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ARTICLE

Complex pathogenesis of Hirschsprung's disease in a patient with hydrocephalus, vesico-ureteral reflux and a balanced translocation t(3;17)(p12;q11)

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Hirschsprung's disease (HSCR), a congenital complex disorder of intestinal innervation, is often associated with other inherited syndromes. Identifying genes involved in syndromic HSCR cases will not only help understanding the specific underlying diseases, but it will also give an insight into the development of the most frequent isolated HSCR. The association between hydrocephalus and HSCR is not surprising as a large number of patients have been reported to show the same clinical association, most of them showing mutations in the *L1CAM* gene, encoding a neural adhesion molecule often involved in isolated X-linked hydrocephalus. L1 defects are believed to be necessary but not sufficient for the occurrence of the intestinal phenotype in syndromic cases. In this paper, we have carried out the molecular characterization of a patient affected with Hirschsprung's disease and X-linked hydrocephalus, with a *de novo* reciprocal balanced translocation t(3;17)(p12;q21). In particular, we have taken advantage of this chromosomal defect to gain access to the predisposing background possibly leading to Hirschsprung's disease. Detailed analysis of the *RET* and *L1CAM* genes, and molecular characterization of *MYO18A* and *TIAF1*, the genes involved in the balanced translocation, allowed us to identify, besides the L1 mutation c.2265delC, different additional factors related to *RET*-dependent and -independent pathways which may have contributed to the genesis of enteric phenotype in the present patient.
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Introduction

Hirschsprung's disease is the major congenital defect affecting the enteric nervous system (ENS). It is characterized by the absence of enteric ganglion cells along a variable length of the intestine. Mutations of the *RET* proto-oncogene, encoding a tyrosine kinase receptor mainly involved in neural crest cells development, were identified in 1994 as being responsible for the disease, and are still considered as the most frequent cause of intestinal aganglionosis (7–35% of sporadic patients).[1] Besides *RET*, nine other HSCR susceptibility genes have been identified, most of them found by studying pedigrees with the

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recurrence of intestinal aganglionosis in association with other clinical signs (syndromic HSCR forms).[2–4] The greatest proportion of these genes has been shown or supposed to be related to three specific pathways that are involved in different cellular programmes crucial for the normal development of the enteric nervous system: the RET pathway (*RET, GDNF, NTN, SOX10, PHOX2B*), the endothelin pathway (*EDN3, EDNRB, SOX10)* and, to a lesser extent, the TGF-β signalling pathways *(ZFHX1B*). However, the great proportion of unexplained cases, incomplete penetrance and the variable expressivity suggest that the disease is the result of complex interactions between known and/or unknown modifier genes. Additional evidence supporting this model is the existence of other genetic syndromes such as trisomy 21, Smith–Nemli–Opitz, and cartilage-hair hypoplasia which show an association with HSCR to a variable extent. All these data suggest that studying syndromic families showing an association of HSCR with other congenital abnormalities might be worthwhile.

In this paper, we have carried out the molecular characterization of a patient affected with HSCR disease, X-linked hydrocephalus, and vesico-ureteral reflux showing a *de novo* structural chromosome anomaly t(3;17)(p12.11;q11.21).

X-linked hydrocephalus has an incidence of 1/30 000 male births and it is characterized by severe mental retardation, spastic tetraplegia and bilateral adducted thumbs, with major additional phenotypes including agenesis of corpus callosum and/or corticospinal tract. The great proportion of cases is ascribed to loss of function mutations of *L1CAM*, a neural cell adhesion molecule located in Xq28 and involved in the development of the ventricular system, axonal tracts and the cerebellum.[5]

The association between hydrocephalus and HSCR is not surprising as brain development is largely controlled by the same neural growth factors acting in the ENS.[6] Until now, mutations of the *L1CAM* gene have been found in seven out of eight patients reported to show association of X-linked hydrocephalus with HSCR disease.[7–9] These results suggest that mutations in *L1CAM* may be involved in HSCR development in association with a predisposing genetic background.

Taking advantage of the chromosomal rearrangement t(3;17)(p12.11;q11.21) detected in our patient, we have identified genes encompassing the breakpoints and evaluated their possible involvement as additional genetic factors which may predispose to syndromic HSCR pathogenesis.

