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Transcriptional regulation of *TLX2* and impaired intestinal innervation: possible role of the *PHOX2A* and *PHOX2B* genes

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TLX2 (also known as *HOX11L1*, *Ncx* and *Enx*) is a transcription factor playing a crucial role in the development of the enteric nervous system, as confirmed by mice models exhibiting intestinal hyperganglionosis and pseudo-obstruction. However, congenital defects of *TLX2* have been excluded as a major cause of intestinal motility disorders in patients affected with intestinal neuronal dysplasia (IND) or pseudo-obstruction. After demonstrating the direct regulation of *TLX2* expression by the homeoprotein *PHOX2B*, in the present work, we have focused on its paralogue *PHOX2A*. By co-transfections, electrophoretic mobility shift assays and chromatin immunoprecipitation, we have demonstrated that *PHOX2A*, like *PHOX2B*, is involved in the cascade leading to *TLX2* transactivation and presumably in the intestinal neuronal differentiation. Based on the hypothesis that missed activation of the *TLX2* gene induces the development of enteric nervous system defects, *PHOX2A* and *PHOX2B* have been regarded as novel candidate genes involved in IND and pseudo-obstruction and consequently analyzed for mutations in a specific set of 26 patients. We have identified one still unreported *PHOX2A* variant; however, absence of any functional effect on *TLX2* transactivation suggests that regulators or effectors other than the *PHOX2* genes must act in the same pathway, likely playing a non redundant and direct role in the pathogenesis of such enteric disorders.

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Introduction

TLX2 (also known as *HOX11L1*, *Ncx* and *Enx*) is a homeodomain transcription factor playing a crucial role in the development of the enteric nervous system, as confirmed by three independent *Tlx2*^{−/−} mouse models displaying an intestinal phenotype ranging from pseudo-obstruction¹

to megacolon with giant enteric ganglia.^{2,3} The phenotype of these latter animal models strongly resembles a human congenital disorder named intestinal neuronal dysplasia (IND), that has been described for the first time by Meier-Ruge in 1971.⁴ Though two different types of IND have been defined (IND type B; OMIM #601223 and IND type A; OMIM #243180), IND has become synonymous with IND type B, that is the most common type and is regarded as a malformation of the enteric nervous system ganglionated plexuses characterized by hyperplastic features. On the contrary, IND type A presents in neonates as bloody diarrhea with intermittent obstructive symptoms and is characterized by severe hypoplasia of adrenergic innervation in the gut wall. Despite formal diagnostic histopathological criteria for IND have been described,⁵ the diagnosis of IND remains not always reliable. For this reason, IND is still a subject of controversy.^{6,7} The observation of a few familial clusters^{8,9} and animal models^{1–3} provide robust pieces of evidence that IND is a real clinical entity. In particular, in addition to *Tlx2*–/– mouse models, a heterozygous endothelin B receptor-deficient rat demonstrated abnormalities of the submucous plexus similar to that observed in human IND.¹⁰ However, no mutation affecting the coding region and 2 kb of 5′-flanking region of *TLX2* gene have been found in a set of IND patients^{9,11} and, similarly, no mutations of the *EDNRB* gene have been detected in a small series of IND and Hirschsprung disease (HSCR)/IND patients.¹²

Although a role of *TLX2* in human intestinal pseudo-obstruction and/or IND seems excluded, variants of upstream regulators of the gene may account for *TLX2* incorrect or absent expression during the development of the intestinal neurons, and therefore for pathological conditions resembling the knockout mice phenotypes. Recently, *TLX2* has been recognized by us as a PHOX2B target, presumably mediating the PHOX2B signal in the developing peripheral nervous system.¹³ *PHOX2B* is a homeobox gene crucial for the differentiation of the autonomic nervous system, whose mutations have been detected in Congenital Central Hypoventilation syndrome,^{14,15} a neurocristopathy often associated with HSCR, the most frequent intestinal innervation defect in human.

In the present work, after demonstrating that *TLX2* is also a transcriptional target of the PHOX2B paralogue PHOX2A gene, we have performed a screening analysis of these *PHOX2* genes in a heterogeneous set of patients affected with impairments of the intestinal innervation including pseudo-obstruction and IND.

