



SHORT REPORT

A new mutation in the six-domain of *SIX3* gene causes holoprosencephaly

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Holoprosencephaly (HPE) is a severe brain malformation which results from incomplete cleavage of the forebrain during early embryogenesis. The aetiology of HPE is very heterogeneous. Among the genetic factors, *SIX3*, which is considered to be the functional orthologue of *Drosophila* genes *sine oculis* (*so*) and *optix*, has been found to be mutated in the homeodomain, in some patients with HPE (HPE2 on chromosome 2p21). We report a new HPE family, presenting a wide spectrum of clinical features, ranging from cyclopia to hypotelorism, in which a mutation was found for the first time in the SIX domain of *SIX3*: a GG insertion creates a frameshift leading to a nonsense mutation downstream in the homeodomain region. *European Journal of Human Genetics* (2000) 8, 797–800.

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Introduction

Holoprosencephaly (HPE) is a severe brain malformation of the forebrain which results from incomplete cleavage of the forebrain during early embryogenesis. Anatomic brain findings have been defined from the most severe form, alobar HPE with a single ventricle, to lobar HPE in which there is separation of most of the cerebral hemispheres. Phenotype is variable ranging from cyclopia to clinically normal individuals. Ophthalmologic features are prevalent ranging in a continuous spectrum from synophthalmia to a mild hypotelorism. The transmission of isolated HPE is compatible with autosomal dominant inheritance with incomplete penetrance (82%).¹

The aetiology is extremely heterogeneous. Among the genetic factors, at least 12 chromosomal loci have been implicated,² including HPE1 in 21q22.3, HPE2 in 2p21, HPE3 in 7q36, and HPE4 in 18p11.3. Many genes have been proposed as candidate genes (see review by Wallis and Muenke³). But to date, only four of them have been confirmed as HPE-causing genes: *Sonic Hedgehog* (*SHH*), *ZIC2*, *SIX3* and *TGIF*. *SIX3*⁴ belongs to the human *SIX* family which contains several genes exhibiting a strong aminoacid

sequence homology, especially for the SIX and homeodomains, as *SIX1/SIX2*, *SIX3/SIX6* and *SIX4/SIX5*.⁵

SIX3 has been isolated from the human critical region HPE2 on chromosome 2p21, owing to the existence of the mouse *Six3* gene on the homologous distal region of mouse chromosome 17. Mouse *Six3* is a homeobox gene expressed in the developing retina, lens, hypothalamus, and pituitary;⁶ it has been initially described as *sine oculis* homologue, a *Drosophila* gene involved in eye development. A new gene, *optix*, also expressed in eye primordia of *Drosophila* embryo, was subsequently identified with a homeodomain 95% identical to the *Six3* protein;⁷ this is the closest paralogue to *SIX3*. Expression of *SIX3* was detected in eye retina in human embryos at 5–7 weeks of gestation until adulthood.⁵

SIX3 was a good candidate gene due to its expression and its map position overlapping the chromosomal region related to HPE2. Thus Wallis *et al*⁸ described four mutations in the homeodomain (one deletion of nine nucleotides and three missense mutations) in patients who had typical HPE facial features.

We have performed a mutational analysis of *SIX3* in 56 HPE patients including those already tested for *SHH*⁹ and *ZIC2*. We found a mutation located for the first time in the SIX domain in a family with severe ophthalmologic symptoms. In the absence of functional data for previous *SIX3* mutations, this novel mutation represents the first loss-of-function *SIX3* mutation reported so far.

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Materials and methods

A total of 56 HPE probands (36 clinically sporadic and 20 familial cases) and two cases of unrelated bilateral anophthalmia were studied.

The genomic structure of the human *SIX3* gene⁴ was used to design primers pairs covering the entire homeodomain and SIX-domain (Figure 1):

Homeodomain: S1 5'-AAG TTC CCG CTG CCA CGC ACC-3' and S2 5'-GCG GGC CCC GCC ACT AAC-3'

Six-domain: S5 5'-TGT TCC AGC TGC CCA CCC TCA-3' and S6 5'-TAC CAC TCC CGC AAC AGG CTC-3'

The PCR reactions were performed in a PTC thermal cycler (MJ Research, Watertown, MA, USA), with an annealing temperature of 62°C for the homeodomain and a temperature gradient from 65°C to 56°C for the SIX domain.