Materials and methods

Cytogenetics investigations

Fluorescent *in situ* hybridization (FISH) was carried out using genomic BAC clones spanning the chromosomal 3p12 and 17q11, two regions selected from the human library RPCI-11 according to the UCSC Human Genome Assembly (March 2006 freeze). Array-CGH was performed using the Agilent Human Genome CGH Microarray Kit 44B (Agilent Technologies).[10]

Gene analysis and profile expression

Screening of *L1CAM* and *RET* coding regions was performed as already described.[6,11] The expression profile of Myosin XVIIIA (*MYO18A)* and TGF-β1-induced anti-apoptotic factor 1 (*TIAF1*) in human tissues was performed on a human cDNA commercial panel (Origene technology Inc., Rockville, MD, USA; www.origene.com). Sequence primers are available under request.

Gene expression levels

Analysis of SNP rs2320786, corresponding to the synonymous substitution p.A1470A of the *MYO18A* gene, was performed on the patient's genomic DNA and corresponding cDNA. Quantitative determination of *MYO18A*, *TIAF1* and *RET* expression levels was performed using the ABI Prism 7500 Sequence Detection System in combination with TaqMan chemistry (SYBR Green PCR Master Mix. PE Biosystems), according to the manufacturer's guidelines. PCR conditions and primer sequences are available under request.

Results

Clinical report

The boy (NF, 10 years) is the first child of healthy non-consanguineous parents. He was electively delivered at 38 weeks of gestational age by cesarean section, with a birth weight of 3620 g, length of 52 cm, and macrocephaly (OFC = 39.5 cm). Ultrasound examination revealed marked ventriculomegaly with reduction of the brain cortex and a shunt was inserted at 8 days after birth. At 16 months he was evaluated for chronic constipation and abdominal distention. Rectal suction biopsy and histological examination confirmed the diagnosis of a short form of HSCR with absence of ganglion cells in the rectosigmoid colon. At 20 months, he underwent a laparoscopic colectomy for short-segment disease. At the age of 9 years, bilateral spastic paraplegia, adducted thumbs and mental retardation were present. He interacts exclusively with a social smile and eye contact, whereas speech is limited to a few words. The patient was also diagnosed with a severe vesico-ureteral reflux (VUR) associated with recurrent urinary infections. For the last 2 years the patient has been treated with continuous low-dose antibiotic prophylaxis.

Karyotype analysis Standard karyotype performed on peripheral blood lymphocytes from the patient showed a *de novo* reciprocal balanced translocation 46,XY,

