ELECTRONIC LETTER

Hirschsprung disease and *L1CAM*: is the disturbed sex ratio caused by *L1CAM* mutations?

R M W Hofstra, P Elfferich, J Osinga, E Verlind, E Fransen, J López Pisón, C E M de Die-Smulders, I Stolte-Dijkstra, C H C M Buys

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HSCR is a congenital disorder characterised by an absence of enteric ganglia over various lengths of the bowel and proliferation of nerve fibres in the distal bowel. The absence of enteric ganglia is thought to be caused by a defective migration of neural crest cells. It results in functional obstruction and life threatening bowel distension shortly after birth with an incidence of 1 in 5000 live births. In about 80% of cases, the rectosigmoid colon is the only part affected and in most of the remaining cases the aganglionosis extends to the ileocaecal junction. In a small percentage of cases, the entire small bowel and colon are aganglionic.[1]

HSCR can be associated with a large number of syndromes, such as Waardenburg syndrome, Smith-Lemli-Opitz syndrome, Goldberg-Shprintzen syndrome, and Ondine's curse. This variety of associated syndromes implies considerable genetic heterogeneity in the aetiology of HSCR, although associations by chance cannot be excluded. Genetic analysis of HSCR has confirmed the heterogeneity. So far, mutations, alone or in combination, have been identified in seven genes, namely *RET*,[2 3] *GDNF*, [4 5] *NTN*,[6] *EDNRB*,[7] *EDN3*,[8 9] *ECE1*,[10] and *SOX10*.[11] It is thought that they account for 50-60% of familial cases and 10-30% of sporadic cases.

Besides an association with several syndromes, a difference in sex ratio has also been observed. A male predominance of 3:1 to 5:1 in Hirschsprung disease has been reported.[12 13] Badner *et al*[13] performed complex segregation analysis of data on 487 probands and their families. They observed an increased sex ratio, 3.9 males to 1 female, with a decrease when the aganglionosis became more extensive. It is this disturbed sex ratio which made us hypothesise that there might well be a HSCR susceptibility gene on the X chromosome.

Some HSCR patients have been reported with a combination of HSCR and X linked hydrocephalus, MASA syndrome.[14–17] MASA derives from Mental retardation, Aphasia, Shuffling gait, and Abducted thumbs[18] and is caused by mutation in the *L1CAM* gene, a gene located on Xq28. In a report on a patient with X linked hydrocephalus and Hirschsprung's disease (HSCR) with a mutation in the *L1CAM* gene, it was suggested that although the disease phenotypes of this patient may well be independent, it cannot be excluded that *L1CAM* mutations contribute to both phenotypes.[14] L1, the protein product of *L1CAM*, is a cell adhesion molecule involved in the development of the nervous system.[19 20] *L1CAM* is most prominently expressed in neurones of both the central and the peripheral nervous system[21–23] and in the enteric neural elements. Positive immunostaining of the *L1CAM* gene was identified in ganglionic segments. In neurofilaments, immunoreactivity was observed in neuronal bodies and fine fibres of the myenteric and submucosal plexuses. In aganglionic segments of HSCR patients, however, *L1CAM* expression is not observed. Instead of intrinsic neurones they contain nerve bundles in the intermuscular space, the submucosa, and the circular muscle layer. No L1 was detected in these nerve bundles. It was suggested, therefore, that *L1CAM* underexpression might contribute to the HSCR phenotype.[23]

Another indication of *L1CAM* involvement comes from the work of Auricchio *et al*.[24] They hypothesised a possible site of a HSCR modifying gene on Xq28, the region in which the *L1CAM* gene is located. They describe the mapping of a disease gene on Xq28 in a family with X linked chronic idiopathic intestinal pseudo-obstruction (CIIP) CIIPX. CIIP can be secondary to several disorders, primarily to those associated with defects of enteric neuronal cells, of which HSCR is the most common one.

To investigate a possible role of the *L1CAM* gene in the development of HSCR, we developed a comprehensive mutation detection assay for the entire coding region and all splice site junctions of *L1CAM*, based on denaturing gradient gel electrophoresis (DGGE), and screened 30 patients for mutations.

