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<tr>
<td><strong>Author(s)</strong></td>
<td>GarciaBarceló, M; Sham, MH; Lui, VCH; Chen, BLS; Ott, J; Tam, PKH</td>
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Association study of PHOX2B as a candidate gene for Hirschsprung’s disease

M Garcia-Barceló, M H Sham, V C H Lui, B L S Chen, J Ott, P K H Tam

Background: Hirschsprung’s disease (HSCR) is a congenital disorder characterised by an absence of ganglion cells in the nerve plexuses of the lower digestive tract. Manifestation of the disease has been linked to mutations in genes that encode the crucial signals for the development of the enteric nervous system—the RET and EDNRB signalling pathways. The PHOX2B gene is involved in neurogenesis and regulates Ret expression in mice, in which disruption of the PHOX2B results in a HSCR-like phenotype.

Aims: To investigate the contribution of PHOX2B to the HSCR phenotype.

Methods: Using polymerase chain reaction amplification and direct sequencing, we screened PHOX2B coding regions and intron/exon boundaries for mutations and polymorphisms in 91 patients with HSCR and 71 ethnically matched controls. Seventy five HSCR patients with no RET mutations were independently considered. Haplotype and genotype frequencies were compared using the standard case control statistic.

Results: Sequence analysis revealed three new polymorphisms: two novel single nucleotide polymorphisms (A→G1364, A→C2607) and a 15 base pair deletion (DEI2609). Statistically significant differences were found for A→G1364. Genotypes comprising allele G were underrepresented in patients (19% v 36%; χ² = 9.30; p = 0.0095 and 22% v 36%; χ² = 7.38; p = 0.024 for patients with no RET mutations). Pairwise linkage disequilibrium (LD) analysis revealed no LD between physically close polymorphisms indicating a hot spot for recombination in exon 3.

Conclusion: The PHOX2B A→G1364 polymorphism is associated with HSCR. Whether it directly contributes to disease susceptibility or represents a marker for a locus in LD with PHOX2B needs further investigation. Our findings are in accordance with the involvement of PHOX2B in the signalling pathways governing the development of enteric neurones.

Hirschsprung’s disease (HSCR), regarded as a multigenic neurocristopathy, is a congenital disorder in which there is an absence of ganglion cells in the nerve plexuses of the lower digestive tract. The condition presents in the neonatal period as failure to pass meconium, chronic severe constipation, colonic distention, secondary electrolyte disturbances, and sometimes enterocolitis and bowel perforation. The estimated population incidence is 1/5000 live births. Males are 3.5–4.0 times more likely to be affected than females. HSCR may occur with other neurodevelopmental disorders such as Waardenburg syndrome type 4 (WS4), and it may also be associated with a variety of other anomalies, including chromosomal abnormalities and syndromes with a Mendelian pattern of inheritance. Approximately 20% of HSCR cases are familial, with a sibling recurrence risk of 4%.

Aganglionosis is due to a disorder of the enteric nervous system (ENS) in which ganglion cells fail to innervate the lower gastrointestinal tract during embryonic development. The extent of the aganglionic segment is variable, and is reflected in the severity of the disease. The genetic signals critical for neural crest migration and differentiation into enteric ganglia include genes encoding neurotrophic factors and their receptors. Substantial evidence has been presented to show that HSCR has a complex genetic aetiology requiring the interaction of several unlinked genes, and possibly environmental factors, to produce the phenotype. It has been postulated that the degree of expression of the receptor tyrosine kinase gene (RET) is critical for the HSCR phenotype. However, RET mutations account for only 20–30% of HSCR cases and lack genotype-phenotype correlation due to low penetrance, indicating that genes other than RET are implicated in the disease. This has led to the search for other susceptibility and modifying genes that could lead to HSCR. Interestingly, HSCR genes identified so far mainly code for protein members of two important signalling pathways involved in the development of enteric ganglia: RET and endothelin receptor B (EDNRB) signalling pathways. Interaction between these two signalling pathways could modify RET expression and therefore HSCR phenotype. Nevertheless, for almost every HSCR gene described, incomplete penetrance and variable expression of the HSCR phenotype has also been observed. Therefore, other genes implicated in RET expression and/or intestinal neurodevelopment should be considered as candidate genes for HSCR.