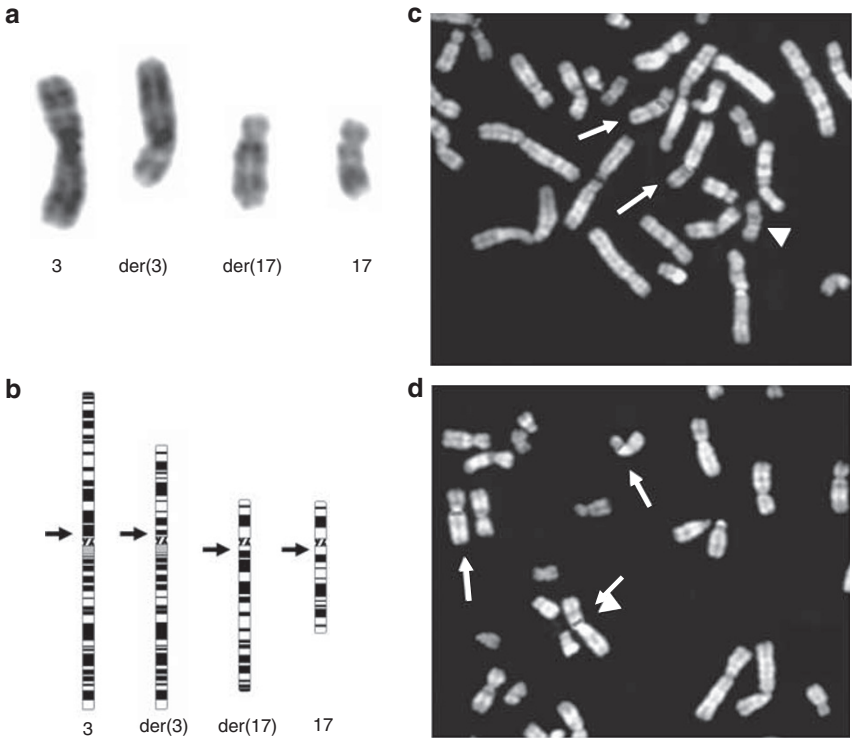


Figure 1 Cytogenetics and FISH results obtained from the patient's lymphocytes. (**a**) Cut-out and (**b**) ideogram of the normal and derivative chromosomes 3 and 17. (**c**) FISH with BAC RP11-331K15 (black signals) shows the chromosome 3 breakpoint on the short arm. Arrowhead indicates the signal on normal chromosome 3 and the arrows indicate the der(3) and der(17) derivative chromosomes. (**d**) FISH with BAC RP11-321A17 demonstrates that the chromosome 17 breakpoint is within this clone. Signals on normal chromosome 17 is indicated by arrowhead whereas der(3) and der(17) derivative chromosomes are indicated by arrows.

t(3;17)(p12.1;q11.21) (Figure 1). The karyotype of both parents was normal.

Molecular screening of *L1CAM* and *RET* genes

L1CAM is a cell adhesion molecule responsible for X-linked hydrocephalus affected males. Molecular testing of the gene revealed a single nucleotide deletion in exon 18 in the patient's DNA, inherited from the healthy mother, which leads to a premature stop codon causing the protein truncation at the level of the third fibronectin type III domain (c.2265delC, p.Pro756Leufs95X) (Figure 2). This kind of mutation is commonly associated with the most severe phenotype of X-linked hydrocephalus.[5] In the attempt to explain the intestinal phenotype of the patient, we performed the mutation analysis of the *RET* gene. Analysis of the 21 exons of the gene did not reveal any mutation in the coding region or in the intron–exon junctions. Interestingly, the patient showed a heterozygous result for the *RET* predisposing haplotype, a specific combination of genetic variants located between the promoter and the second exon of the gene, largely reported to be over-represented in HSCR patients with respect to controls.[12] This haplotype has been associated with reduced gene expression and, accordingly, demonstrated in strong linkage disequilibrium with an intronic nucleotide substitution which has been shown to impair an *RET* transcriptional enhancer *in vitro*.[13]

The molecular analysis of *L1CAM* and *RET* genes is fully in agreement with the previous screening performed on eight patients with the association of hydrocephalus and HSCR disease, most of which showed severe *L1CAM* defects but no *RET* coding mutations.[7–9] The presence of a *L1CAM* mutation fully accounts for the neurological phenotype, whereas the molecular defect causing HSCR disease remains unknown. The main hypothesis proposed to explain the co-occurrence of the two diseases is that the *L1CAM* mutation itself may confer a predisposing background on which additional factors may act causing the disease phenotype.

Breakpoint characterization

To define the translocation at the molecular level, we constructed two genomic contigs with BACs covering the region containing the breakpoints on both chromosomes. For chromosome 3p12.1, the breakpoint mapped to the