MATERIAL AND METHODS

Patients

We screened 28 HSCR patients, 18 sporadic and 10 familial male HSCR patients for whom we had previously not found a *RET* or *GDNF* mutation,[25] and two patients with both HSCR and hydrocephalus. Mutation analysis of *EDN3* and *EDNRB* was not performed in all cases. In those cases in which the genes were screened, no mutation was found (unpublished data). The familial cases came from families with only male HSCR patients. Among the 18 sporadic cases, nine had long segment and nine short segment HSCR.

We also included two patients with a combination of HSCR and hydrocephalus. The first of these two patients is a 6 year old male, the second child of non-consanguineous, healthy, Spanish parents. Since the newborn period, he has had relative macrocephaly, with head circumference on the 50th centile, and weight and length below the 3rd centile. He has complete agenesis of the corpus callosum, adducted thumbs with camptodactyly of the remaining fingers, spastic paraplegia, especially of the legs, and hydrocephalus, together with Hirschsprung disease, pyeloectasia, and left testicular hypoplasia. He is mentally retarded without speech development, but has acceptable social contact and comprehension. The karyotype is normal, 46,XY, and cardiological and ophthalmological features were normal.

The second of these two patients was the second child of healthy, unrelated, Dutch parents. His older brother was normal. The boy was born by caesarean section after an uncomplicated pregnancy. Apgar scores were 7 and 8 at one and five minutes, respectively. Birth weight, length, and head circumference were within normal limits. After a period of severe and untreatable constipation, the diagnosis of Hirschsprung disease, short segment type, was made at the age of 3

Abbreviations: HSCR, Hirschsprung disease; DGGE, denaturing gradient gel electrophoresis; CIIP, chronic idiopathic intestinal pseudo-obstruction

Table 1 Primers used for DGGE analysis of the *L1CAM* gene. Shown are the primer sequences, the amplicon size, and the pools in which the DGGE products can be analysed

Exon	Pool	Length (bp)	Primer	Primer sequence
1	II	151	LC-1F	GTGGCTGTGCTGCGCGGTGC
