Electronic letter

Novel mutations of *SOX10* suggest a dominant negative role in Waardenburg-Shah syndrome

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EDITOR—Waardenburg syndrome (OMIM 193500) is a rare disorder (1 in 40 000 live births) characterised by distinctive facial features, pigmentary disturbance (white forelock, heterochromia iridis, white eyelashes, leucoderma), and cochlear deafness.[1] Waardenburg-Shah syndrome combines the features of Waardenburg syndrome and Hirschsprung's disease (also called Waardenburg-Hirschsprung disease, Waardenburg syndrome type IV, WS4) (OMIM 277580). In addition to having white forelock, eyebrows, and eyelashes, the patients present in the neonatal period with intestinal obstruction, characteristic of Hirschsprung's disease.[2–4] Mutations in three different genes have been identified in WS4 patients. These genes include the endothelin-B receptor gene (*EDNRB*),[5] the gene for its ligand endothelin-3 (*EDN3*),[6 7] and the *SOX10* gene. It has been observed that heterozygous mutations of *EDNRB* or *EDN3* are found in Hirschsprung's disease alone[8–12] and only homozygous mutations of either gene are found in WS4. Therefore, when resulting from *EDN3* or *EDNRB* mutations, WS4 is inherited as an autosomal recessive trait.

Among the WS4 patients studied so far, 10 out of 37 have been reported to have had *SOX10* mutations.[13–16] Interestingly, when *SOX10* mutations are involved, WS4 is inherited as an autosomal dominant condition. The presence of *SOX10* mutations in the Waardenburg-Shah patients suggest that the *SOX10* gene could be involved in regulatory and signalling pathways for the normal development of the neural crest cell lineages which differentiate into melanocytes and enteric ganglia. The involvement of *Sox10* in the development of enteric neurones has also been reported in the *Dom* (*Dominant megacolon*) mouse model of Hirschsprung's disease. It was shown that a single base insertion in the mouse *Sox10* gene was responsible for the megacolon phenotype of the *Dom* mutant.[17 18] Interestingly, in the Waardenburg-Shah patients as well as the *Dom* mutant mouse model, the intestinal aganglionosis phenotype appears to originate from heterozygous *SOX10*/*Sox10* mutations, suggesting that the phenotype could be the result of haploinsufficiency.

In this study, we examined mutations in three WS4 patients, two of Chinese origin and one English. We identified two novel *SOX10* mutations among three WS4 patients we investigated. We also reviewed all the *SOX10* mutations reported so far to correlate *SOX10* mutations with the aganglionosis phenotype observed in the patients.

Patients

Patient 1 is a Chinese girl, born at term, who was admitted to hospital because of failure of passage of meconium within the first 24 hours of life, followed by vomiting and a distended abdomen. She has bilateral hearing loss, light brown hair, and vivid blue irides with grey speckles. The diagnosis of Hirschsprung's disease was made on the basis of history, physical findings, and barium enema results, and was confirmed by the pathological findings of the resected specimen. At operation, the aganglionic segment was found to involve the distal sigmoid colon and rectum. A pull through procedure was performed. There is no family history.

Patient 2 is a Chinese boy, born at term, who developed abdominal distension and vomiting on day 2 of life with delayed passage of meconium. He had dysmorphic facial features with ptosis of the right eye. He is profoundly deaf and suffers from mental and global developmental retardation. He has blue eyes on both sides and one strand of white hair over the forehead. All these are features of Waardenburg syndrome. On laparotomy, frozen section histology of intestinal biopsies showed total colonic aganglionosis and ileostomy was performed. Subsequently, total colostomy and Duhamel pull through was performed. There is no family history.

Patient 3 is an English boy, born at term, who presented with failure of passage of meconium, bile stained vomiting, and a distended abdomen on day 1. Rectal biopsy confirmed the diagnosis of Hirschsprung's disease. He had rectosigmoid aganglionosis. Temporary colostomy was performed initially and definitive pull through operation done subsequently. The presence of a white forelock and deafness led to the diagnosis of Waardenburg syndrome. There is no family history.

