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TITLE PAGE

Title: Involvement of *SOX10* in the pathogenesis of Hirschsprung disease: report of a truncating mutation in an isolated patient.

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Abstract

SOX10 protein is a key transcription factor during neural-crest development. Mutations in *SOX10* are associated with several neurocristopathies such as Waardenburg syndrome type IV (WS4), a congenital disorder characterized by the association of hearing loss, pigmentary abnormalities and absence of ganglion cells in the myenteric and submucosal plexus of the gastrointestinal tract, also known as aganglionic megacolon or Hirschsprung disease (HSCR). Several mutations at this *locus* are known to cause a high percentage of WS4 cases, but no *SOX10* mutations had been ever reported associated to isolated HSCR patient. Therefore, non-syndromic HSCR disease was initially thought not to be associated to mutations at this particular *locus*. In the present study, we describe the evaluation of the *SOX10* gene in a series of 196 isolated HSCR cases, the largest patient series evaluated so far, and report a truncating c.153-155del mutation. This is the first time that a *SOX10* mutation is detected in an isolated HSCR patient, which completely changes the scenario for the implications of *SOX10* mutations in human disease, giving us a new tool for genetic counselling.

Key Words: SOX10, Hirschsprung disease, neurocristopathology, Waardenburg syndrome, enteric nervous system.

Introduction

SOX10 encodes a 466-amino-acid transcription factor belonging to an evolutionary conserved protein family, which contains a central high mobility group (HMG) DNA-binding/DNA-bending domain and a C-terminal transactivation domain [1, 2]. In a manner similar to all members of this family, *SOX10* exerts its function through binding to the promoters or enhancers of its target genes, alone or in association with other transcription factors [3, 4]. *SOX10* is a key transcription factor during neural-crest derived cells migration and differentiation, and *MITF*, *TYR*, *TRP2*, *MPZ*, *GJB1*, *RET*

and *EDNRB* are known to be target genes for *SOX10* regulation [3, 4, 5, 6, 7, 8, 9, 10, 11]. *SOX10* modulate gene expression of pluripotent neural-crest cells that migrate from the neural tube throughout the embryo along several pathways during embryogenesis. Those precursors give rise to enteric neurons and glia, some of the craniofacial skeletal tissue, melanocytes of the skin and inner ear, in addition to other cell types [12].

The first pathogenic mutations described for *SOX10*, was associated with the **Waardenburg syndrome type IV** (WS4, OMIM 277580) [13], a congenital disorder characterized by hearing loss and pigmentary abnormalities due to an abnormal proliferation, survival, migration, or differentiation of neural-crest-derived melanocytes, combined with absence of ganglion cells in the myenteric and submucosal plexus of the gastrointestinal tract, also known as aganglionic megacolon or Hirschsprung disease (HSCR, OMIM 142623) [14]. The association of WS to intestinal pseudo-obstruction instead of HSCR disease is also observed in some WS4 patients [15]. **Several subtypes of WS are defined on the basis of the presence of additional symptoms, being known as WS2 the presentation of clinical features of WS alone.** WS4 phenotype has been reported to be caused by *SOX10* point mutations **as well as gross deletions, presented** in 45-55% of patients [16]. Most of such *SOX10* point mutations responsible for WS4 generate premature stop codons arising as *de novo* events in the patients [17]. Of note, no *SOX10* mutations have been ever reported to date associated to isolated HSCR patient.

On the other hand, heterozygous *SOX10* mutations are associated with a large range of phenotypes beyond WS4, including WS2 (pigmentation defects and deafness but no HSCR), and clinical features that reflect an involvement of the central and/or peripheral nervous system regrouped under the name of PCWH (Peripheral demyelinating neuropathy, Central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease; OMIM 609136) [18]. Taking the phenotype variability observed so far, *SOX10* may be regarded as an interesting candidate gene

in other neurodegenerative disorders and neurocristopathies. In this sense, the implications of *SOX10* in the pathogenesis of HSCR have been largely discussed. A correlation between HSCR phenotype in WS4 patients and severity of *SOX10* mutations has been observed [19]. However, since no *SOX10* mutations could be detected in a series of patients with isolated HSCR, non-syndromic HSCR disease was initially thought not to be associated to mutations at this particular *locus* [13]. In the present report we have sought to completely determine if *SOX10* plays any kind of role in the pathogenesis of HSCR, by a screening of both point mutations and gene-dosage anomalies of its coding sequence in a series of 196 HSCR patients, the largest patient series evaluated so far.

