Evaluation of the \textit{HOX11L1} gene as a candidate for congenital disorders of intestinal innervation

Editor—Homeobox (Hox) gene products are characterised by the presence of a highly conserved 20 amino acid region (homeodomain) known to bind specific DNA sequences. They promote the expression of downstream genes, thus controlling morphogenesis and cell differentiation in the developing embryo.\textsuperscript{1} \textit{Hox11l1} is also named \textit{Ncx},\textsuperscript{2,3} \textit{Enx},\textsuperscript{4} \textit{pmnur6},\textsuperscript{5} and reported as \textit{Tlx-2} in the Mouse Genome Database (MGD, http://www.informatics.jax.org). \textit{Hox11l1} is a member of the \textit{Hox} 11 unclustered homeobox genes, a family characterised by a novel homeodomain with threonine replacing isoleucine or valine at a critical site (residue 48 in the third helix); this alters the DNA recognition motif, which may identify a unique set of target genes. \textit{Hox11l1} expression is detectable in mice in the E9.5 to the E12.5 stages of development and involves dorsal root ganglia, cranial (V, IX, X) and enteric nerve ganglia, adrenal glands in embryos, and adrenal glands and intestine in adult mice. Since its expression is limited to tissues derived from neural crest cells, \textit{Hox11l1} may play a role in the proliferation or differentiation of neural crest cell lines.\textsuperscript{2}

Two different \textit{Hox11l1} knock out mouse models have been generated.\textsuperscript{3,4} In both cases homozygous mutant mice were viable but developed megacolon at the age of 3-5 weeks. Histological and immunohistochemical analysis showed hyperplasia of myenteric ganglia, a phenotype similar to that observed in a human congenital intestinal disorder named intestinal neuronal dysplasia (IND).\textsuperscript{6,7}

There are two clinically and histologically distinguishable subtypes of IND. Type A (IND type A, MIM 243180), occurring in less than 5% of cases, is characterised by congenital aplasia or hypoplasia of the sympathetic innervation and presents acutely in the neonatal period with episodes of intestinal obstruction, diarrhoea, and bloody stools. Type B (IND type B, MIM 601223) is characterised by a malformation of the parasympathetic submucous plexus\textsuperscript{8} and is clinically indistinguishable from Hirschsprung disease (HSCR, MIM 142623), a congenital disorder characterised by megacolon because of absence of intramural ganglia along a variable length of the colon.

The observation in the \textit{Hox11l1} knock out mice\textsuperscript{3} of an IND-like phenotype suggested an involvement of the human homologue of this gene in the pathogenesis of congenital disorders of intestinal innervation.

As both HSCR and IND are neurocristopathies causing developmental and functional defects of the enteric nervous system,\textsuperscript{9} and the IND phenotype has been reported in 25-35% of patients affected with Hirschsprung disease,\textsuperscript{10,11} we selected a total of 48 patients with either disease to test a possible involvement of \textit{HOX11L1} in the pathogenesis of their defective phenotype. In every case the histochemical diagnosis was performed both preoperatively using acetylcholinesterase (AChe), lactate dehydrogenase, and NADPH-diaphorase techniques, and intraoperatively using rapid AChe and alpha-naphthylesterase (ANE). The histochemical diagnosis of IND met the Borchard criteria.\textsuperscript{5}

Both sporadic and familial cases were analysed as summarised in table 1.

So far, a wide range of mutations of the \textit{RET} proto-oncogene have been found in 30-50% of familial and sporadic HSCR cases,\textsuperscript{12-14} while other genes like \textit{GDNF},\textsuperscript{15,16} \textit{SOX10},\textsuperscript{17} \textit{ECE 1},\textsuperscript{18} \textit{EDN3},\textsuperscript{19,20} and \textit{EDNRB}\textsuperscript{21,22} are responsible for a total of 5-10% of cases. These observations, along with evidence of a complex, multifactorial inheritance, suggest the existence of additional susceptibility or modifier HSCR genes, which could account for the molecular defect of cases either still unsolved or already known to carry \textit{RET} mutations and showing variable expressivity/incomplete penetrance of the phenotype. For this reason, among the 22 HSCR patients selected for \textit{HOX11L1} mutational screening, eight carried a \textit{RET} mutation while none of the 26 IND cases had been analysed for this gene. All the IND patients were affected with the B form, with the exception of one who showed the more severe A form. Two IND B patients showed associated anomalies; one had megacystis and one thalassaeemia. Finally, two of the six IND familial cases belonged to two large pedigrees which were used to perform linkage analysis (see below).

The three exons, corresponding to the coding portion of \textit{HOX11L1}, were amplified, using primers designed on the sequences retrieved from GenBank (accession numbers AJ002607, AJ002608, AJ002609, and NM001534), subjected to single strand conformation polymorphism (SSCP) analysis, and visualised by silver staining. Details of the PCR reactions and SSCP conditions are reported in table 2. In order to improve the resolution of the screening,\textsuperscript{25} the largest PCR products were analysed in two segments by either using two pairs of primers, as in the case of exon 1, or digesting the PCR product with a restriction enzyme, as in the case of exon 2 (table 2).

