and mutant alleles were amplified in separate tubes using betaine (0·75 mol/l) to increase the efficiency of the reaction.

Modified PCR for detection of α°-deletions

A modified PCR, as previously described (Tan *et al*, 2001), was used to detect the α°-deletions commonly found in Southern Asia and Africa). The reaction was performed in a final volume of 25 μl on a Perkin Elmer GeneAmp PCR System 2400 thermal cycler 1·5 U of Taq DNA Polymerase (Geneii, Bangalore, India) and 200-ng genomic DNA was used in each multiplex PCR assay. After amplification, the entire reaction mix was electrophoresed on a 2·5% agarose gel, stained with ethidium bromide solution and visualized wit an ultra-violet transilluminator.

Restriction enzyme digestion for the detection of 5′-UTR substitution

A 1085-bp fragment was specifically amplified including the α2-globin gene using primers C1 and C3 as described previously (Dode *et al*, 1990). This product was then digested for 18 h with NcoI according to the manufacturer's directions (Fermentas, Vilnius, Lithuania). A mutation in the vicinity of the initiator AUG codon abolishes the NcoI recognition site, resulting in an undigested product, whereas the normal allele digests into two fragments of sizes 239 and 845 bp.

Sequencing of PCR products

The α-globin2-specific PCR products were amplified as described previously using primers C1 and C3 (Dode *et al*, 1990). Both these primers were used for sequencing the 5′- and 3′-ends of the α2-globin gene respectively. Additional internal primers used for sequencing were designed in the second exon of the α-globin gene. The sequences are as follows: Alp2F: 5′-gcttctccccgcaggatgtt-3′ at position 6901 and Alp2R: 5′-gccgtggctcaggtcgaagt-3′ at position 6975 (nucleotide positions are according to GenBank accession no. J000153). The 1085-bp C1/C3 PCR product was sequenced from both the 5′- and 3′-ends using the primers C1 and C3 and the internal primers Alp2F and Alp2R (Fig. 1). Sequencings were carried out manually using a sequencing kit (United States Biochemicals, Cleaveland, OH, USA) and 6% denaturing polyacrylamide gel. Electrophoresis was carried out on Bio-Rad Sequi-Gen® sequencing cell apparatus (Bio-Rad, Hercules, CA, USA).

Molecular modelling studies of H13 Ala130 → Pro130

Initial model building of the H13 Ala130 → Pro130 mutation at both the α-chains was carried out using the Bioplymer module of INSIGHTII software (Accelerys, San Diego, CA, USA) running on a Silicon Graphics O_2 workstation. The native Hb structure was obtained from protein data bank (1A3N). The mutated Hb was subjected to energy minimization using CHARMM (version 28) and CHARMM 22 force field. The protein was then subjected to two molecular dynamics simulation runs, at 300 and 500 K, keeping the β-chains fixed. The time-averaged structures obtained from the two simulations were superimposed on the mutated crystal structure of Hb. The root mean square deviations of the backbone atoms were analysed to understand the effect of the mutation on the quaternary structure of the Hb molecule.

Results

Haematological and clinical data

Proband 1 was a girl from the Bardhaman district of West Bengal, India who became symptomatic at 5 years with weakness, highly coloured urine and anaemia. At the age of 7 years she was transfused with two units of packed red cells when her Hb dropped to 5·4 g/dl. Later, she was again investigated for anaemia and her haematological profile was: Hb, 8·1 g/dl; PCV, 29·4%; MCV, 73·5 fL; MCH, 20·2 pg and MCHC, 27·5%. Total differential and platelet counts were within normal limits. Hypochromia, polychromasia, tear cells and occasional normoblasts were detected on histological examination of the blood film. Hb electrophoresis showed normal HbA and HbA_2 levels. HPLC, using the beta-short programme, showed no HbH peak and HbF < 1%. The electrophoretic pattern of the parents was normal. At this stage the patient was referred to us with suspected α-thalassaemia. Then her Hb has been maintained at 8·1 g/dl with infrequent transfusions. Although the age of menarche was 15 years and 9 months there has been no other problems with endocrinological functions.

The heterozygous proband 2 for Hb Sun Prairie, a 35-year-old male, presented with persistent unexplained anaemia and a normal Hb electrophoresis pattern. No other clinical records were available.

Genetic analysis

Southern hybridization and polymerase chain reaction studies ruled out the presence of the α^+ deletions, $-\alpha^{3.7}$, $-\alpha^{4.2}$, $-\alpha^{7.9}$ and the α° deletions, $-^{SEA}$, $-^{FIL}$, $-^{Thai}$, $-\alpha^{SA}$ and $-^{20.5}$ deletions. Direct sequencing of a Pfu polymerase-amplified PCR product showed the presence of Hb Sun Prairie (Cod130 GCT → CCT, Ala → Pro) in both chromosomes (Fig. 2A) in one patient and in one chromosome in the other (Fig. 2B).