			LC-1Rgc	(40GC)CATAGCGGCGAAGGTAGGCG
2	V	107	LC-2Fgc	(40GC)TCTCTCCATTCCCTCCCTTG
			LC-2R	TACCCTTCTCCTTGGCCTTC
3	IV	287	LC-3Fgc	(40GC)GTGCTGAGGCTATGACACCA
			LC-3R	GAAGTTAGGCAGTCCAGGGA
4	X	376	LC-4F1gc	(40GC)CACCTGTCCCTCCAGCCTGT
			LC-4R	CACAATCCCACACGAACTCC
5	IV	197	LC-5Fgc	(55GC)GAGGAGAGTGTCAGCCCGTC
			LC-5R	TAACACCCCGACCCCACGAG
6	IX	279	LC6F	GTGTCTTCCTGGACGGGGTC
			LC-6Rgc	(40GC)AAGGGCCATGCCTGAGGGTG
7	V	278	LC-7Fgc	(55GC)AATTCTGGGGTGGAGGGAAG
			LC-7R	TGGTCTGAGCTCCCTGCTAG
8	VIII	303	LC-8F	CCCTTCTGCTCTCTTCCCTT
			LC-8Rgc	(40GC)CATGACAGTGGGCATCACAG
9	III	349	LC-9F	CCTGTGATGCCCACTGTCAT
			LC-9Rgc	(40GC)TTCCTCACCCTCCTCTTCCT
10	IX	316	LC-10F	GAAGAGGAGGGTGAGGAAGG
			LC-10Rgc	(40GC)AGTGGGTGCAGGGACAGACT
11	II	311	LC-11Fgc	(40GC)AGCAGGGAACCAAGATTTGC
			LC-11R	ACACGACACTCACCACTACC
12A	I	287	LC-12AF	GTAGTGGTGAGTGTCGTGTC
			LC-12ARgc	(40GC)AGCCATGATGGTAACATTGT
12B	VII	195	LC-12BF	AATGACACCGGACGCTACTT
			LC-12BRg	(40GC)TTTTTTTTTTTTTGTCACTCTGTTGTCCTCCAG
13	VI	281	LC-13Fgc	(40GC)ACAACAGAGTGACTTCCCCA
			LC-13R	TTCCAGCTTTTCCCACTCTG
14	I	228	LC-14Fgc	(40GC)GGGAGGGATTGGGAGGGGAG
			LC-14R	CCCGGCCTTCTGGAGTGGAG
15	VII	317	LC-15F	TGGGCCCTTTCAAGCACCGA
			LC-15Rgc	(40GC)TTCCCATGAGGAACCGTGAG
16A	X	232	LC-16Afgc	(40GC)CCCAAAGCCACATGCTGATC
			LC-16AR	ACCACAGTCTCAGAGACCGG
16B	XI	153	LC-16BFgc	(GC)ACACCTTTAGGGTTACTGCC
			LC-16BR	TCTAAGCCCTCCCTTCACAG
17	VI	220	LC-17Fgc	(40GC)TCCTCGTGGCTCTCCAAAAG
			LC-17R	CCCAGGTCATGGCCTCATGT
18	V	405	LC-18F	GGGGGGGCCAAAGAATGCTGGTGTT
			LC-18Rgc	(40GC)ATGCTGAGAGGTGTGGACAT
19	VIII	309	LC-19Fgc	(40GC)TTCTCTGTGTGTAGGGGCTT
			LC-19R	GGGGGGCTCACCATCCTGTCGCTTTA
20	VIII	416	LC-20Fgc	(40GC)GAGAGGAGGGTCCCCATTAA
			LC-20R	ACAGAACCGAGTGGCAGGTA
21	VI	228	LC-21Fgc	(55GC)TACCTGCCACTCGGTTCTGT
			LC-21R	CTCCACCTCCCTTCCCTGCT
22	II	273	LC-22F	CACAGCCAACCCCTGTCTGT
			LC-22Rgc	(40GC)TCCCTGTTGGCAGGTCATTC
23	IV	296	LC-23Fgc	(40GC)TAAACCTTAGACTTCCTGGCAGCT
			LC-23R	TGAGTCGGAGTGCCAGGCAA
24	X	331	LC-24Fgc	(40GC)TAAATTGCCTGGCACTCCGACTCA
			LC-24R	GGGCCTTCAGGGGACAGAAGGACAT
25	VII	357	LC-25F	GGGGCCAGGGTCCCAACTTTAAGAGC
			LC-25Rgc	(40GC)ATCCAGGAGGCCTTGCAGAA
26/27	IX	383	LC-26/27Fgc	(40GC)GCTGTTGAGACAGAGTGCTG
			LC-26/27R	AGGCGCACATTGTCTATAGG
28	III	429	LC-28F	AAACAAATGGAAGGCAGGCG
			LC-28RGC	(40GC)AGACAGCAAGTTCTCCTCTG