Methods

DNA was extracted from peripheral blood samples collected from patients and their family members using QiaAmp Blood Kit (Qiagen)

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Table 1 Sequences of PCR primers for amplification of SOX10 genomic fragments covering the entire coding region and intervening exon-intron junctions

Exon	*Primer sequence*	*Product size (bp)*
2	forward 5'GCC TGG AGG CTC CAC CTT CTG C-3' reverse 5'-TCC CTC AGC CTG CCC CAA CG-3'	443
3	forward 5'-GGC TGT GCC CAC GTC CTG TCT C-3' reverse 5'-CAG TCC CGC TCT GAG GTG CAG G-3'	689
4	forward 5'-AGT CCA CAA ATC ATA GGG CAC A-3' reverse 5'-TAG AGT CCA GGG TCT CAT TGC C-3'	527
5a	forward 5'-CAT GCT GCC AAA ATG TGA AAC T-3' reverse 5'-ATG GTC AGA GTA GTC AAA CTG G-3'	661
5b	forward 5'-CAG ATC GCC TAC ACC TCC CTC A-3' reverse 5'-GGG CAT GTC AGA CCC TCA CTA T-3'	696
5c	forward 5'-ACC ACT CCT ATG ACT CCT GTT T-3' reverse 5'-AGA GGG GAC TAC TGA GAT AAA T-3'	749

or by phenol chloroform extraction method. Six sets of primers (sequences summarised in table 1) were used to amplify *SOX10* gene fragments covering exons 2 to 5 and intron-exon junctions by PCR. The PCR products were purified and the DNA sequences determined by an automated sequencer (ABI Prism 310) after cycle sequencing reactions (dRhodamine kit). All sequences were determined from both forward and reverse orientations (table 1). Mutations were confirmed by sequencing duplicate PCR templates from separate reactions.

Results

Two different novel *SOX10* mutations were identified in the two Chinese WS4 patients. Patient 1 was diagnosed as having typical short segment HSCR; her intestinal aganglionosis extended to the rectosigmoid region. Sequence analysis showed that she had a heterozygous frameshift mutation in exon 3 of the *SOX10* gene and a single nucleotide (G) at nucleotide position 168 from the start codon is deleted (fig 1B). This mutation would lead to a frameshift in the mutant protein producing a truncated SOX10 protein of 107 amino acids (fig 1E). This truncated mutant protein, which lacks the HMG DNA binding and the C-terminal transactivating domains, is likely to be non-functional. No *SOX10* mutation could be detected in her parents, indicating that the 168delG mutation in patient 1 was sporadic.

Patient 2 was diagnosed as having total colonic aganglionosis. Analysis of the *SOX10* sequence showed that he had a heterozygous mutation at the stop codon in exon 5. A transversion of T to A (fig 1D) at the first base of the stop codon changed it to a codon for lysine. The mutant *SOX10* open reading frame encodes an extra 86 amino acid long proline rich peptide at the C-terminus (fig 1E). Examination of the *SOX10* sequence of his parents