Interestingly, the mutation-bearing chromosome in both the patients harboured a C → T transition two bases upstream from the translation starting ATG site (Fig. 3A). Presence of the transition (C → T) described here abolished a restriction endonuclease site (NcoI) that was used for demonstrating the heterozygosity of the mutation (Fig. 3B).

Primers	Position on the α-globin gene	DNA Strand	Sequence 5′ → 3′
C1	6464	Sense	tggagggtggagacgtcctg
C3	7548	Antisense	ccattgttggcacattccgg
Alp2F	6901	Sense	gcttctccccgcaggatgtt
Alp2R	6975	Antisense	gccgtggctcaggtcgaagt

Fig 1. Scheme of the α-globin gene. The primers used for DNA sequencing are indicated by arrows below and above the gene respectively. Primers C1 and C3 have been used both for amplification and sequencing. The sequence position numbers, according to GenBank accession no. J000153, are reported in the table. The positions of the substitutions are indicated in the gene by asterisks.

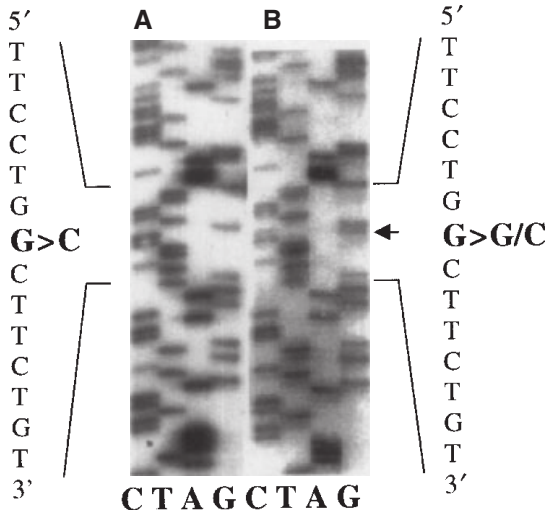

Fig 2. (A) Scanned autoradiogram showing 5′-CTGGCT-3′ → C, homozygous mutant at alpha2 130,H13 (proband1). (B) Scanned autoradiogram showing 5′-CTGGCT-3′ → C/G, heterozygous mutant at alpha2 130,H13 (proband2).

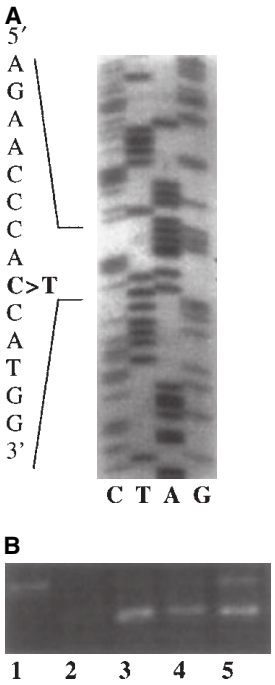

Fig 3. (A) Scanned autoradiogram showing 5-′CCCACCATG-3′ → T, two bases upstream from the initiation codon of α2-globin in proband1. (B) NcoI digested α2-specific PCR product; lane 1: undigested product, lanes 3 and 4: normal samples, lane 5: hetorozygous mutant at −2.

Screening of 17 other unrelated chromosomes by direct sequencing did not identify the presence of any other Hb Sun Prairie containing chromosome.

Molecular modelling analysis

In both patients, molecular modelling studies, performed by subjecting the protein to two molecular dynamics simulation runs at 300 and 500 K, showed that the root-mean square deviations of the backbone atoms was <3Å even at a higher temperature. This indicated that the mutation did not affect the structure of the protein significantly (Fig. 4A). The helix H was found to retain the native structure even at 500 K. The mutated residue H13 was near the central cavity (Fig. 4B), and is not likely to affect α–β-interface interactions.

Discussion

Many features of an mRNA contribute to the efficiency of its translation. The most important of these are the 5′m^7 GpppG cap, length of the 5′-UTR, start-site consensus sequences, presence of secondary structure, upstream AUG, upstream ORF and internal ribosome entry sites. Mutations at these sequences are known to modulate the expression of the genes concerned. Some of these mutations, such as those at +22 (Oner *et al*, 1991) and +33 (Ho *et al*, 1996b) of the β-globin gene, are known to depress transcription and effects of some changes are not certain (Oner *et al*, 1991). A change at +10 in the β-globin 5′-UTR has been shown to affect translation (Athanassiadou *et al*, 1994). The initiation complex recognizes the AUG starting codon in the context of a consensus sequence 5′-A/GCCAUGG-3′, known as the Kozak sequence. It was shown (Kozak, 1984) that a 93% reduction in translation