months. In the first instance, a partial posterior myectomy of the rectal muscles was done, with insufficient result, followed by resection of the affected bowel segment. He had severe and long lasting postoperative urination problems. At 6 months, length and weight were on the 50th centile, whereas his head circumference was between the 75th and 90th centile. At 9 months, macrocrania was found to be progressive and the head circumference (49.5 cm) was now above the 97th centile. An MRI scan of the brain showed dilated cerebral ventricles, without structural brain anomalies. At 12 months, a ventriculoperitoneal drain was inserted. At 13 months, he was operated on for bilateral inguinal hernias. Pigment anomalies of the hair, which was uncombable, and diffuse alopecia was noted in the second year of life. Furthermore, he had a small depigmented area on his face and one on his lower left leg. His psychomotor development was normal with normal IQ test at 1 and 2 years of age. Karyotype was normal, 46,XY.

DNA analysis

To analyse the 28 exons of the *L1CAM* gene, we designed 28 amplicons, as we previously described.[26] Primers used for amplification, amplicon sizes, melting temperatures, and pools

of PCR products used for loading on DGGE gels are listed in table 1. All fragments were amplified using a single PCR programme and DGGE analysis was performed under a single set of experimental conditions. Amplification was carried out in a 50 μl reaction mixture containing 100 ng genomic DNA, 0.25 mmol/l dNTP, 10 pmol of each primer, and 0.125 U *Taq* DNA polymerase. The PCR programme started with denaturation at 94°C for three minutes followed by five cycles of denaturation, one minute at 94°C, primer hybridisation, one minute at 56°C, and elongation, two minutes at 72°C, then by five cycles of one minute at 94°C, one minute at 53°C, two minutes at 72°C, and 25 cycles of one minute at 94°C, one minute at 5°C, and two minutes at 72°C. Eventually, an elongation step was added at 72°C for five minutes. PCR products of the patients were mixed with those of a control. The amount of control PCR products added was approximately half of the amount of patient PCR products. After mixing the PCR products, a heteroduplex step was performed: the samples were denatured at 96°C for 10 minutes followed by renaturation at 50°C for one hour. Subsequently, the PCR products were pooled. The pooled PCR products were applied to a 1 mm 9% polyacrylamide (PAA) gel (acrylamide:bisacrylamide 37.5:1) in 0.5 × TAE (1 × TAE = 40 mmol/l Tris, HAC pH 8.0, 20 mmol/l NaAc, 1 mmol/l Na_2EDTA) containing a denaturing gradient of 45-80% urea-formamide (100% urea-formamide (UF) contains 7 mol/l urea and 40% deionised formamide). Gels were run at 100 V/19 cm and 58°C for 16 hours. After staining the DNA with ethidium bromide, the gel pattern was documented. Using an ABI PRISM 377 DNA sequencer (Perkin Elmer), direct sequencing of independently amplified PCR products was performed in both sense and antisense directions, with the same primers as used for DGGE, but without GC clamp whenever an aberrant DGGE pattern was found.

RESULTS

Out of 30 patients examined only two *L1CAM*-DGGE variations were identified. In a long segment HSCR patient, we detected a C to T change (C362T), which did not result in an amino acid change (Ser120Ser) and was therefore considered a non-pathogenic variant. The second DGGE variant was present in one of the two patients with HSCR and MASA/hydrocephalus. Sequencing showed that a T to G tranversion caused the DGGE variant 6 bp after the splice donor site of exon 5 (IVS5+6 T>G). To test the possible splicing effect of this tranversion, we isolated RNA from lymphocytes of the patient. cDNA was made and a first PCR was performed using primers of exons 3 and 8 (L1Ffor 5′-TCACGGAAC AGTCTCCACGG-3′ and L1Frev 5′TGATGGTGGGCGTGGGA AAG-3′). This gave a PCR product of 714 bp. A nested PCR reaction were performed with primers in exons 4 and 6 (L1Nfor 5′-TCACGGGCAACAACAGCAC-3′ and L1Nrev 5′-ATGATGGTCCTGGTGCCTGG-3′). This gave a PCR product of 370 bp. The PCR product of the patient gave the expected 370 bp long PCR product. A normal sequence of the fragment was confirmed by sequencing, making this variant most likely not pathogenic.

DISCUSSION

A substantial role of the L1 protein in the development of HSCR explaining the excess of affected males is very unlikely, as we found no pathogenic mutations in 28 male HSCR patients. We cannot, however, totally exclude involvement of *L1CAM*, as we might have missed mutations by the DGGE system used or more likely missed mutations in the non-scanned regions of the gene; most of the intronic sequences were not screened nor were the regulatory sequences of the 3′ and 5′ untranslated regions of the gene. A third possibility, in particular for the two patients with the combined phenotype, might be involvement of another gene.

- Hirschsprung disease (HSCR) is a congenital disorder characterised by intestinal obstruction owing to an absence of intramural ganglia along variable lengths of the colon. HSCR occurs more often in males than in females (4:1) and can be found in combination with MASA syndrome (Mental retardation, Aphasia, Shuffling gait, and Adducted thumbs), an X linked syndrome caused by mutations of the *L1CAM* gene.
- We hypothesised a possible involvement of *L1CAM* in HSCR. We performed mutation scanning of *L1CAM* in 28 male HSCR patients (10 familial and 18 sporadic cases) and two HSCR/MASA patients. No mutations were found, making an involvement highly unlikely.

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Authors' affiliations

R M W Hofstra, P Elfferich, J Osinga, E Verlind, I Stolte-Dijkstra, C H C M Buys, Department of Medical Genetics, University of Groningen, The Netherlands
E Fransen, Department of Biochemistry, University of Antwerp, Belgium
J López-Pisón, Sección Neuropediatría, Hospital Infantil Miguel Servet, Zaragoza, Spain
C E M De Die-Smulders, Department of Clinical Genetics, Academic Hospital, Maastricht, The Netherlands

Correspondence to: Dr R M W Hofstra, Department of Medical Genetics, Ant Deusinglaan 4, 9713 AW Groningen, The Netherlands; R.M.W.Hofstra@medgen.azg.nl

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