No sequence variant, either causative missense mutation or neutral substitution, was detected in any of the 48 patients considered.

Taking advantage of the recent mapping of \textit{HOX11L1} to chromosome 2p12-p13,\textsuperscript{24} we verified the possible presence of molecular defects of the non-coding, regulatory regions of this gene by haplotype reconstruction and linkage analysis in the two largest families among the six pedigrees available, characterised by recurrence of IND B (fig 1). These two families had already been tested for possible linkage to 10q11.2 and no evidence of an involvement of the \textit{RET} proto-oncogene was obtained.\textsuperscript{23}

The four microsatellites flanking the \textit{HOX11L1} locus used in the linkage analysis are included in the Génethon map\textsuperscript{26} and span about 11 cm of genomic DNA. In particular, marker D2S286 is located on YAC 909_g_10, a clone already known to contain the \textit{HOX11L1} gene.\textsuperscript{24}

Two point lod score analysis was carried out in the two IND pedigrees using the MLINK program included in the LINKAGE package,\textsuperscript{27} while the GENEHUNTER program was used for multipoint analysis.\textsuperscript{28} These analyses were performed assuming the disease to be a dominant disorder with incomplete penetrance (70%) with a frequency of 1/4000. However, in order to avoid possible

### Table 1 Features of patients analysed

<table>
<thead>
<tr>
<th>Occurrence of disease</th>
<th>HSCR</th>
<th>IND</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic</td>
<td>16</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>Familial</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>26</td>
<td>48</td>
</tr>
</tbody>
</table>
One hundred and fifty ng of DNA were amplified in 30 µl reaction containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, di
coding sequence, corresponding to the distal portion of intron 1.

Ten µl of exon 2 PCR product were digested with

The probability of detecting conformational variants.

Seven µl of each PCR reaction were mixed with 3 µl of denaturing solution, kept for five minutes at 95
°
C, and loaded on a 6% non-denaturing polyacrylamide gel

Expansion cycle at 72
°
C.

Table 2  Conditions used for PCR-SSCP analysis of the three coding exons of HOX11L1

<table>
<thead>
<tr>
<th>PCR size (bp)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>T anneal (°C)</th>
<th>MgCl2 (mmol/l)</th>
<th>Glycerol (%)</th>
<th>Watt/time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1A 269</td>
<td>HOX11L1 1AF</td>
<td>HOX11L1 1AR</td>
<td>58</td>
<td>0 4°C</td>
<td>3 h</td>
<td>45 W</td>
</tr>
<tr>
<td></td>
<td>5'-ctggtgccgctcaggg-3'</td>
<td>5'-cagcgggcaagggag-3'</td>
<td></td>
<td>5 4°C</td>
<td>5 h</td>
<td>45 W</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>RT 35 W 10+ o/n 4 W</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 1B 290</td>
<td>HOX11L1 1BF</td>
<td>HOX11L1 1BR</td>
<td>58</td>
<td>0 4°C</td>
<td>3 h</td>
<td>45 W</td>
</tr>
<tr>
<td></td>
<td>5'-gatacagagcgcctgag-3'</td>
<td>5'-aaatgagagcgctgag-3'</td>
<td></td>
<td>5 4°C</td>
<td>5 h</td>
<td>45 W</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>RT 35 W 10+ o/n 4 W</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2‡ 395</td>
<td>HOX11L1 2F</td>
<td>HOX11L1 2R</td>
<td>62</td>
<td>0 4°C</td>
<td>3 h</td>
<td>45 W</td>
</tr>
<tr>
<td>(32+166+197</td>
<td></td>
<td></td>
<td>5</td>
<td>4°C</td>
<td>5 h</td>
<td>45 W</td>
</tr>
<tr>
<td>after digestion with BstNI</td>
<td></td>
<td></td>
<td>10</td>
<td>RT 35 W 10+ o/n 4 W</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 3 327</td>
<td>HOX11L1 3F</td>
<td>HOX11L1 3R</td>
<td>60</td>
<td>0 4°C</td>
<td>3 h</td>
<td>45 W</td>
</tr>
<tr>
<td></td>
<td>5'-cggctgccgccgctgctc-3'</td>
<td>5'-cggctgccactagaccatgg-3'</td>
<td></td>
<td>5 4°C</td>
<td>5 h</td>
<td>45 W</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>RT 35 W 10+ o/n 4 W</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*One hundred and fifty ng of DNA were amplified in 30 µl reaction containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, different concentrations of MgCl2, 200 µmol/l dNTPs, 5% DMSO, and 1.25 U Taq polymerase with 1 µmol/l of each appropriate PCR primer. Samples were amplified for 35 cycles each consisting of one minute at 95°C, one minute at the above reported annealing temperature, and one minute at 72°C. An initial denaturation step at 94°C for five minutes and a final extension cycle at 72°C for seven minutes were also performed.

†Seven µl of each PCR reaction were mixed with 3 µl of denaturing solution, kept for five minutes at 95°C, and loaded on a 6% non-denaturing polyacrylamide gel (acylamide: bis-acylamide ratio 49:1) with different glycerol concentrations in TBE 0.5 x buffer. Each exon was tested under at least two SSCP conditions to increase the probability of detecting conformational variants.