Methods

Constructs and transfections

Cloning of *TLX2* 5′-regulatory region and of both *PHOX2A* and *PHOX2B* wild-type cDNA sequences has already been

reported.^{11,16,17} The *PHOX2A* mutant construct was generated by PCR starting from the wt corresponding construct, as described previously.¹³ Oligonucleotides used for site-directed mutagenesis were: 5′-GCCAAGGGCGCGGCG GGCGCCAAAAAGGG-3′ and 5′-CCCTTTTGGCGCCCG CCGCGCCCTTGGCGCTGG-3′.

SK-N-BE neuroblastoma and HEK293 cells (10⁵) were plated on 35 mm Petri dishes 1 day before transfection. Constructs under analysis and *Renilla luciferase* reporter plasmid pRL-CMV (Promega), used as a transfection efficiency control, were co-transfected using Eugene 6 (Roche). In particular, 160 fmoles of the expression constructs were mixed with 40 fmoles of constructs containing *TLX2* regulatory region and 20 fmoles of pRL-CMV and added to 3 µl of Eugene 6. The empty pcDNA3.1 vector was co-transfected with the promoter–reporter construct as negative control.

PHOX2A binding study

Electrophoretic mobility shift assays (EMSA) were performed using SK-N-BE and IMR32 nuclear extracts (NE), prepared as described previously.¹³ Six micrograms of NE were incubated with the ^γ32P-labeled probe 5′-GGGGAAGGTAATGTAATTCGGCCCC-3′ for 20 min at room temperature in binding buffer (hepes 20 mM pH 7.9, glycerol 20%, EDTA 1 mM, KCl 50 mM, DTT 1 mM, PMSF 0.5 mM) with 2 µg of poly (dI-dC). For supershift assays, antibodies were incubated with the NE mix for 30 min on ice before adding the specific probe. A polyclonal antibody against a peptide corresponding to a sequence in the carboxy-termini of PHOX2A was produced in chicken egg yolk.¹⁸ Chromatin immunoprecipitation assay (ChIP) was performed using formaldehyde cross-linked and sonicated chromatin from IMR32 cells as already described.¹⁸ The *TLX2* cell-specific promoter region was amplified by PCR using the primers 5′-CGGGAAC CAGCAGGATGGAG-3′ and 5′-GAGAAGGGAGGTGGG AAAGAC-3′ as already reported.¹³

Expression analysis

Endogenous levels of PHOX2A protein has been analyzed on NE by means of Western blot experiments using the above PHOX2A polyclonal antibody. The filter has been stripped and re-probed with an anti-Sp1 antibody (Upstate Biotechnology), used as a control for the integrity of the NE. The expression of PHOX2A protein after transfection, with the corresponding expression constructs has been confirmed on total lysates from transfected cells. A β-actin antibody (Sigma) has been used as control.

Patients and controls

A total of 22 patients with both sporadic and familial IND and 4 patients affected with chronic intestinal pseudo-obstruction were analyzed. Histochemical diagnosis of the IND cases, collected from 1991 to 2004, was performed

both preoperatively and intraoperatively according to the reported criteria.⁵ The clinical diagnosis of chronic intestinal pseudo-obstruction was made following the criteria already reported.¹⁹

Eighty individuals of Italian origin were analyzed as controls. DNA was extracted from either blood samples or cell pellets as already described.⁹

The present study has been approved by the Gaslini Institute ethics committee (Genova, Italy).

Mutation screening

PHOX2A coding sequence was analyzed for mutations by PCR under the conditions reported in Table 1 followed by denaturing high-performance liquid chromatography (DHPLC) analysis (Transgenomics), according to instructions of the supplier. Samples showing anomalous chromatographic profiles were sequenced by using BIG DYE v1.1 and 3130 automated sequencer (Applied Biosystems).

The 9 nt deletion thus identified was confirmed by cloning the PCR product obtained from patient's and father's DNAs in TA-cloning vector (Invitrogen) and subsequent sequencing of several bacterial colonies.

Results

Transactivation of the human *TLX2* gene by *PHOX2A*

Starting from previous results demonstrating the capability of *PHOX2B* to specifically bind two 'ATTA' repeats located

in the *TLX2* promoter, thus inducing its transcription in neural crest-derived cells,¹³ we have focused on the possible role of its paralogue *PHOX2A* in the same pathway.