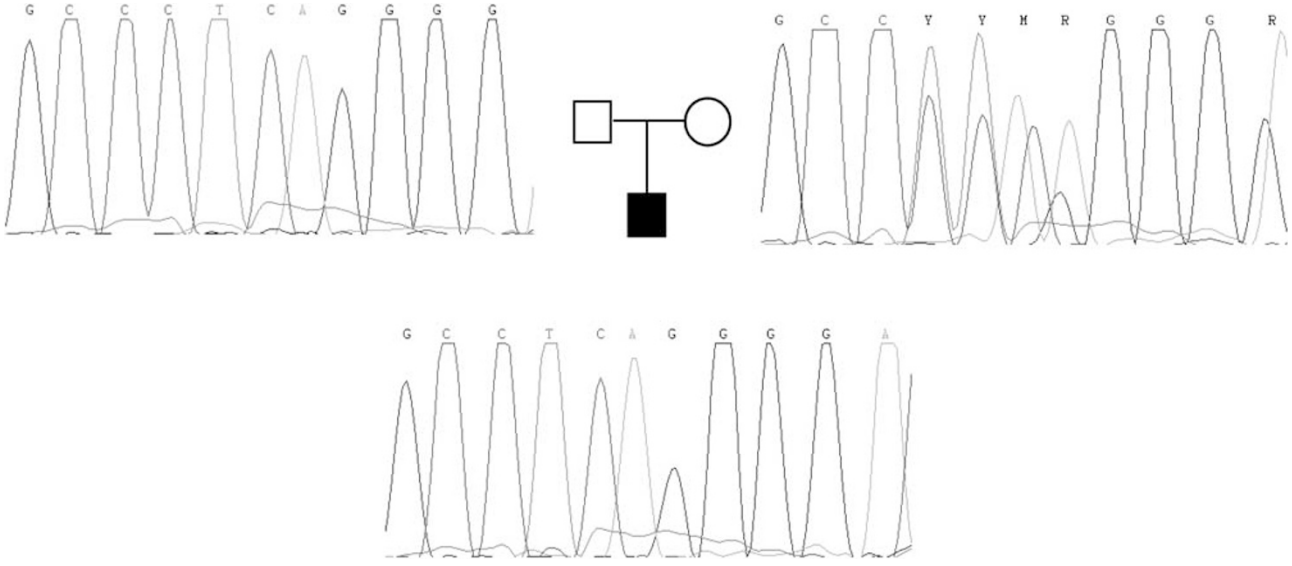


Figure 2 Sequence analysis of patient's DNA, showing the c.2265delC (p.Pro756fs) mutation in the L1CAM gene. The sequence of the parental DNAs demonstrates that the mutation is inherited from the heterozygous mother.

RP11-331K15 clone, giving signals on normal chromosome 3, on derivative der(3), and on derivative der(17).

Using a similar strategy, we were also able to map the chromosome 17q11.2 breakpoint to the BAC clone RP11-321A17 (AC024619). Comparative genomic hybridization (CGH) array analysis of patient DNA allowed us to exclude the presence of further deletions or duplications elsewhere in the genome with a resolution of about 75 Kb.

Somatic cell hybrids from the patient's lymphoblasts were generated to separate the normal chromosomes 3 and 17 and the two derivative chromosomes der(3) and der(17). DNA from these hybrids was used in a PCR-based strategy with selected non-polymorphic markers spanning the region defined by BAC clones (not shown). We therefore localized both breakpoints, amplified and sequenced both junction fragments thus providing the exact molecular definition of the translocation (Figure 3). The breakpoint on der(3) lacks one nucleotide from each parental chromosome, whereas the der(17) breakpoint contains seven additional bases, probably derived from a partial duplication of a nearby sequence (Figure 3). There is no apparent homology between the two sequences, and no repeats are involved.

The chromosome 3 breakpoint is located at 390 kb far from the 3′UTR of the *CADM2(IGSF4D)* gene and 480 kb far from the 3′ terminal of the *VGLL3* gene. Comparative genomic analysis performed with the Genome Vista browser ((http://pipeline.lbl.gov/index.html) did not reveal any significant homology with the mouse sequence, thus making unlikely the presence of a regulatory region in this genomic fragment.

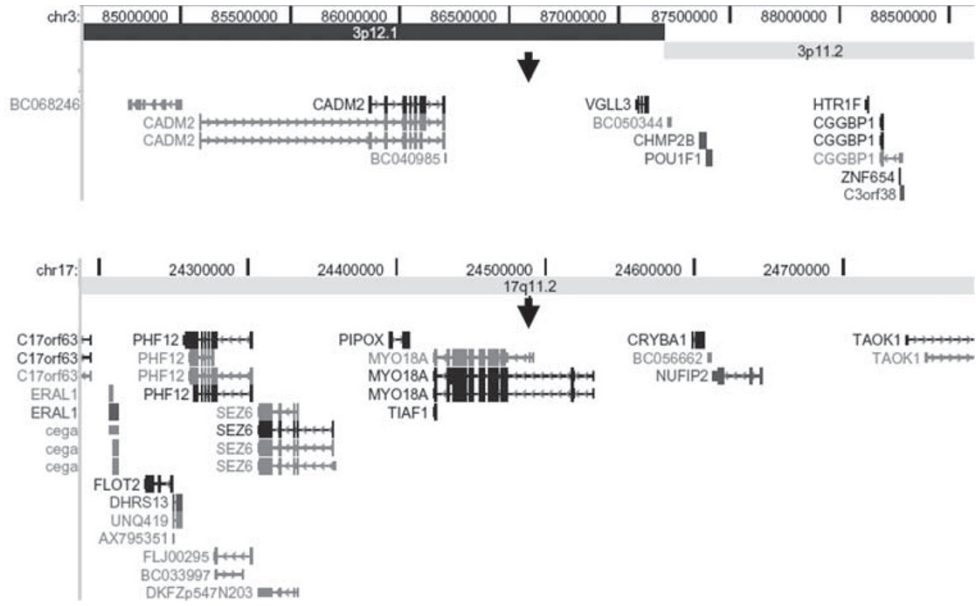
On chromosome 17 the breakpoint occurred in a region containing two genes, *MYO18A* and *TIAF1*. Myosin XVIIIA is encoded by 40 exons with the production of two alternative isoforms. The protein has recently been demonstrated as a high affinity receptor (SP-R210) for surfactant protein A (SP-A), a highly versatile immune molecule, which contributes significantly to surfactant homeostasis and pulmonary immunity.[14]