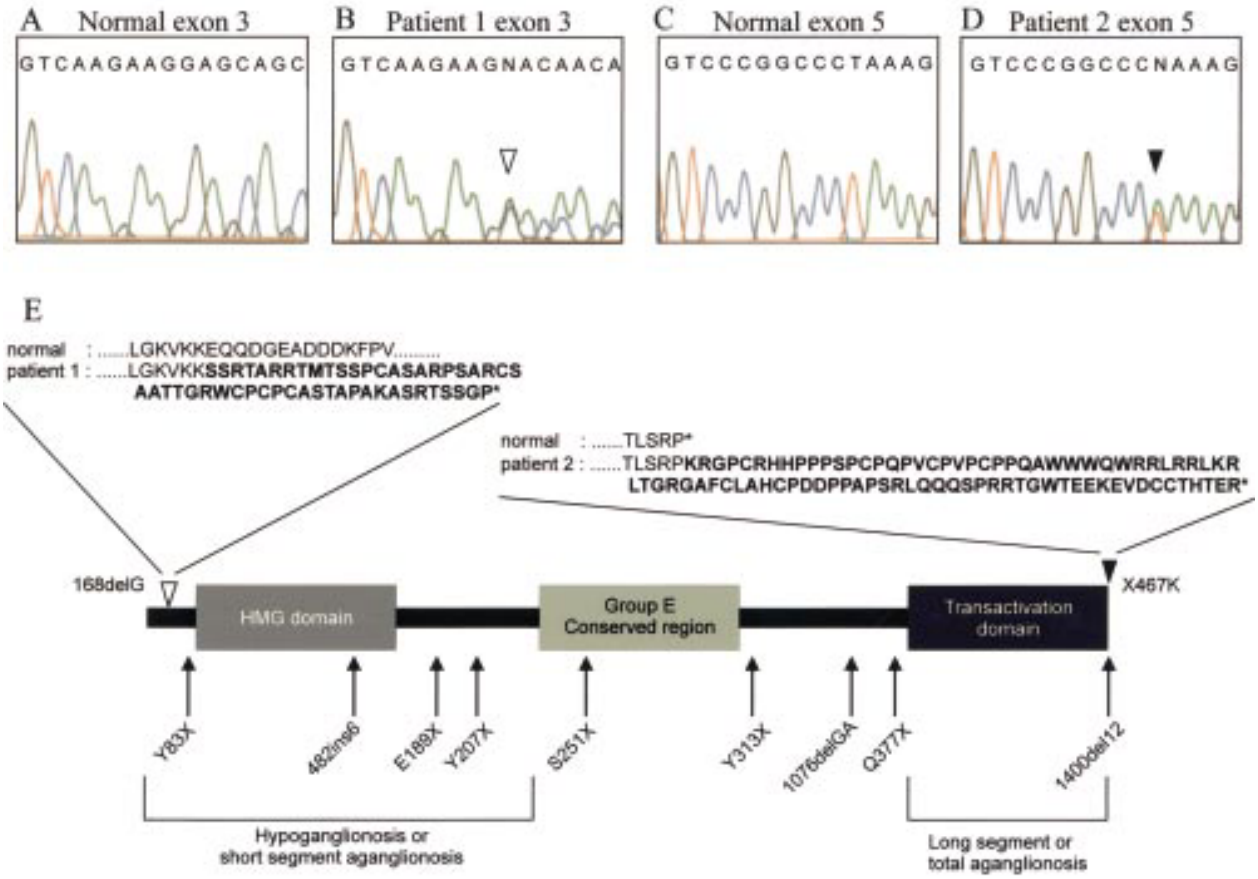

Figure 1 Analysis of SOX10 mutations in WS4 patients. DNA sequence analysis of (A) normal exon 3 and (B) exon 3 of patient 1 shows that there is a deletion of a G residue at nucleotide position 168 (open arrowhead). Analysis of (C) normal exon 5 and (D) exon 5 of patient 2 shows a T→A transversion at the first base of the stop codon (filled arrowhead), changing it into a codon for lysine. (E) A diagram illustrating the sequences for the mutant SOX10 peptide encoded by the frameshift mutation 168delG from patient 1 and the proline rich C-terminal peptide encoded by the X467K mutation from patient 2. The normal functional domains of the SOX10 protein and all reported SOX10 mutations in WS4 patients are also summarised here, illustrating that mutations around the HMG domain all led to hypoganglionosis or short segment aganglionosis, and mutations in the transactivation domain all led to long segment or total aganglionosis.