Co-transfection in SK-N-BE neuroblastoma cells of the *PHOX2A* expression construct, able to correctly induce expression of *PHOX2A* protein as demonstrated in Figure 1b, together with the *TLX2* promoter construct, bearing the *luciferase* cDNA, showed an increased gene reporter activity of approximately two fold, in comparison with the empty pcDNA3.1 expression vector (Student's *t*-test $P < 0.05$). As already observed for *PHOX2B*, specific mutations at the identified 'ATTA' repeats in the promoter sequence prevented the transactivation by *PHOX2A* (Figure 2a). To determine whether *PHOX2A* and *PHOX2B* could have either overlapping or opposite effects on *TLX2* promoter, co-transfections of both expression constructs were carried out in SK-N-BE cells. The ability of the same *PHOX2B* expression construct to induce levels of *PHOX2B* protein has previously been demonstrated by Western blot analysis.¹⁷ As displayed in Figure 2a, after double co-transfection, the reporter gene resulted approximately 5.8-fold induced compared to the empty pcDNA3.1 expression vector. This activation level was not statistically different from that obtained transfecting *PHOX2A* or *PHOX2B* constructs alone,¹³ as shown by the Student's *t*-test.

The direct interaction between *PHOX2A* and the 'ATTA' repeats was investigated by EMSA. Previously, the specificity of the complex formed by IMR32 and SK-N-BE NE with

Table 1 PCR conditions

	PCR size (bp)	Forward primer	Reverse primer	Protocol ^b	PCR conditions ^a MgCl ₂ (mM)	Enhancer
Exon 1	346	5'-acctccacccggaccccgac-3'	5'-agcgggccagggattc-3'	95°C 1' 60°C 1' 72°C 2' 40 cycles	1	DMSO 5%
Exon 2	296	5'-cgggttgaaactctgcttctcac-3'	5'-catgcgcactctcgtaacac-3'	95°C 1' 60°C 1' 72°C 2' 40 cycles	1	—
Exon 3A	227	5'-gatctcactcgagccttgc-3'	5'-ctgcacgtggactccttga-3'	95°C 30" 62°C 30" 72°C 45" 35 cycles	1.5	Glycerol 10%
Exon 3B	269	5'-cgggccaaagtccgcaaacaggag-3'	5'-agtgcgcccttgagcggctgtgg-3'	95°C 30" 62°C 30" 72°C 45" 35 cycles	1.5	Glycerol 10%
Exon 3C	272	5'-gctgccgctgcactgggctcc-3'	5'-ggacgtctctggggcaggctcgga-3'	95°C 1' 62°C 1' 72°C 1'30" 40 cycles	1	Glycerol 10%

^aSixty nanograms of DNA were amplified in 25 µl reaction containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 200 µl dNTPs and 1.25 U *Taq* polymerase (TaqGold, Applied Biosystem) with 1 µM of each appropriate PCR primers. For exon 1 amplification, Taq Platinum (Invitrogen) was used in the same conditions.

^bAn initial denaturation step at 95°C for 10 min (2 min in case of exon 1) and an extension cycle at 72°C for 7 min at the end were performed.

Figure 1 Analysis of proteins expression. PHOX2A expression has been assayed by Western blot analysis on NE to assess the endogenous protein level (a) on total lysates of transfected SK-N-BE (b) and HEK 293 (c) to confirm the functionality of all expression constructs used in the study. Empty = lysate from cells transfected with empty pcDNA 3.1 expression vector; 2A = lysate from cells transfected with wild-type PHOX2A expression construct; 2A mut = lysate from cells transfected with mutant PHOX2A expression construct; 2A + 2B = lysate from cells transfected with both wild-type PHOX2A and PHOX2B expression constructs.

a probe containing the wt *PHOX2* binding site had already been proven by using wt and mutated cold competitors.¹³ In the current experiment, direct binding at the sequence under analysis has been demonstrated following pre-incubation of PHOX2A expressing IMR32 and SK-N-BE NE (Figure 1a) with a specific polyclonal antibody α -PHOX2A (Figure 2b, lanes 2 and 4). In particular, complex A was preserved and, in addition, a prominent, slightly supershifted band was formed, as already reported for PHOX2A binding to sequences in the dopamine β -hydroxylase promoter.²⁰