TGF-β1-induced antiapoptotic factor 1 (*TIAF1*) is an intronless gene, which resides within the 3′ untranslated region of the *MYO18A* gene. Identified and cloned in 1998, *TIAF1* was shown to participate in the transforming growth factor β1-mediated growth regulation by both increasing the expression of p53, Cip1/p21, and Smad proteins, suppressing ERK phosphorylation, and altering TGF-β1-mediated Smad2/3 phosphorylation.[15–16] It has also been shown that *TIAF1* and p53 functionally interact in regulating apoptosis, and *TIAF1* is likely to participate in the nuclear translocation of activated p53.[17]

The role of *TIAF1* in preventing cellular apoptosis is very intriguing in the light of the patient's HSCR phenotype as it has been hypothesized that one of the causes of HSCR pathogenesis is a defect in normal survival of enteric neurons and/or premature apoptosis.[1]

The expression profile of the *MYO18A* and *TIAF* genes performed on a panel of 24 human tissues is in agreement with literature data, showing a quite ubiquitous presence of the two genes with a preferential expression in the muscle (data not shown).

Considering the role of *TIAF1* as an antiapoptotic factor, we decided to carry out a genetic analysis of this gene to



Chrom.	Position	Sequence		
17	24488584 - 24488643	ATAGTGAACTCAATGCCTGGGT **TTGCA**GG	G	AGGGCAAGGACACTGaaattgcttagtagc
3 (rev)	86590820 - 86590761	TTTCATTACCATTTATCTTTTTATTCTTAA	C	ACCTATAATAGCATATTTCCTAAAAGATA
der(3)		TTTCATTACCATTTATCTTTTTATTCTTAA		AGGGCAAGGACACTGaaattgcttagtagc
der(17)		ATAGTGAACTCAATGCCTGGGT **TTGCA**GG	CA**TTGCA**	ACCTATAATAGCATATTTCCTAAAAGATA

Figure 3 Map of the translocated regions. The breakpoint on chromosome 3 is located between the CADM2(IGSF4D) and VEGL3 genes. The breakpoint on chromosome 17 interrupts the MYO18A and TIAF1 genes. The sequences at the breakpoints are reported in the table at the bottom.

determine if it may affect the survival of enteric neurons, playing a role in the normal development of the intestine. To this end, we screened the gene in four additional patients showing the association of X-linked hydrocephalus with HSCR disease. An analysis of 1256 bp of the *MYO18A* 3′UTR of gene, containing the whole coding region of *TIAF1*, did not show any alteration in these individuals, with the exception of a silent polymorphism (rs1049848) in one patient. To further investigate the role of *TIAF1* in favouring the intestinal phenotype in syndromic HSCR, we analysed a selected group of 17 HSCR patients showing aganglionosis in association with other clinical symptoms (six multiple malformations, five limb defects, three dismorphisms, one renal hypoplasia, one mental retardation, and one cardiopathy). We did not find any mutation or polymorphic variant of the *TIAF1* gene in these patients, a circumstance which does not support the effect of mutations of this gene as a major event in HSCR disease.