The paired mesoderm homeobox 2b gene (PHOX2B) encodes a transcription factor (homeodomain protein) which is involved in the development of several noradrenergic neurone populations. In mice, PHOX2B expression starts as soon as enteroblasts invade the foregut mesenchyme and is maintained throughout the development into enteric neurones. Homozygous disruption of the Phox2b gene results in absence of enteric ganglia, a feature which is reminiscent of HSCR. Furthermore, there is no Ret expression in Phox2b mutant embryos. This indicates that regulation of Ret by Phox2b could account for failure of the ENS to develop.

The human PHOX2B gene has been cloned and sequenced. Its coding region consists of 945 base pairs (bp) allocated in three exons, yielding a homeodomain protein of 314 amino acid residues.

The compelling evidence of the murine model has made the human PHOX2B gene an attractive target for exploration as a potential candidate gene for Hirschsprung’s disease.

Abbreviations: HSCR, Hirschsprung’s disease; ENS, enteric nervous system; LD, linkage disequilibrium; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; WS4, Waardenburg syndrome type 4; TCA, total colonic aganglionosis; bp, base pair; EH, estimating haplotype frequencies.
PHOX2B (unpublished data). To better evaluate the relevance of
assessed. Association analysis was therefore performed on
sequences) of PHOX2B′ committee.

i na2 5 µl of each nucleotide); 2.5 µl of all four nucleotides (final concentration 0.2 mM for each
appropriate primer; 1 mM MgCl2; and 1.25 U of AmpliTaq Gold
polymerase (Applied Biosystems, Foster City, California, USA). Due to its high GC content, amplification of exon 3 was
performed using the GC-RICH PCR System (Roche Molecular
Biocahemicals, USA). Exon 3 PCR was performed in a 25 µl reaction volume containing 100 ng genomic DNA; a
mixture of all four nucleotides (final concentration 0.2 mM for each
nucleotide); 5 µl of 10× reaction buffer; 0.5 µM of each
appropriate primer; 1 mM MgCl2; and 1.25 U of AmpliTaq Gold
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nucleotide); 5 µl of 10× reaction buffer; 0.5 µM of each
appropriate primer; 1 mM MgCl2; and 1.25 U of AmpliTaq Gold
polymerase (Applied Biosystems, Foster City, California, USA).

apotential human HSCR gene. HSCR results from mutations and/or single nucleotide polymorphisms (SNPs) in several
genes acting either alone or in combination. Thus the hypothesis underlying this study is that mutations or polymor-
phisms in the PHOX2B gene may be associated with the HSCR phenotype by directly affecting expression of the PHOX2B gene
product, by being in linkage disequilibrium (LD) with unknown causative mutations or polymorphisms, or by becoming patho-
genetic in the presence of mutations and/or polymorphisms in other genes. Our data contribute to the molecular dissection of
HSCR and enable an understanding of how genetic interactions lead to the HSCR phenotype. To our knowledge, this is the first
association study between PHOX2B and HSCR.

MATERIAL AND METHODS

Patients and controls

A total of 91 ethnic Chinese patients, diagnosed with HSCR between 1984 and 2001, were included in this study. Diagnos-
sis was made at Queen Mary Hospital, Hong Kong SAR, and
was based on histological examination of either biopsy or sur-
gical resection material for absence of enteric plexuses. Five
patients (one with total colonic aganglionosis (TCA), two with
long segment aganglionosis, and two with short segment
aganglionosis) had affected relatives (two unrelated families
in total). Eighty six patients were affected with sporadic HSCR with the following phenotypes: four individuals with TCA;
seven with long segment aganglionosis; and 75 with short
segment aganglionosis. Nine patients with sporadic HSCR were also affected with the following associated anomalies: five with Down’s syndrome (short segment); one with
Waardenburg syndrome (TCA); one with renal agenesis (short
segment); one with parathyroid adenoma (short segment); and
one with desmoid tumour (short segment). Seventy five of 91 patients had no causative mutations in the RET
gene (unpublished data). To better evaluate the relevance of
PHOX2B in HSCR, these 75 patients were also independently
assessed. Association analysis was therefore performed on
HSCR patients regardless of their RET mutation status and on
the group of 75 patients with no RET mutations.

Normal controls (71 individuals) were unselected unrelated
ethnic Chinese subjects from Hong Kong without a diagnosis of
HSCR.