To confirm that the interaction between PHOX2A and the *TLX2* promoter occurs *in vivo*, we performed chromatin immunoprecipitation using formaldehyde cross-linked and

sonicated chromatin from IMR32 cells and the antibody used in the EMSA experiments. IMR32 cells have been used in this case since they express both PHOX2A (Figure 1a) and PHOX2B,¹³ a circumstance that allowed to investigate whether *in vivo* binding of one of these two factors can exclude binding of the other one. Results of immunoprecipitated DNA amplification, represented in Figure 2c, showed that, similarly to the known interacting PHOX2B antibody used as positive control, the PHOX2A-specific antiserum effectively immunoprecipitated the *TLX2* regulatory sequence bearing the 'ATTA' repeats, while the control assay performed with normal chicken IgY did not present any amplified DNA.

Screening of *PHOX2A* and *PHOX2B* coding sequences in patients

Starting from the observations that (1) the *PHOX2* genes are regulators of the *TLX2* gene expression and (2) the *TLX2* gene is involved in the development of correct intestinal innervation, we have hypothesised a role of the *PHOX2* genes in enteric nervous system disorders. To this end, we have undertaken the study of the coding region of *PHOX2A* and *PHOX2B* genes, in a series of 22 patients affected with IND and 4 patients affected with intestinal pseudo-obstruction. IND patients have been recruited according to criteria already described⁵ which, though hotly debated,²¹ are those currently used for the diagnosis of IND.²²

The majority of these patients had already been tested for possible nucleotide changes in the coding and promoter sequences of the *TLX2* gene, without finding any alteration.^{9,11}

We have performed a mutation screening of the three exons spanning the entire coding sequence of the *PHOX2A* gene by means of PCR amplification and DHPLC analysis (Table 1). In the exon 1 of an IND patient, we have found an already reported synonymous heterozygous nucleotide substitution c.156C>T of the Leu52 codon.²³ Moreover, another IND patient presented a heterozygous G>A nucleotide change in the 5'-UTR, 19 bp upstream of the first ATG codon. This has resulted to be a common polymorphism, being detected also in 3 of 30 control individuals. The screening of exon 2 did not reveal any coding variant but a common polymorphism consisting of the insertion of a C nucleotide within a poly-C stretch at the distal end of intron 1, present in 6 of our patients and 5 controls among 30 individuals. Finally, a patient affected with IND showed a heterozygous in-frame deletion of 9 nt in the third exon, leading to the deletion of three amino acids close to the C terminus of the protein. In particular, this mutation determines the lack of a duplicated Glu-Ala-Ala peptide sequence, 10 amino acids downstream of the homeodomain. Analysis of the patient's parents has revealed that the deletion was inherited from the asymptomatic father (Figure 3). This deletion has resulted absent in 160 control alleles.

Figure 2 Transactivation of the human *TLX2* gene by PHOX2A. (a) *Luciferase* activity displayed by either wt or 'ATTA' mutant *TLX2* promoter-reporter construct measured on lysates from SK-N-BE cells co-transfected with both the empty pcDNA 3.1 and the pcDNA 3.1-PHOX2A expression construct; this latter in particular was assayed either alone or together with the pcDNA 3.1-PHOX2B expression construct. Values represent fold induction *versus* activity derived from transfection of the corresponding *TLX2* promoter-reporter construct alone and have been obtained by three independent experiments performed in duplicate. (b) EMSA assays performed incubating NE from IMR32 and SK-N-BE cells with radiolabeled double-stranded oligonucleotide corresponding to 'ATTA' repeats in the *TLX2* promoter sequence in the absence (lanes 1 and 3) or in the presence of the antibody α -PHOX2A (lanes 2 and 4). (c) ChIP assays performed on chromatin derived from IMR32 cells. Primers specific for *TLX2* promoter sequence bearing 'ATTA' repeats were used to amplify DNA from complexes immunoprecipitated with an α -PHOX2A antibody. Chicken IgY and the antibody α -PHOX2B were used as negative and positive controls, respectively. Input = fragmented DNA before immunoprecipitation.