Expression profile of the patient

After investigating the presence of informative alleles of single nucleotide polymorphisms (SNPs) of the *MYO18A* and *TIAF1* genes among those reported in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/), the patient showed a heterozygous result C/T for rs2320786 (p.A1470A) in the coding region of *MYO18A*. RT-PCR performed on patient lymphoblasts mRNA could confirm the presence of the only C allele at the SNP rs2320786 locus, suggesting a monoallelic expression of the *MYO18A* gene as a likely result of the translocation (Figure 4a). To exclude a physiological monoallelic expression of this gene, we checked if the *MYO18A/TIAF1* gene(s) and the 17q21 region have previously been implicated in imprinting (http://igc.otago.ac.nz/home.html), finding negative results. Moreover, we also performed Real-time RT-PCR for the two genes involved in the translocation, testing the gene expression level in the patient compared with a normal control individual (Figure 4b). *MYO18A* and *TIAF1* show a reduction in their expression of approximately 50% in patient's mRNA with respect to the double expression dose detected in the control, thus confirming a monoallelic expression because of the translocation.

It has been proposed that the presence of a *RET* predisposing haplotype can increase the risk of aganglionosis above the threshold. For this reason, we performed real-time RT-PCR on the *RET* gene using lymphoblasts' mRNA of our patient, who carries one copy of the *RET* predisposing 5′haplotype, and compared results to that obtained using mRNA from a control homozygote

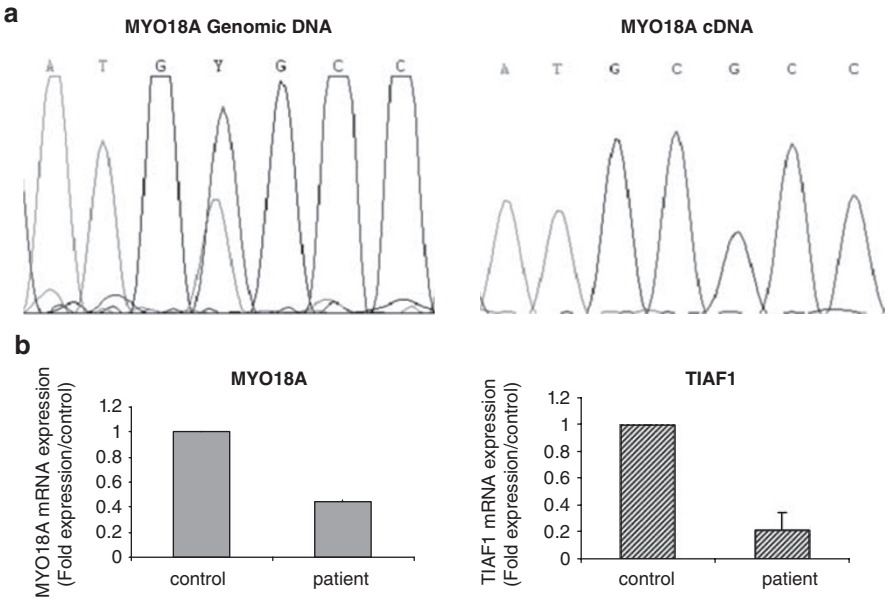


Figure 4 Expression profile of the patient. (**a**) Monoallelic expression at an informative polymorphic marker locus of the MYO18A gene, determined by comparing the sequence of the patient's genomic DNA with his corresponding lymphoblasts cDNA. (**b**) Quantitative determination of the expression levels of MYO18A and TIAF1 in patient's lymphoblast mRNA. Data are normalized on the control's gene expression levels.

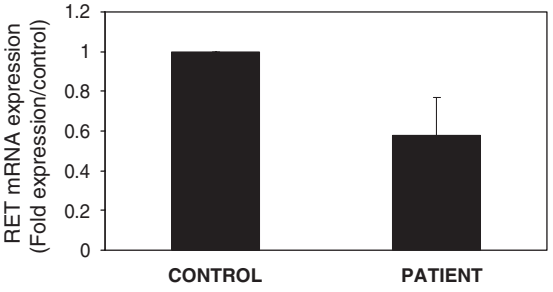


Figure 5 RET expression in lymphoblastoid cells. RET expression levels are reduced in the patient carrying one copy of the predisposing haplotype compared with a normal wild-type control.