Materials and Methods

Patients and control subjects

In this study we have included a total of 196 patients presenting with HSCR (22% female, 78% male), in one of those patients HSCR phenotype was associated to Waardenburg–Shah syndrome, while in the remaining 195 cases were isolated HSCR. 176 were sporadic cases, while 20 were familial cases belonging to 13 different families.

In addition, we have also analyzed a group of 150 normal controls comprising unselected, unrelated, race, age, and sex-matched individuals. An informed consent was obtained from all the participants for clinical and molecular genetic studies. The study conformed to the tenets of the declaration of Helsinki.

PCR, dHPLC analysis, sequence analysis, and QMF-PCR

Genomic DNA was extracted from peripheral blood leukocytes from patients and healthy controls, using standard protocols. Primers were designed for the mutational screening of the *SOX10* coding region, the intron/exon boundaries and the 5' and 3' untranslated regions (UTRs). Primers and PCR conditions are available on [supplementary table 1](#). The mutational screening was carried out on an automated

dHPLC equipped with a DNA separation column using the WAVE DNA Fragment Analysis system (Transgenomic, Omaha, NE). Those samples with aberrant wave profiles were subjected to sequence analysis using an automated sequencer ABI 3730 and the software SeqScape Ver.2.5. (Applied Biosystems, Foster City, CA). When a novel sequence variant was detected, the exon harbouring the change was also screened in a group of 150 normal controls, as well as in all available family members of the proband, in order to evaluate the putative pathogenicity of the sequence variant underlying such profile.

To evaluate gene-dosage anomalies within *SOX10* coding sequence we used a modification of the QMF-PCR method described elsewhere [16]. We amplified in a multiplex reaction the three coding exons of *SOX10*, exon 4 of *POLR2F*, and a region located 5' of *SOX10*, using QIAGEN multiplex PCR kit (Qiagen, Germantown, MD). In addition, two control amplicons were used: *DSCR1*, located on chromosome 21, and *F9*, located on chromosome X. Primers and PCR conditions are available on [supplementary table 2](#). Fragment analysis was performed using the 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and for data analysis we used GeneMarker v 1.6 (Softgenetics L.L.C) comparing patients tested with controls. Dosage quotients under 0.6 were considered as indicating potential deletions.

Plasmids, Cell Culture, and Transfection

The pECE-SOX10, pECE-SOX10-E189X, pECE-PAX3, pECE-EGR2, pGL3-MITFdel1718, and pGL3-Cx32 vectors were described elsewhere (Bondurand et al., 2000, 2001). To generate pCMVMyc-SOX10, the human *SOX10* cDNA (GenBank accession number AJ001183) was amplified by PCR using the primers 5'-TTGCTAAGAATTCATGGCGG-AGGAGCAGGATATC-3' and 5'-AATAATGCGGCCGCCTTCTCCTCTGTCCAGCCTG-3' and pECE-SOX10 as a template. The PCR product was then cloned in the pCMV-Myc (Clontech, Mountain View, CA) after *EcoRI* and *NotI* digestion. The mutations c.112_131del and c.153-155del were introduced independently within the pECE-SOX10 and pCMV-Myc-SOX10

constructs by site-directed mutagenesis using the Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The nucleotide sequence of each construct was verified by direct sequencing as described above.

HeLa cells were grown in DMEM supplemented with 10% fetal calf serum and transfected using Lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA).

Reporter Assays

Cells were plated on 12-well plates and transfected 1 day after with 0.150 μg of each effector and reporter plasmid. The total amount of plasmid was kept constant by addition of empty pECE vector. Twenty-four hours after transfection, cells were washed twice with PBS, lysed and extracts were assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI) as described before [5, 6, 20]. **As far as competition assays are concerned, increasing amount of mutant SOX10 plasmids (0.150, 0.300 or 0.600 μg) were mixed with a fixed amount of wild type SOX10 (0.150 μg) and the reporter pGL3-Cx32 plasmid (0.150 μg). DNA per well was kept constant by adding empty pECE vector.**