Table 2 Summary of SOX10 mutations identified and phenotypes of affected patients

Sex	*SOX10 mutation*	*Effect on protein sequence*	*Affected colon phenotype*	*Reference*
F	nt168delG	Frameshift→truncation before HMG	Short segment HSCR	This study (patient 1)
M	Y83X (C→A at nt249)	Truncation before HMG domain	Hypoganglionosis*	14 (family 2)
NA	nt482ins6	Duplication of LR in HMG domain	Short segment HSCR	14 (family 3)
NA	E189X (G→T at nt565)	Truncation after HMG domain	Short segment HSCR	14 (family 1)
M	Y207X	Truncation after HMG domain	Short segment HSCR	13 (family 140)
M	S251X (C→A at nt752)	Truncation after HMG domain	Total colonic aganglionosis	16 (family 3)
M	Y313X (C→A at nt939)	Truncation after HMG domain	Short segment HSCR	16 (family 2)
M	Y313X (C→A at nt939)	Truncation after HMG domain	Total colonic aganglionosis	16 (family 1)
M	nt1076delGA	Frameshift→37aa added at 359	NA	14 (family 4)
M	Q377X	Truncation of C-terminal domain	Variable diagnosis, ranging from hypoganglionosis to long segment HSCR	13 (family 192)
F	nt1400del12 (X467C)	82aa added at stop codon	Long segment HSCR	15
M	X467K	86aa added at stop codon	Total colonic aganglionosis	This study (patient 2)

NA: data not available from the reference.
*Hypoganglionosis was based on description cited in the reference.

confirmed that the X467K mutation in patient 2 was also sporadic.

Patient 3 has typical features of Waardenburg-Shah syndrome and rectosigmoid aganglionosis. Our mutation analysis on the *SOX10* gene showed no nucleotide change in the entire coding region and in the intron-exon junctions.

Discussion

The mutation identified in patient 1 (168delG) would lead to the production of a truncated protein lacking the DNA binding and the putative transactivation domains (fig 1E). It is likely that the small polypeptide produced from the mutant allele is non-functional. Moreover, the mutant mRNA produced from this mutant allele might be unstable and easily degraded within the cell.[19] Therefore, if the 168delG allele was malfunctional, the phenotype displayed by patient 1 would result from a heterozygous null mutation. This would support the hypothesis that Waardenburg-Shah syndrome is the result of haploinsufficiency of a functional *SOX10* allele.

The other novel mutation we identified (X467K), which affected the stop codon of *SOX10*, would produce a larger protein with an extra C-terminal proline rich domain of 86 amino acids long (fig 1E). Interestingly, a mutation which would produce a similar but shorter mutant protein has been identified in a patient with complex syndromes in addition to WS4; in that case a 12 bp deletion at the stop codon led to the production of an extra 82 amino acid domain.[15] It has been postulated that the C-terminal proline rich domain of this mutant SOX10 protein has a dominant negative effect. In terms of phenotype of the colon, both patients who have similar mutations (patient 2 in this study and that described by Inoue *et al*[15]) have more severe intestinal aganglionosis (total colonic aganglionosis and long segment aganglionosis). However, patient 2 in this study does not have the dysmyelinating features and the other complex syndromes described by Inoue *et al*.[15] Whether the C-terminal proline rich domain contributed to a more severe intestinal aganglionosis in the patients would require further experimental support, possibly in an animal model.