for a wild-type version of the *RET* gene. Our patient showed a marked reduction of *RET* mRNA (58 *vs* 100% of the control, *P*-value = 0.019) (Figure 5), a finding in agreement with previous data showing an impaired *RET* expression associated with this haplotype.[18]

Our data support the hypothesis of a major role played by the association of L1CAM mutation and the *RET* predisposing haplotype in the occurrence of the two phenotypes, as previously reported in eight patients.[7–9] Moreover, based on present results, the hypothesis that the impaired expression of the *MYO18A/TIAF1* genes represents an additional risk factor, acting together with *L1CAM* and *RET* defects in our patient to the occurrence of the Hirschsprung's phenotype, cannot be excluded.

Discussion

In this paper we describe a patient associated with HSCR and X-linked hydrocephalus, who shows a mutation in the *L1CAM* gene, a decreased *RET* expression and haploinsufficiency of two genes involved in a balanced translocation (3;17)(p12;q21).

Of eight patients reported so far with co-occurrence of HSCR and hydrocephalus, seven showed mutations in the *L1CAM* gene, which was proposed as a candidate HSCR gene.

L1CAM may be implicated in HSCR for several reasons. First of all, the 4:1 male/female bias in the incidence of HSCR has suggested, for a long time, a role of an X-linked gene in the disease aetiology; however, linkage studies have failed to yield any X chromosome locus and, to date, *L1CAM* is the only X-linked gene known to associate with HSCR.[19] Second, *L1CAM* expression has been shown to be under tight regulation in mouse ret k-/k- intestine [20] and a selective deficit in *L1CAM* immunostaining has been reported in sections of bowel from patients with HSCR.[21] Third, *L1CAM* is expressed exclusively by neural crest-derived cells in the developing mouse gut and *L1CAM*-deficient mice showed a small but significant reduction in enteric neural crest cell migration throughout the developing gut.[22]

Although these observations suggest an important role for *L1CAM* in the migration of neural crest cells in the developing gut, the low incidence of HSCR disease in patients with *L1CAM* mutations (about 3%) and the absence of *L1CAM* mutations in males affected with

isolated HSCR suggest that *L1CAM* is not an HSCR causative gene but provides a predisposing genetic background, likely acting as an X-linked modifier gene for the development of HSCR.[7–9] Additional factors may act on this background promoting the occurrence of Hirschsprung's disease, among which the *RET* predisposing haplotype, found in our infant affected with HSCR and hydrocephalus and carrying a *L1CAM* mutation. In agreement with the presence of one copy of this low-expressed *RET* haplotype, we found a marked decrease of the *RET* gene expression in our patient compared with a normal control. Though still in need of further validation in a larger number of patients, these data seem fully in agreement with the model proposed for other syndromes also showing association with HSCR disease, such as the congenital central hypoventilation syndrome (CCHS), Bardet-Biedl Syndrome and Down syndrome, and sharing the *RET* predisposing haplotype as a risk factor for the intestinal phenotype.[23]

With respect to the *de novo* balanced translocation carried by the patient, we considered two different scenarios: (i) the *de novo* balanced translocation can be non-pathogenetic and only by chance associated with the disease phenotype; ii) the genes involved in the translocation may act as modifier genes in HSCR and/or in other phenotypes observed in the patient. *MYO18A*, encoding for the receptor of collectin SP-A, seems not to be involved in HSCR disease but may play a pivotal role in the immunity response of the lung. However, with the exception of a hospitalization for bronchiolite at 9 months of age, until now our patient does not show any susceptibility to recurrent pulmonary infections or asthma and allergic reactions. More interestingly, the second gene involved in the translocation, *TIAF1,* may play a role as predisposing HSCR factor by acting in the TGF-β-signalling pathway as an antiapoptotic factor. One of the known HSCR genes, *ZFHX1B*, is also involved in the TGF-β/BMP/Smad-mediated signalling cascade as a transcriptional repressor of smad proteins. Indeed, mutations of the *ZFHX1B* gene results in Mowat–Wilson syndrome, a multiple congenital anomaly characterized by mental retardation, microcephaly and, at variable extent, Hirschsprung's disease, congenital heart disease, genitourinary anomalies and hypospadias[5].