Immunostaining

Cells were plated on 24-well plates and transfected 1 day after with 350 μg of each SOX10 construct. Twenty-four hours after transfection, cultures were fixed in 4% PFA for 10 minutes at RT. After washing twice in PBS + 0.1% Triton X-100 (PBT), they were incubated with blocking solution (PBS + 1%BSA +0.15%glycine) at 4°C overnight. Primary antibodies were diluted in blocking solution as follows: SOX10-N20 (goat; Santa Cruz Biotechnology, Santa Cruz, CA) 1:50, c-Myc (mouse; BD Biosciences, Palo Alto, CA) 1:100. Incubation with primary antibody was performed at 4°C overnight. After several washes with blocking solution, secondary antibodies were added for 2 hours at RT at the following dilutions in blocking solution: anti-goat Alexa Fluor 568 (Invitrogen, Carlsbad, CA) 1:1/500, anti-mouse Alexa Flour 568 (Molecular Probes, Eugene, OR) 1:100. Cells were mounted in Vectashield medium containing DAPI

(Vector Laboratories, Burlingame, CA) and fluorescence images were examined with a Leica DMR epifluorescence microscope. Alternatively Cells were counter stained with TO-PRO-3-iodide (Molecular Probes; 1:5000 in PBS), mounted using Vectashield without DAPI, and examined with Zeiss Axioplan 2 confocal microscope. Images were analysed using Metaphor software package.

Results

We have analysed the coding region of *SOX10* in 196 HSCR patients, using dHPLC and QMF-PCR technology. We failed to detect any gene-dosage anomalies in our HSCR cases; however a total of 12 sequence variants were detected (Table 1). 10 out of 12 variants consisted on synonymous, intronic changes, or variants located in the untranslated regions of the gene. Because those variants generate no alteration at the protein sequence level, it was more probable that their pathogenic mechanism, if any, would affect transcript stability or RNA splicing. However, we found no evidences for those variants to produce any alteration of transcriptional and/or splicing processes when they were submitted to several Splice Sites and Transcription Factors Binding sequences prediction interfaces (http://www.fruitfly.org/seq_tools/splice.html; http://www.fruitfly.org/cgi-bin/seq_tools/promoter.html; <http://www.ebi.ac.uk/asd-srv/wb.cgi>).

The most interesting findings in the present study are two novel *SOX10* mutations, c.112_131del and c.153-155del. Both of those heterozygous mutations cause a frameshift that alters the mRNA sequence generating a totally different protein sequence starting at position 38 and 52 respectively. In addition the mutations introduce premature termination codons at positions 58 and 108 respectively. As a result, both of those truncating proteins are lacking the HMG DNA binding domain, the two independent nuclear localization signals within this domain, and the C-terminal transactivating domain (Figure 1), essential for the protein function. One of the two mutations (c.112_131del) was detected, as a *de novo* event, in a female WS4 patient.

More interesting is the finding of the mutation c.153-155del in a male patient presenting with isolated short-segment HSCR. As long as this is the first report of a truncating *SOX10* mutation in an isolated HSCR case, a more detailed inspection of the whole clinical data of the patient at the age of fifteen was fulfilled. This examination failed to detect any other phenotypic features such as pigmentary abnormalities, chronic intestinal pseudo-obstruction, microcornea, coloboma, nerve lesions, peripheral demyelinating neuropathy, or central dysmyelinating leukodystrophy associated with *SOX10* mutations [17, 18]. Heterochromia irides were also discarded as both eyes were brown. To rule out any slight loss of hearing in this patient with no auditory problems an auditory brainstem response was performed demonstrating no sensorineural deafness in this patient. Mutation c.153-155del was inherited from his unaffected mother. No additional point mutations were detected in this patient in other HSCR susceptibility genes; namely *RET*, *GDNF*, *NRTN*, *PSPN*, *ARTN*, *EDNRB*, *EDN3*, *NTF3*, *NTRK3* or *PHOX2B*.