DNA was extracted from peripheral blood as previously
described. All patients and controls assented to molecular
analysis. The study was approved by the local ethics
committee.

Polymerase chain reaction and DNA sequencing

Polymerase chain reaction (PCR) was used to amplify the three coding regions (including their 5′ and 3′ flanking sequences) of PHOX2B. For exons 1 and 2, PCR was performed in a 25 µl reaction volume containing 100 ng genomic DNA; a
mixture of all four nucleotides (final concentration 0.2 mM for each
nucleotide); 2.5 µl of 10× reaction buffer; 0.5 µM of each
appropriate primer; 1 mM MgCl2; and 1.25 U of AmpliTaq Gold
polymerase (Applied Biosystems, Foster City, California, USA).

RESULTS

Polyomorphism sites

By direct sequencing of the entire coding region and portions of 5′ and 3′ flanking regions, we found two new SNPs and one
deletion in the PHOX2B gene (fig 1). Nucleotide positions are
defined in relation to the first nucleotide of the start codon, which is designated position +1.

The first polymorphism identified was an A→G transition (referred as A→Gc2607) in codon 253 of the PHOX2B exon 3.
Table 2  Estimated haplotype frequencies in patients and controls determined by estimating haplotype frequencies

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Intron 2</th>
<th>Exon 3</th>
<th>Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>A→G1364</td>
<td>A→C2607</td>
<td>DEL2609</td>
<td>Patients</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>ND</td>
<td>0.855</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>D</td>
<td>0.029</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>D</td>
<td>0.000</td>
</tr>
<tr>
<td>A</td>
<td>ND</td>
<td>NM</td>
<td>0.056</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>D</td>
<td>0.009</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>ND</td>
<td>0.049</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>D</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Allergic variation between patients and controls

To determine the significance of these three novel polymorphisms in HSCR, we conducted a case control association study to investigate whether they were directly related to the HSCR phenotype. Differences in allele frequencies between the patient and control groups were not statistically significant for any of the polymorphisms (A→G1364, χ²=2.41; p=0.12; A→C2607, χ²=1.62; p=0.20; DEL2609, χ²=1.06; p=0.3). Also, there were no statistically significant differences when the 75 patients with no RET mutations were compared with controls. Within each group, allele frequencies of both sexes were compared and no statistically significant differences for any of the identified polymorphisms were found.

Genotype frequency distribution in patients and controls

Comparison of the genotype frequencies of the three new polymorphisms between patients and controls (table 1) revealed statistically significant differences for A→G1364 (χ²=9.30; p=0.0095). An association was also found when 75 patients with no mutations in RET were compared (χ²=7.38; p=0.024). For this particular polymorphism, genotypes comprising G were underrepresented in both patient groups: 19% (91 patients) versus 36% (71 controls) and 22% (75 patients) versus 36% (71 controls). Within each group, no statistically significant differences in genotype frequencies for any of the identified polymorphisms were found between the

Table 1  Genotype frequency distribution of the PHOX2B gene polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Patients HSCR +/− RET mutations n=91†</th>
<th>HSCR − RET mutations n=75 †</th>
<th>Controls n=71</th>
</tr>
</thead>
<tbody>
<tr>
<td>A→G1364</td>
<td>0.81 (n=73)</td>
<td>0.77 (n=56)</td>
<td>0.64 (n=46)</td>
</tr>
<tr>
<td>AA</td>
<td>0.16 (n=15)</td>
<td>0.19 (n=14)</td>
<td>0.36 (n=25)</td>
</tr>
<tr>
<td>AG</td>
<td>0.03 (n=3)</td>
<td>0.03 (n=3)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td>GG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A→C2607</td>
<td>0.90 (n=82)</td>
<td>0.88 (n=64)</td>
<td>0.83 (n=59)</td>
</tr>
<tr>
<td>AA</td>
<td>0.10 (n=9)</td>
<td>0.12 (n=9)</td>
<td>0.17 (n=12)</td>
</tr>
<tr>
<td>AC</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEL2609</td>
<td>0.92 (n=79)</td>
<td>0.90 (n=66)</td>
<td>0.87 (n=62)</td>
</tr>
<tr>
<td>NN</td>
<td>0.08 (n=7)</td>
<td>0.10 (n=7)</td>
<td>0.13 (n=9)</td>
</tr>
<tr>
<td>DD</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td>A→G1364</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A→C2607</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A→G1364</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HSCR patients regardless of their RET mutation status.
†HSCR patients with no RET mutations.
‡IV no deletion.
§D deletion.