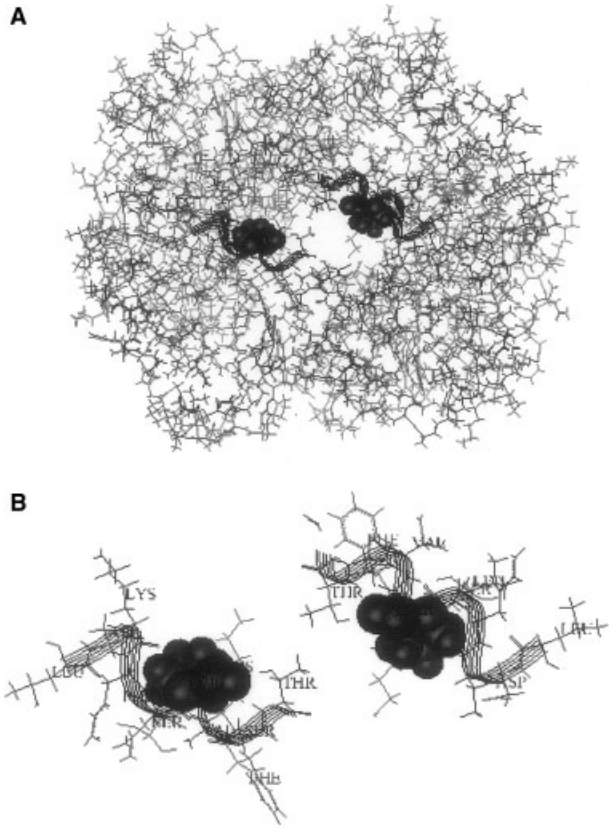

Fig 4. (A) Modelled figure of the quaternary structure of Hb Sun Prairie obtained using the Bioplymer module of INSIGHTII software (Accelerys Inc). The mutant residue highlighted close to the central cavity of the molecule is represented by space filling representation. Part of the H-helix is shown representation as ribbon. (B) Modelled figure showing the two mutant residues (cod130 Apa → Pro) in both α-globins. The interaction of the mutant residue, shown in space filling representation embedded in the H-helix, is limited to residues in the same helix. The surrounding interacting residues are indicated.

efficiency occurred as a result of an A → C change, three bases upstream from the initiation codon of the rat preproinsulin gene. The most conspicuous conserved feature in the 5′-UTR is the presence of a purine (mostly A) three nucleotides upstream from the initiating AUG codon, as shown in a tabulation of 211 mRNA from higher eucaryotic cells. Interestingly there is a predominance of C in positions −1, −2, −4 and −5, just upstream of the initiator codon. From the study, the sequence CCA/GCCAUG (G) emerged as the consensus sequence for eucaryotic initiation sites.

Changes in this sequence are known to modulate the expression level of the gene concerned in humans as well. Several such instances leading to a disease or disease susceptibility has been reported. A C → T change at −4 produces a reduction in coagulation factor XII (Kanaji *et al*, 1998). A T → C change at −5 in lbα-glycoprotein (Afshar-Kharghan *et al*, 1999) and a C → T change at −1 in the annexinV gene (Gonzalez-Conejero *et al*, 2002) has been reported to alter disease susceptibility through alteration of protein level. A recent report showed a 30% reduction in translation efficiency because of a G → C mutation at −6 position of β-globin 5′-UTR (Angioletti *et al*, 2004).

A severe form of α-thalassaemia, because of the deletion of the nucleotides two and three bases preceding the initiation codon of α-globin, in an Algerian patient homozygous for the rightward 3·7 kb α-globin gene deletion and displaying HbH disease, has been detected (Morle *et al*, 1986). It has also been shown that this deletion leads to a 30–45% reduction in the translation efficiency of synthetic α-globin mRNA in rabbit reticulocyte lysate (Morle *et al*, 1986). In other experiments, the same group has also shown that COS cell (African green monkey kidney cell line COS 7) transfected with constructed α/G_γ-hybrid globin genes, in which the 3′-end of the normal or mutated α-globin genes downstream to the ATG initiation codon was substituted by the 3′-part of a G_γ-globin gene, synthesized a similar amount of α/G_γ-hybrid mRNA but 50% less G_γ-globin when transfected with the α/G_β-hybrid gene carrying the deletion (Morle *et al*, 1986).

Our result is the second report of a change in Kozak sequence in the human α-globin gene. Incidentally, no other report of a change at the −2 position of the Kozak sequence is available to date.

Molecular modelling studies did not indicate any severe structural distortion in the Hb molecule because of the substitution of the alanine at the 130th position of the α-chain by proline. The conformation of the H-helix remained largely unchanged and all the residues that are within the sphere of influence of cd130 are within the H-helix itself (Fig. 4B). As this residue is facing the central cavity of the molecule and does not touch any other chain, it is unlikely to have a drastic effect on the conformation of the molecule. However, the clinical picture of the patient was quite severe, and not in keeping with the molecular modelling data.

In the light of these results, along with the inversion of α/β-mRNA ratio noted by other authors (Ho *et al*, 1996a), we suggest that the rather severe phenotype associated with the Hb Sun Prairie mutation might be caused by the additional depression of expression because of the transition two bases upstream from the translation start site.

It would be interesting to investigate whether the presence of Hb Sun Prairie in the Indian subcontinent is always associated with the change at 5′-UTR.

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