To test the functional consequences of the two mutations reported here, we first attempted to visualize the localization of the protein *SOX10*, wild-type (wt) and mutants (mt) within the cell. We introduced the two mutations independently into the *SOX10* cDNA and performed immuno-fluorescence experiments on HeLa cells transiently transfected with wt and each of the mt construct. The results revealed aberrant localization of both mutant proteins into the cytoplasm, in contrast to wt protein located only in the nucleus (Figure 2). Due to the small protein size of the two mutants, and in order to avoid a possible misrecognition of the antibody used, we replicate the experiments using the myc-*SOX* construction and the same results were observed (Figure 2). Mutant proteins could be also localized in the nucleus, as it was expected due to small protein size. Proteins below 40 kDa are known to cross the nuclear pores complexes via passive diffusion [21]. The molecular mass of our two mutant were predicted to be 5.76 and 10.67 kDa respectively by Compute pI/Mw tool Expasy (http://expasy.org/tools/pi_tool-ref.html), explaining the presence of the mutants in the

nucleus. To confirm that the mutant proteins present in the nucleus were not functional, we analysed the transactivation capacity of both mutants on two promoters previously shown to contain monomeric or dimeric SOX10 binding sites, MITF and Cx32 respectively [5, 6]. Cotransfection of either promoter with wild-type, c.112_131del or c.153-155del SOX10 mutants and/or SOX10 cofactors (PAX3 and EGR2) revealed that both mutants failed to transactivate these reporter construct, alone or in synergy with the cofactors, as promoter activation was drastically reduced (Figure 3A, B). To determine the ability of SOX10 mutant proteins to interfere with wild-type SOX10 function, we also we carried out competition assays by co-transfecting mutant and wild-type SOX10 expressing vectors together with the pGL3-Cx32 reporter plasmid. As previously described, E189X mutant decreased the transcriptional activity of wild-type protein (Inoue et al., 2004) in a dose dependent manner. However, the two mutants reported here showed no such effect (Figure 3C). Their lack of dominant negative activity may explain why they are associated with a weaker phenotype.

Discussion

All the members of the Sox protein family contain a HMG box as their DNA-binding domain. This HMG box contains three α -helical regions arranged in a twisted L shape for sequence-specific recognition, leading a large conformational change in the DNA. Two independent nuclear localization signals in the protein are located at the extremities of this DNA-binding domain [3]. SOX10 protein also includes a transactivation domain, a sequence of 113 amino acids in the carboxyl-terminal region, required for transcriptional activation [3]. Several *in vitro* studies have shown the functional consequences of different SOX10 mutations located all over its coding sequence, as no hot spots have been found for mutations at this *locus* [3, 17, 18, 22, 23]. Most of nonsense mutations affecting the first few exons of the gene encode small aberrant mRNAs that are eliminated before translation, generating haploinsufficiency due to nonsense mediated decay of truncated mRNA, leading to WS4 phenotype [17,

18, 23]. In contrast, mutations affecting the last exon, which encode the transactivation domain, escape this mechanism and show a dominant negative effect resulting in more severe PCWH phenotype [18]. However, the recent observation of whole SOX10 deletions in patients presenting with PCWH suggests that mechanisms other than NMD might explain phenotype variability [16].

The two frameshift mutations reported here are lacking all functional domains, since wild-type sequence protein expands only to position 37 and 52 respectively. Our *in vitro* functional assays demonstrate not only that mutant proteins have an aberrant cytosolic localization, but also that transactivation **and dominant negative activities are** absent in the two mutants tested. Therefore, we propose haploinsufficiency as the most plausible mechanism leading to WS4 and isolated HSCR in the two respective patients.

The association between syndromic HSCR and SOX10 gene has been well established, since most of the patients harbouring heterozygous mutation at this locus present with HSCR as a part of their clinical features. In fact, very few patients with a mutation at SOX10 not presenting with HSCR, or other enteric phenotypes, have been reported [13, 16, 22, 24, 25, 26], **although SOX10 mutations show a wide phenotypic heterogeneity**. Interestingly, for these patients with isolated WS features (WS2), tissue-specific compensation in the ENS by other SOX family proteins has been suggested, so that SOX10 mutations could only affect a certain portion of neural-crest-derived cells. **Other genes, responsible for WS4 phenotype, have shown to produce HSCR phenotype alone, as it occurs in patients with heterozygous mutations in EDNRB and EDN3 genes (Svensson et al., 1999; Attie et al., 1995; Edery et al., 1996; Hofstra et al., 1996; Verheij et al., 2002). Nonetheless, no mutation at SOX10 gene has been found in an isolated HSCR patient until now. This finding suggests that SOX10 mutations responsible for central and peripheral myelin disorders without clinical features of WS or enteric alteration might be found in those patients despite the negative results found so far (Pigault et al., 2001).**