The patient described here shows haploinsufficiency for *TIAF1*, but further molecular analyses allowed us to detect mutations neither in four patients showing hydrocephalus and HSCR disease nor in 17 additional HSCR patients characterized by different syndromic associations; therefore, no definitive conclusion can be drawn regarding the role of *TIAF1* in HSCR. Nonetheless, in the present patient, we hypothesize that *TIAF1* haploinsufficiency may have perturbed an alternative, *RET*-independent HSCR pathway, namely the TGF-β-signalling pathway, thus increasing not only the risk of developing intestinal aganglionosis but also, based on the control that TGF-β variants seem to exert on the pathogenesis of urinary tract infection and vesicoureteral reflux,[24] the risk of developing VUR. In particular, we propose a complex mechanism in which one copy of the *RET* predisposing haplotype and the *L1CAM* mutation

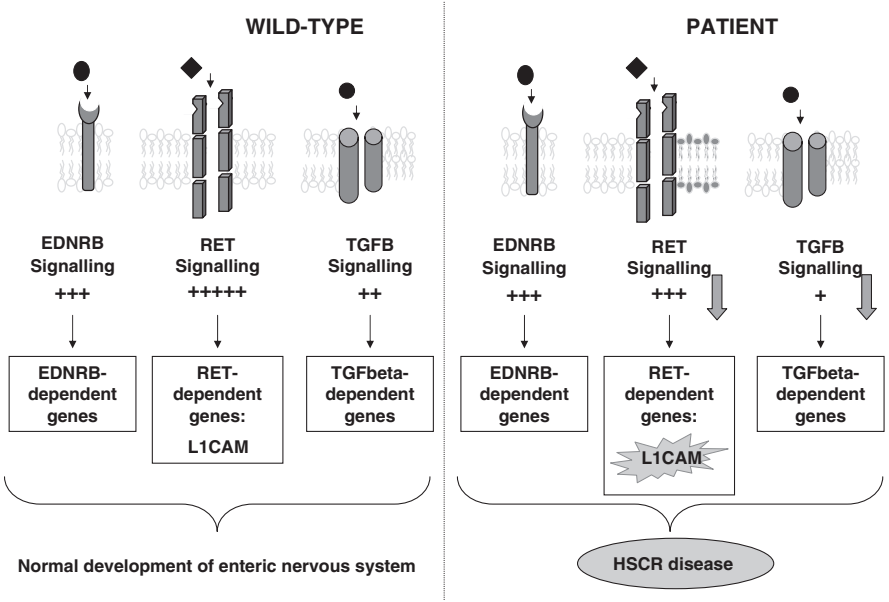


Figure 6 Hypothetical model of Hirschsprung pathogenesis in our patient. The three major pathways involved in the normal development of the enteric nervous system are shown. RET decreased expression and TIAF haploinsufficiency can concur together with L1CAM mutation to HSCR pathogenesis.

play a critical role affecting the *RET*-mediated cellular program, whereas *TIAF* haploinsufficiency may concur to HSCR disease and VUR pathogenesis by altering a *RET*-independent-signalling pathway (Figure 6). However, the pathogenetic mechanism we have postulated in the present patient, while confirming the complex genetic etiology of syndromic HSCR, cannot be easily generalized to other cases.

Interestingly, both the pathways mediated by TGF-β and Ret may concur to the development of the VUR observed in our patient. VUR has often been reported to be associated with TGF-β signal-dependent Mowat–Wilson syndrome (38%) and TGF-β1 polymorphisms have been recently shown to be associated with the susceptibility to VUR.[5,24]

On the other hand, the *RET* gene plays a critical role in kidney development and it has been found involved in isolated congenital anomalies of the kidney.[25] Considering that, like HSCR, vesico-ureteral reflux is a genetically heterogeneous trait which may occur as a result of the complex inheritance, we suggest that contemporary defects in the two pathways (decreased *RET* expression and *TIAF1* haploinsufficiency) may be critical for the onset of vesico-ureteral reflux in our proband and that these different pathways may be involved in other cases of either syndromic or isolated vesico-ureteral reflux or renal malformations.

Further screening of the *TIAF1* gene in other HSCR patients with association of vesico-uretral reflux or other renal malformations may be helpful to better understand the molecular basis of the disease.

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Conflict of interests

None.

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