A mutation screening of the three exons spanning the entire coding sequence of the *PHOX2B* gene has been performed by PCR and direct DNA sequencing analysis, as already reported.¹⁵ Two patients affected with IND presented an already described synonymous heterozygous nucleotide substitution c.762C>A, involving the Ala254 codon.^{24,25} A contraction of seven alanines in a stretch of 20 Ala residues, already reported in a patient affected with schizophrenia²⁵ and rarely found in controls,¹⁴ was shown in another IND patient.

Functional analysis of the mutant PHOX2A
To assess a possible role of the newly identified *PHOX2A* mutation in impaired intestinal motility of the correspond-

ing carrier, we assayed the ability of this variant to induce trans-activation of the regulatory region of *TLX2*.
To this end, an expression construct corresponding to *PHOX2A* containing the observed deletion was co-transfected together with a *luciferase* reporter construct containing the *TLX2* regulatory region, into two different cell lines: SK-N-BE neuroblastoma cells, already used as a recipient to assay the PHOX2A and PHOX2B physiological capability of *TLX2* transactivation,¹³ and HEK293, a cell line from embryonal kidney, thus presumably lacking of the expression of neural-specific factors, including PHOX2A (Figure 1a). In both cases, the mutant protein was expressed at the same level of the wt protein (Figure 1b and c) and the *luciferase* level induced by co-transfecting the *TLX2* promoter with the mutant construct has resulted

Figure 3 PHOX2A 9nt in-frame deletion. In the upper box, sequence from patient's DNA is represented. Sequences of the single alleles, obtained after PCR cloning, are provided underneath.

comparable to the value obtained with the corresponding wt construct (Figure 4).

Discussion

Despite *TLX2* has been proposed as a candidate gene for the IND by two independent research groups,^{2,3} anomalies in its coding and promoter regions have been excluded in appropriate sets of patients.^{9,11} Nonetheless, the phenotype of *Tlx2*^{-/-} mice suggest that missed activation of the gene could induce an impairment in the intestinal development. In this light, the identification of genes acting upstream *TLX2* during the ontogenesis could provide candidates likely involved in IND and/or in other genetic defects of intestinal innervation, in addition to possible diagnostic markers. Pursuing such a goal, we have already identified PHOX2B as a specific activator of the *TLX2* transcription in cells of neural origin.¹³

Observations on PHOX2A and PHOX2B binding identical consensus sequences and increasing the dopamine β -hydroxylase promoter activity with comparable efficiency had suggested that *PHOX2A* and *PHOX2B* may be functionally redundant.²⁶ By contrast, the generation of two knock-in mutant mice, in which *Phox2a* is replaced by

Figure 4 Functional analysis of the mutant PHOX2A. Luciferase activity driven by the *TLX2* promoter sequence was measured on lysates from both SK-N-BE and HEK 293 cells co-transfected with expression plasmids containing wt or deleted *PHOX2A*. Values represent fold induction versus activity derived from transfection of the pGL3 basic vector together with the corresponding pcDNA3.1 expression vector.

the *Phox2b* coding sequence, and *vice versa*, indicates that *Phox2a* and *Phox2b* are not functionally equivalent, as only *Phox2b* can fulfil the role of *Phox2a* in the structures that depend on both genes.²⁷ Consistently, *PHOX2A* is expressed, like *TLX2* and *PHOX2B*, in enteric and cranial (VII, IX, X) nerve ganglia during embryogenesis.²⁸ The *PHOX2B* involvement in a gene expression cascade including *TLX2* and leading to enteric neuron differentiation does not surprise considering the already known absence of autonomic innervation displayed by the *Phox2b*^{-/-} mice²⁹ and the involvement of this gene in human neurocristopathies.^{14,15} On the other hand, mice *Phox2a*^{-/-} fail to develop the locus coeruleus, the anterior parasympathetic ganglia and the cranial sensory ganglia.³⁰ Moreover, a few specific mutations of *PHOX2A* have been found associated with familial cases of Congenital Fibrosis of the Extra Ocular Muscle type II, a human disorder giving rise to strabismus.³¹ Nonetheless, a wider role of *PHOX2A* in the development of neural-crest cells toward autonomic neuronal lineages has recently been demonstrated. In particular, while, on one hand, the transcriptional components of the cAMP pathway, CREB and CBP, has been demonstrated to induce *PHOX2A* transcription, on the other, the cAMP-dependent protein kinase A (PKA) has been proven to regulate the activity of a Ser/Thr PP2A-like phosphatase responsible for the *PHOX2A* activation. *PHOX2A* thus results to be central in sympatho-adrenal cell specification.³² Since inhibition of PKA in murine enteric neurons causes lethal intestinal pseudo-obstruction,³³ we are tempted to hypothesize that, in this case, impaired intestinal phenotype can derive from the lack of *Tlx2* expression due to missed PKA-mediated *Phox2a* activation. Based on these observations, we decided to test whether *PHOX2A*, like its paralogue *PHOX2B*,¹³ may be involved in the transcriptional regulation of the human *TLX2*.