The c.153-155del mutation presented here is the first *SOX10* mutation reported in an isolated HSCR patient without any other additional WS feature. It is difficult to explain how the mutation in this patient might affect the portion of neural-crest-derived cells colonizing the gut, responsible for ENS formation, and not other neural-crest-derived tissues. The possibility for the patient to be a mosaic seems unlikely, as the mutation was inherited from his healthy mother, who shows no clinical features of WS or HSCR phenotypes after a careful anamnesis and detailed clinical examination. The lack of clinical symptoms for the mother could be explained by the sex-dependent penetrance of mutations generally observed in HSCR. Another possibility to explain this phenomenon could be a germline mosaicism for the mother, although the mother refused to be tested for that possibility, in any case this phenomenon would not explain the penetrance of her condition but rather affect the recurrence risk of the family.

The lack of symptoms suggestive of WS in the HSCR patient harbouring a *SOX10* mutation might be also explained by the nature of the mutation itself. It was demonstrated that mutations leading to a truncation of *SOX10* protein before the HMG domain rather than having a dominant negative effect, are functionally equivalent to a null mutation and generate haploinsufficiency in the cell [18]. These mutations tend to be correlated with milder HSCR and WS phenotypes, and this could be the case for the c.153-155del. Haploinsufficiency could be more dramatic in the ENS development than in other neural-crest derivatives (melanocytes of the skin or inner ear), in which the reduced amount of normal protein might be supplied by a redundant function in other members of *SOX* protein family with a differential expression pattern, such as *SOX8*, *LSox5* or *SOX22* [27]. In fact, functional redundancy between *SoxE* group of genes (*SOX8*, *SOX9* and *SOX10*) has been proposed. Moreover, loss of function of *Sox8* gene in *Sox10* heterozygous mouse embryos increases penetrance and severity of colon aganglionosis [28]. This is the first time that a *SOX10* mutation do not give rise to any feature suggestive of WS, so that additional phenomena might be occurring in this patient to explain the distinct phenotype observed, such as an enhanced expression of

the wild type allele in the non affected tissues. In addition, given that Hirschsprung disease is the paradigm of a complex and polygenic disease, we could also propose an involvement of other modifier genes still unidentified, that together with SOX10 would act to produce isolated enteric phenotype.

In summary, the most relevant result of our study has been the detection of a SOX10 mutation in a patient presenting with isolated HSCR, which points out the association of this gene with the pathogenesis of HSCR *per se*, not only as a part of a syndromic trait. This finding gives a new perspective of the SOX10 role in HSCR and constitutes a new step towards the dissection of the polygenic nature of this disease.

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Tables

Table 1. *SOX10* sequence variants detected in the present study.

Figure Legends

Figure 1. SOX10 Protein. Representation of SOX10 protein with the transactivation domain and the HMG box, which shows the sequences for the two frameshift mutations found in a WS4 and isolated HSCR patients respectively.

Figure 2. Subcellular localization of wild-type and mutant SOX10 proteins in transfected cells. SOX10 expression is detected in red and DAPI fluorescence reveals nuclei in blue in HeLa cells transfected with wild-type or mutant (c.112_132del and c.153-155del) Myc-SOX10 and SOX10 expression vectors.

Figure 3. Transactivation capacity of wild-type and mutant SOX10 proteins. The MITF promoter (pMITF) (A) or the GJB1 (pCx32) promoter (B) luciferase reporters were transfected in HeLa cells in combination with wild-type (WT) or mutant SOX10 proteins (c.112_132del, c.153-155del and E189X), and/or PAX3 (A) or EGR2 (B). Competition assays were performed by increasing amounts (1X, 2X and 4X) of mutant SOX10 expression plasmid (E189X, c.112_132del, and c.153-155del), that were mixed with a fixed amount of wild-type SOX10 expression plasmid and cotransfected with the GJB1 promoter luciferase reporter plasmid (C). Reporter-gene activations are presented as fold induction relative to the empty expression vector (pECE). Results represent the mean \pm SEM from three experiments, each performed in duplicate.