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Haplotypes. Within each group, each of the three polymorphic sites was in Hardy-Weinberg equilibrium.

**Haplotype frequencies and linkage disequilibrium among the three polymorphic sites**

We were also interested in assessing the level of association between HSCR disease and the genotypes involving two or three polymorphic sites. When we used contingency tables to compare frequencies of haplotypes generated by the combination A→G1364, A→C2607, and DEL2609, statistically significant differences between patients and controls were found for both haplotype combinations (χ² = 12.41; p = 0.014 and χ² = 9.71; p = 0.04, respectively). Contributions to χ² were dissected and demonstrated that the major contribution was that of genotypes of the polymorphic site A→G1364. In contrast, no statistically significant difference in frequencies was found for the combination A→G1364 + DEL2609 (χ² = 3.26; p = 0.19). When haplotypes comprising the three polymorphisms were described in this study were analysed for differences in frequencies between patients and controls, no association was found (χ² = 11; p = 0.08, 6 df). Similar results were obtained for the 75 patients with no RET mutations.

Using the EH program we estimated the haplotype frequencies of the three polymorphisms combined (table 2). The two most common haplotype combinations for both cases and controls were A-A-ND and G-A-ND (allele designations as shown in table 1). When haplotype frequencies were compared, none of the haplotype combinations showed statistically significant differences between patients and controls (table 3). Using this program, we also assessed differences in frequencies of haplotype combinations comprising only two polymorphic sites. No statistically significant differences were found. Similar results were found for the group of 75 patients with no RET mutations (data not shown). Overall, these findings are in agreement with the data obtained when contingency tables were used: the association found was driven by A→G1364.

Significant evidence for LD was found among the three polymorphisms for both patients and controls, either analysed independently or combined. These findings were not surprising given the close physical proximity of the markers. We also assessed LD between two polymorphisms (table 4). The A→G1364 polymorphism was in LD with A→C2607 in both patients and controls, either analysed independently or combined. Surprisingly, no LD was found between A→G1364 and DEL2609 nor between A→C2607 and DEL2609 despite being only 2 bp apart. Similar results were obtained for the group of 75 patients (data not shown). These data strongly suggest that only the A→G1364 polymorphism is associated with HSCR disease and that the discrepancies in the analysis of haplotype frequencies are due to incomplete LD.

**DISCUSSION**

In this study, we sequenced the entire coding region of the PHOX2B gene as well as intron/exon boundaries of ethnically matched patients and controls in searching for mutations or polymorphisms that could contribute to the clinical manifestation of HSCR disease. We identified two new SNPs (A→G1364, A→C2607) and one deletion (DEL2609).

A→G1364 is a silent transition that does not alter the amino acid sequence. This polymorphism was also present in the control population with no significant allele or genotype frequency differences. Interestingly, DEL2609 showed no association with HSCR despite causing the loss of five alanine residues in the PHOX2B protein. In both patients and controls, DEL2609 was always heterozygous. The functional consequences of this deletion are unclear at the moment. In mice, heterozygous disruption of the Phox2b homeodomain shows no obvious phenotype, but homozygous mutants are embryonically lethal. In those individuals carrying DEL2609, any functional consequence caused by the DEL2609 polymorphism may be compensated by the normal allele.

For A→G1364 we found statistically significant differences in genotype distribution when patients were compared with controls, indicating an association of the A→G1364 polymorphism with HSCR. The association found could be due to a direct contribution of A→G1364 to the HSCR phenotype or that A→G1364 is in LD with another susceptibility locus. Direct involvement of A→G1364 in HSCR disease would be through alteration of intrinsic sequences crucial for splicing and/or regulation of expression of the PHOX2B gene. Based on our branch site sequence analysis, it is unlikely that the A→G1364 polymorphism is involved in alternative splicing of PHOX2B transcripts. As cis acting regulatory elements for the PHOX2B gene have not been defined, at present it is still unclear whether the A→G1364 polymorphism could directly affect PHOX2B gene expression. It is possible that either the PHOX2B A→G1364 polymorphism or other mutations in PHOX2B need to occur together or act in combination with other mutations in the RET and/or EDNRB signalling pathway genes to produce the HSCR phenotype. A combination of specific variations in multiple genes of these signalling pathways may confer either protection or susceptibility to the disease and even modify the phenotype. Coexistence of RET mutations with mutations or polymorphisms in GDNF and EDNRB genes have been described in patients with HSCR.14 The association found when patients with no mutations in RET coding regions were examined indicate that A→G1364—or a locus in LD—could directly contribute to the HSCR phenotype. Nevertheless, A→G1364 may act in combination with any of the many polymorphisms of the RET gene which are present in both the healthy population and affected individuals, or RET mutations could be in non-coding regions. Mutation analyses of the EDNRB, EDN3, and GDNF genes in the same group of patients are now in progress to investigate coexisting mutations with