Despite a previous study had already defined a DNA sequence of 20 nucleotides located in the 5' flanking region of murine *Tlx2* as sufficient to maintain tissue-specific expression of the gene, without investigating the molecular details underlying such an activity and claiming for additional specific nuclear factors to control lineage-restricted expression,³⁴ we have more recently identified *PHOX2B* as one of the factors able to induce *TLX2* expression by binding a conserved tandem 'TAAT/ATTA' enhancer element, proximal to the above murine sequence, in the human *TLX2* gene.¹³

Following the hypothesis that *PHOX2A* might play a similar role, co-transfection assays and EMSA experiments have confirmed that *PHOX2A* specifically enhances the reporter gene transcription, through the physical interaction with the human 'ATTA' sequences in the *TLX2* promoter, previously identified as actively involved in *PHOX2B*-mediated activation.¹³ To determine whether the two paralogue *PHOX2* transcription factors could have either additive or opposite effects on *TLX2* promoter, co-

transfections of *PHOX2A* and *PHOX2B* together were also carried out in neuroblastoma cells. In this case, the gene reporter activity did not result statistically different from that obtained transfecting each of the two expression constructs alone, thus excluding any possible antagonistic or additive effect. ChIP assay has confirmed that the interaction between *TLX2* promoter and both *PHOX2A* and *PHOX2B* occurs *in vivo*, suggesting that these transcription factors play a similar role in IMR32 cells. Nevertheless, our study could not rule out that (a) *PHOX2A* and *PHOX2B* play distinct physiological roles in different body district or developmental stages; (b) the two factors interact with each other; and (c) the two factors interact with different proteins.

Since we have provided evidence that *PHOX2A*, like *PHOX2B*,¹³ is a regulator of *TLX2* cell-specific expression, these two *PHOX2* genes can be reasonably considered as candidates in the search for the genetic basis of IND and other defects of enteric innervation. Indeed, starting from the observation that *Phox2b*^{-/-} mice lack the entire autonomic nervous system,²⁸ *PHOX2B* has already been taken into consideration as a possible candidate in HSCR pathogenesis, though no mutation could be demonstrated in patients.²⁴ IND and chronic intestinal pseudo-obstruction are very different from HSCR, since tissues from patients with these disorders do not display absence of ganglion cells, rather they may show morphological and functional alterations of enteric neurons. This is indeed the phenotype of the *Tlx2*^{-/-} mice, suggesting that enteric nervous system defects can be accounted for by the *TLX2* inability to work or to be expressed. After excluding abnormalities in the coding and promoter sequence of *TLX2* in patients,^{9,11} we have focused on its positive developmental regulators *PHOX2A* and *PHOX2B*. Their coding sequences have been investigated, thus identifying one inherited heterozygous in-frame *PHOX2A* deletion in a patient characterized by vesical dysfunction and megacystis, known to be rare associated anomalies in IND.³⁵ Despite absence in a set of control individuals suggested a possible causative role, no impaired *TLX2* transactivation in SK-N-BE and HEK293 cell lines could be demonstrated. Nonetheless, a functional effect of the examined mutation on still unknown targets involved in enteric neuron differentiation processes or a possible role of such a variant in the development of associated anomalies cannot be excluded.

In conclusion, in the present work we have identified *PHOX2A* as a novel *TLX2* regulator, thus providing a new piece of information in the complicate pathways leading to the enteric neuron differentiation. Moreover, exclusion of a major causative role of the *PHOX2* genes in the development of intestinal innervation defects different from HSCR confirms that these disorders still represent a challenge from both the clinical and the genetic point of view.

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