Among the WS4 patients studied so far (including this study), 12 out of 40 have been identified to have *SOX10* mutations.[13–16] Waardenburg-Shah syndrome is a rare malformation, yet mutations in the three known causative genes (*EDN3, EDNRB, SOX10*) have so far only explained some of the patients. On the other hand, *SOX10* mutation per se might not be sufficient to cause WS4. A *SOX10* missense mutation (S135T) has been reported in a case of Yemenite deaf-blind hypopigmentation syndrome.[20] The patient described had characteristics of hypopigmentation, but not all the features of Waardenburg syndrome nor intestinal aganglionosis. In another case, a single nucleotide deletion (795delG) in the *SOX10* gene has been reported in a patient with peripheral neuropathy, hypomyelination, deafness, and chronic intestinal pseudo-obstruction, which are not features of WS4.[21] WS4 patients who have *SOX10* mutations also displayed different severity in the extent of intestinal aganglionosis. Table 2 summarises the 12 cases of WS4 patients with *SOX10* mutations reported so far and their associated intestinal phenotype. Generally, there seems to be a correlation between the specific location of the mutation in the *SOX10* sequence and the severity of intestinal aganglionosis (fig 1E). The reported mutations can be grouped into four categories. (1) Mutations that lead to a truncation of SOX10 protein before the HMG domain (Y83X, 168delG), or that affect the HMG domain directly (482ins6), are functionally equivalent to null mutations, as the mutant proteins would not have any DNA binding activity. These mutations tend to be correlated with a milder phenotype, that is, hypoganglionosis or short segment HSCR. (2) Mutations that lead to a truncation of SOX10 protein shortly after the HMG domain (E189X, Y207X) would produce a mutant protein that potentially would have DNA binding activity but would lack the other functional domains. These mutations have also been shown to cause milder short segment HSCR. (3) Mutations which affect the C-terminal putative transactivation domain (Q377X, 1400del12, X467K) tend to have a stronger effect, leading to long segment or total aganglionosis. (4) The other mutations which cause truncation of the SOX10 protein in between the HMG domain and the C-terminal domain (S251X, Y313X) have more variable phenotypic consequences. In particular, the Y313X mutation detected in two different families actually led to a different intestinal phenotype.[16] A region between the

HMG domain and the transactivation domain of the SOX10 protein is highly conserved between the group E SOX proteins (96% homology)[22] and it could be involved in protein-protein interaction,[23] such that when its structure is affected by mutation it could lead to more severe biochemical consequences.

Among the 12 cases reported, half of the mutations are nonsense mutations resulting in a truncation of the SOX10 polypeptide along its length (table 2). The truncated mutant mRNA produced by the mutant alleles could be subjected to rapid decay because of the mRNA surveillance mechanisms within the cells,[19 24] rendering a haploinsufficient condition. In the case of the Y313X mutation, varied mutant mRNA stability in the two patients might account for the different intestinal phenotype observed.[16] In view of the structure of the SOX10 protein and its organisation of functional domains, we postulate that the WS4 features could be the result of a dominant negative effect of the mutant protein, but not necessarily of haploinsufficiency of normal *SOX10*. Particularly in those cases where mutations of *SOX10* occurred in the C-terminal transactivation domain, the mutant SOX10 protein could gain novel functions and contribute to a more severe phenotype.

As also observed in other studies, there remain a large number of Waardenburg-Shah patients who do not have any *SOX10* mutations within the coding region and splice junctions. Therefore, other mutations within the *SOX10* gene locus or other genes may be responsible for their hypopigmentation and intestinal aganglionosis phenotype. We also found silent and polymorphic DNA changes in the *SOX10* gene of Hirschsprung's disease patients without Waardenburg syndrome (Sham *et al*, unpublished data). Although these nucleotide changes do not affect the amino acid sequence of SOX10 protein, given the multigenic nature of Hirschsprung's disease, the importance of these silent and polymorphic changes in the aetiology of Hirschsprung's disease has yet to be evaluated.

- Two novel *SOX10* mutations were identified in two of the three WS4 patients we examined. One of the mutations was a single nucleotide deletion in exon 3 (168delG), which led to a frameshift mutation producing a truncated SOX10 protein. The other mutation was at the stop codon in exon 5, which led to a mutant protein with an extra 86 amino acid long, proline rich peptide at the C-terminus. Both of these mutations were sporadic.
- We analysed all the reported *SOX10* mutations in WS4 patients and found that mutations around the HMG domain would lead to hypoganglionosis or short segment HSCR; mutations that affect the C-terminal transactivation domain would lead to long segment or total aganglionosis.
- *SOX10* mutations affecting the C-terminal transactivation domain might produce mutant proteins that have a dominant negative effect, which account for a more severe intestinal aganglionosis phenotype in the patients.

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