‡Ten µl of exon 2 PCR product were digested with BstNI in a final volume of 20 µl. The digestion product was precipitated with 1/10 volume of NaAcetate 3M followed by 2.5 volumes of absolute ethanol and resuspended in 7 µl of sterile water. Among the restriction fragments obtained, the 32 bp segment does not contain any coding sequence, corresponding to the distal portion of intron 1.

![Pedigree diagram](image_url)

**Figure 1** (Left) Pedigrees and haplotype reconstruction for microsatellites D2S2110, D2S286, D2S2114, and D2S139. Hatched symbols indicate patients diagnosed on the basis of clinical symptoms only. (Right) Marker order and corresponding combined lod scores at θ = 0 are shown at the top, while the results of the multipoint analysis are displayed at the bottom. Primers for markers D2S286 and D2S139, already included in the ABI-PRISM Linkage Mapping Set, were amplified according to the manufacturer’s instructions. Primers for D2S2110 and D2S2114 were synthesised by an Oligo 1000-M DNA Synthesizer (Beckmann), labelled with 6-FAM fluorescent amidite, and purified with OPC columns (ABI, Perkin-Elmer) according to the manufacturer’s instructions. PCR was carried out under standard conditions. PCR products were loaded in an automated sequencer ABI373A and allele sizes defined using the Genescan software.
errors, all affected subjects were defined as affected, all spouses as unaffected, and all unaffected patients at risk of carrying the disease allele as unknown.

As shown in fig 1, negative lod scores <−2 were obtained for markers D2S286 and D2S2114 by the two point analysis. Accordingly, both haplotype reconstruction and multipoint analysis confirmed the exclusion of this chromosomal region in the pathogenesis of the IND phenotype recurring in the two pedigrees analysed. In particular, the three affected brothers (II.1, II.2, and II.3) and the two affected sisters (III.4 and III.5) of pedigree 1 inherited different haplotypes from the two affected mothers (II.2 and II.5). Furthermore, in pedigree 2, the common haplotype present in the three affected subjects II.4, III.1, and III.3 was absent in II.6, the carrier father of patients II.4 and III.5.

In conclusion, mutation screening of the whole HOX11L1 coding region, performed by PCR-SSCP analysis in 48 patients affected with IND or HSCR, did not show any novel sequence variant, either causative missense mutation or neutral substitution. In addition, linkage analysis excluded any other molecular defect of HOX11L1, possibly impairing its expression, in affected members of two IND pedigrees.

In recent years, several studies have been undertaken to define the role of cooperative interactions of the Hox genes and their regulatory regions, like promoters and retinoic acid responsive elements. Moreover, the recent observation in Drosophila of a posterior Hox protein which, if overexpressed, can suppress the function of a more anterior member of the cluster, confirms that the correct expression of Hox genes can be impaired by a variety of different molecular mechanisms.

Overall, these data suggest that further investigation of non-coding regions of HOX11L1 could be useful to exclude the involvement of this gene in intestinal innervation disorders.

The first two authors contributed equally to this work. We are grateful to Giuseppe Santamaria and Francesco Caroli for their technical assistance. We thank Professor Schärli and Dr Weber for allowing us to study the two IND pedigrees. The financial support of Italian Telethon (grant E791) is gratefully acknowledged. This work was also supported by the Italian Ministry of Health (Grant 3692) and the Italian Telethon (Grant E791). We acknowledge the assistance of Giuseppe Santamaria and Francesco Caroli for their technical assistance. We are grateful to Giuseppe Santamaria and Francesco Caroli for their technical assistance. We thank Professor Schärli and Dr Weber for allowing us to study the two IND pedigrees.

Correspondence to: Dr Ceccherini, isa.c@unige.it
Genetic Cancer Susceptibility Unit, IARC, Lyon, France

*Laboratorio di Genetica Molecolare, Istituto G Gaslini, Largo G Gaslini 5, 16148 Genova, Italy
‡Divisione e Cattedra di Chirurgia Pediatrica, Istituto G Gaslini, Genova, Italy
†Genetic Cancer Susceptibility Unit, IARC, Lyon, France

Evaluation of the \textit{HOX11L1} gene as a candidate for congenital disorders of intestinal innervation

MARCELLA COSTA, MONICA FAVA, MARCO SERI, ROBERTO CUSANO, MONICA SANCANDI, PAOLA FORABOSCO, MARGHERITA LERONE, GIUSEPPE MARTUCCIHELLO, GIOVANNI ROMEO and ISABELLA CECCHERINI

\textit{J Med Genet} 2000 37: e9
doi: 10.1136/jmg.37.7.e9

Updated information and services can be found at:
http://jmg.bmj.com/content/37/7/e9

These include:

References
This article cites 28 articles, 7 of which you can access for free at:
http://jmg.bmj.com/content/37/7/e9#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections

- Reproductive medicine (519)
- Epidemiology (630)
- Genetic screening / counselling (886)
- Immunology (including allergy) (604)
- Molecular genetics (1254)
- Neurogastroenterology (21)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/