SSCA was performed with a Genephyor system (Pharmacia Biotech, Uppsala, Sweden) in two conditions of migration (20°C for 2 h and 5°C for 4 h); the polyacrylamide gels were silver-stained. Heteroduplex PCR products were separated overnight on 50 cm MDE™ (FMC corporation, Rockland, ME, USA), and ethidium bromide stained.¹⁰

When abnormal SSCA or heteroduplex band shifts were observed, sequencing of the PCR products was performed on the ABI 373A DNA sequencer (Perkin Elmer, Roche, Foster City, CA, USA). Mutations were confirmed by PCR restriction.

Results

Of the 56 HPE cases for the six and homeodomain of *SIX3*, anomalies in SSCA and HA were found in one familial case. Direct sequencing showed a GG insertion between nucleo-

tides 557 and 558. This mutation creates a frameshift leading to a nonsense mutation (stop codon TAG) 63 aminoacids downstream in the homeodomain region. This mutation was confirmed by PCR restriction with *ApaI* and *NlaIV*.

In this family, three cases with typical features of HPE were described (Figure 2). Patient II1 had synophthalmia and proboscis (Figure 3); the autopsy revealed a single optic nerve with coloboma of the retina and confirmed alobar HPE. Patient II2 (Figure 4) had cebocephaly with alobar HPE. Foetus II5 (Figure 5) was aborted as a result of a single cerebral ventricle revealed by ultrasound scan, and cebocephaly which was confirmed by post-mortem examination.

The mutation was identified in a child (II4) presenting with a phenotype typical of the HPE spectrum (single central upper incisor, ocular hypotelorism, mild mental retardation). This DNA alteration was inherited from her father who had only an isolated mild hypotelorism. MRI scans showed no brain malformation in patient II4 or her parents. Unfortunately, no DNA or tissue blocks were available for patients II1, II2 and II5.

The mutation was not found in the mother and in the only normal child (II3), nor in 100 normal chromosomes from unrelated Caucasian individuals. In this family, no mutation was identified in *SHH* and *ZIC2* genes.

No SSCA or HA anomalies were visualized in the other HPE cases, neither in SIX or homeodomain nor in the two anophthalmic patients.

Discussion

We have identified a new mutation for the first time in the SIX domain of *SIX3* gene, in one HPE familial case.

The anticipated effect of this mutation could also be associated with HPE for additional reasons: truncation of the



Figure 1 Organisation of the human *SIX3* gene (from Wallis *et al*⁶). Primer sets are indicated by arrows: S5–S6 are used to amplify the SIX domain and S1–S2 for the homeodomain. Bold arrow indicates the position of the stop codon created in the homeodomain by the frameshift mutation 557–558insGG, in the SIX domain.

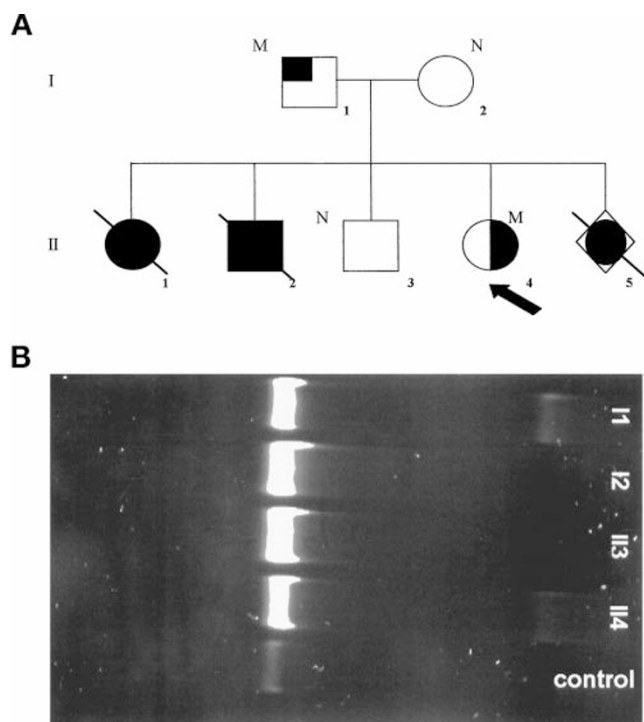


Figure 2 A Pedigree of the HPE family associated with the *SIX3* mutation. Filled symbols indicate alobar HPE with cyclopy (II1) or cebocephaly (II2 and II5). Half-filled symbol (II4) indicates minor manifestation with hypotelorism, single upper incisor and mental retardation. Quarter-filled symbol (I1) indicates isolated hypotelorism. M: *SIX3* mutation carrier; N: no *SIX* mutation. B Heteroduplex analysis of the *SIX* domain of *SIX3* in the HPE family.

corresponding protein resulting from the stop codon in the homeodomain; localization in a highly conserved region through evolution of the animal species;⁸ and segregation between the mutation and the associated disease phenotype. An effective functional assay for *SIX3* should be developed to confirm this association, but this novel mutation represents the first truncating mutation and confirms the involvement of *SIX3* in HPE.