<table>
<thead>
<tr>
<th>Group</th>
<th>n*</th>
<th>ln(L)</th>
<th>χ²</th>
<th>p*</th>
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</thead>
<tbody>
<tr>
<td>Patients</td>
<td>91</td>
<td>90.84</td>
<td>36.54</td>
<td>0.42</td>
</tr>
<tr>
<td>Controls</td>
<td>71</td>
<td>-97.86</td>
<td>21.99</td>
<td></td>
</tr>
<tr>
<td>All subjects</td>
<td>162</td>
<td>-192.24</td>
<td>56.49</td>
<td></td>
</tr>
</tbody>
</table>

*Number of individuals.
†The relevant test statistic is $\ln(L) + \ln(n_{cases}) - \ln(n_{controls})$.
**PHOX2B** polymorphism. Recently, Gabriel and colleagues described two loci—3p21 and 19q12—as RET dependent modifiers segregating in families affected with short segment aganglionic. The **PHOX2B** gene maps to the 4p12 region, which is not a predicted modifier loci for RET in familial HSCR. The patients reported here were mainly sporadic cases of HSCR for whom mutations in RET accounted for only 15% of cases whereas approximately 50% of familial HSCR cases were due to RET mutations. Nevertheless, the phenotypic effect of the **tPHOX2B** contribution could depend on the number, severity, and co-occurrence of polymorphisms and/or mutations in RET. Our study provides evidence of the possible contribution of **PHOX2B** to HSCR acting either alone or in combination.

The underrepresentation of the A→G substitution polymorphism may indicate that the transition A→G is relatively recent in evolutionary terms, as shown by the relatively low frequency of the allele G in the population. If the minor allele G conferred a protective effect, its frequency in the population would be rising to become the major allele. We found LD among the three polymorphisms that spread over 1.2 kb, which was expected given their physical proximity. Interestingly, the pairwise disequilibrium test showed lack of LD between A→G versus DEL2609 and between A→C versus DEL13, which are only two base pairs apart. This indicates that the DEL13 polymorphism is probably situated in a recombination hot spot. Detailed sequence examination of exon 3 (from nucleotides 2290 to 2805) in which this polymorphism is located showed that there is a high GC content and these are runs of CGG repeats which could serve as loci for recombination. Recombination across the hot spot would explain the results obtained in the pairwise disequilibrium test and haplotype analysis. This suggests that if there are other polymorphisms or mutations in and around the **PHOX2B** gene which are also contributing to HSCR, they should be located upstream of the A→G substitution polymorphism. These other possible susceptibility loci would not be in LD with the polymorphism (DEL13) found in the recombination hot spot that defines the breakpoint of a LD block. Therefore, the DEL13 polymorphism should not be used as a marker in further linkage based association tests. As the marker by marker approach ignores the polygenic nature of HSCR disease and does not consider possible interactions among susceptibility genes, it would be interesting to investigate sets of SNPs in the different susceptibility loci described for HSCR. Therefore, it would be necessary to assemble an extensive catalogue of SNPs in these candidate genes and perform association studies considering all of the candidate genes together. Dissection of the genetic aetiology of HSCR disease will help us in understanding other polygenic complex disorders and congenital malformations considered multifactorial in origin.

**ACKNOWLEDGEMENTS**

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**ELECTRONIC DATA BASE INFORMATION**

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim/ (for HSCR [MIM 142623]; RET [MIM 164761]; EDNBR [MIM 121244]; **PHOX2B** [MIM 603851]; WS4 [MIM 277580]; and GDNF [MIM 600837]).

EH software was downloaded from http://www.ncbi.nlm.nih.gov/SNP.


**REFERENCES**