Interestingly, this 2G insertion leads to a stop codon downstream in the homeodomain where the four previous mutations (one 9-bp deletion, three missense mutations), had been already reported.⁸ The first stop codon described was located in the helix 3 and predicted a change in the DNA-binding helical structure. Wallis *et al* reported only one missense mutation in helix 3 of the homeodomain, associated with ocular signs (a sporadic case with semi-lobar HPE and associated iris coloboma and micro-ophthalmia). In the family we studied, patients had also ocular features ranging from cyclopia to mild hypotelorism. No specific genotype-phenotype association was evident due to the wide variability in these clinical features. Furthermore, such ophthalmologic signs have been described, associated with *SHH* mutations.¹¹

The variable expression of the *SIX3* mutations might be explained by environmental factors and/or potential modifying genes. This hypothesis was supported by Nanni *et al*¹¹ who reported three patients with both an *SHH* mutation and abnormalities in *ZIC2* or *TGIF* genes. In the family we



Figure 3 Patient II1: cyclopia with synophthalmia and proboscis.



Figure 4 Patient II2: cebocephaly with alobar HPE.

studied, mutational analysis for *SHH* and *ZIC2* genes was negative.

We encountered some recurrent problems during DNA sequencing. The concomitant amplification of another sequence, such as *SIX6*, was suggested. This gene, belonging to the *SIX* family is closely related to *SIX3*.⁵ In the end the involvement of *SIX6* was excluded by two enzymatic digestions. Furthermore, expression of *SIX6* in the human embryonic eye starts after 12 weeks of gestation.⁵ As human proencephalic brain cleaves into two parts (telencephale and diencephale) at an earlier stage (5 weeks), alterations of *SIX6* should not be involved in HPE. In contrast, *SIX6* haploinsufficiency was described in some anophthalmic patients with pituitary anomalies.⁵

The identification of this *SIX3* mutation made genetic counselling more reassuring for the normal male (II3), who

was not a carrier. This *SIX3* mutation, found in a panel of 56 unrelated HPE patients, confirms the well-known genetic heterogeneity of the disease, as five mutations were found in *SHH*,^{9,12} three in *ZIC2* and a missense mutation in *SIX3* (data not shown). Similar frequencies were described by the previous reports.^{8,13} Finally, this new report is a striking example of the extremely variable expression of the HPE spectrum raising the possibility of modifying genes or environmental factors.

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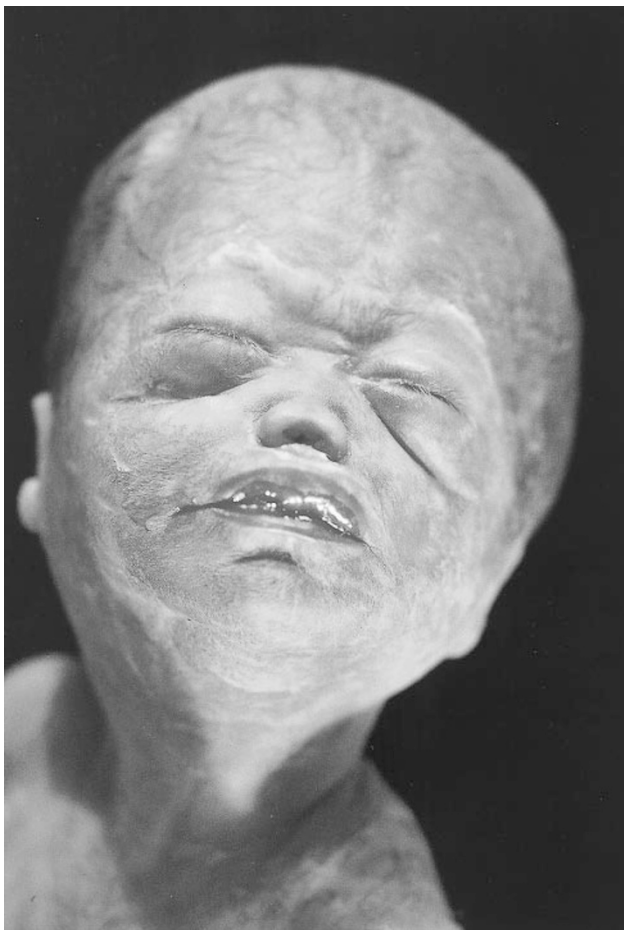


Figure 5 Case II5: medical abortion as a result of single cerebral ventricle. Cebocephaly with alobar HPE.