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<td><strong>Author(s)</strong></td>
<td>GarciaBarceló, M; Sham, MH; Lui, VCH; Chen, BLS; Ott, J; Tam, PKH</td>
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<tr>
<td><strong>Citation</strong></td>
<td>Gut, 2003, v. 52 n. 4, p. 563-567</td>
</tr>
<tr>
<td><strong>Issued Date</strong></td>
<td>2003</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 and P K H Tam

Gut 2003;52:563-567
doi:10.1136/gut.52.4.563

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Notes
Association study of PHOX2B as a candidate gene for Hirschsprung’s disease

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

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.

The compelling evidence of the murine model has made the Phox2b gene an attractive target for exploration as a 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 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 polymorphisms 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 pathogenic 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. Diagnosis was made at Queen Mary Hospital, Hong Kong SAR, and was based on histological examination of either biopsy or surgical 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 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). Due to its high GC content, amplification of exon 3 was performed using the GC-RICH PCR System (Roche Molecular Biochemicals, 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 5× GC-RICH PCR reaction buffer; 2.5 µl of 5 M GC-RICH resolution solution; 0.5 µM of each appropriate primer; and 1.25 U of a blend of Taq DNA Polymerase and Tgo DNA Polymerase. Primers used to amplify the three coding regions, including intron-exon boundaries, were derived from the PHOX2B gene sequence available in GenBank (accession No AB015671). The primers were as follow (size of amplified DNA is in parentheses; forward primer is shown first in each set): exon 1: GACCTCGAAGGAGCATTCA and AATTAC-CCCTCTCTGAATC (586 bp); exon 2: CTGCGTATGACCT-GACCTT and ACAGCACAACAAATCCAGT (442 bp); and exon 3: ACCCTAAACGGTTGCTTTTTCT and ACAATAAGCTT-GGGCTAAC (687 bp). The cycling conditions for the three exons were 95°C for eight minutes followed by 35 cycles of 95°C for one minute, 62°C for one minute, and 72°C for 45 seconds. A final 10 minute extension was included at the end of the 35 cycles. Prior to sequencing, PCR products were column purified (Life Technologies, UK) to remove reaction buffer and unincorporated primers, and visualised by running 5 µl of each sample on 2% agarose gels. PCR products were screened for mutations by direct sequencing using a dye terminator cycle sequencing kit (ABI PRISM Big Dye Terminator v 2.0 Cycle sequencing kit; Applied Biosystems) and an ABI 3100 automated sequencer (Applied Biosystems). For those samples in which DNA sequence variation had been observed, PCR amplification from genomic DNA and sequencing using both forward and reverse primers were repeated up to five times.

Statistical analysis

Allele and genotype frequencies for each polymorphism in the control group and in unrelated patients were calculated. Allele and genotype frequency comparisons between the control and patient groups were performed using χ² tests. χ² tests were also performed to determine whether each polymorphism was in Hardy-Weinberg equilibrium within each group.

The level of association between genotypes involving two or three polymorphic sites and HSCR disease was assessed using both χ² contingency tables and the estimating haplotype frequencies (EH) program. EH is a linkage utility program to test and estimate linkage disequilibrium between different markers or between a disease locus and markers. The EH program uses the method of gene counting, which provides maximum likelihood estimates of the haplotype frequencies. Haplotype frequencies are estimated considering allelic association/linkage disequilibrium (H) among markers and without (H0). The EH program also provides log likelihood, χ², and the number of degrees of freedom under hypotheses H0 and H. EH is only applicable to unrelated individuals. To test whether haplotype frequencies were significantly different between cases and controls, we performed three separate analyses using the EH program: on case subjects alone, on control subjects alone, and on the combination of case and control subjects. The three log likelihoods obtained (lnLcase, lnLcontrol, and lnLcombined) were used to calculate the expected statistic test T= [lnL(Leave) + lnL(Combine)] - lnL(Combined)]. Twice this value gives an approximate χ² distribution with a number of degrees of freedom (df) equal to the number of haplotypes estimated under the hypothesis that allelic association is allowed. The EH program, which assumes Hardy-Weinberg equilibrium, was also used to estimate LD between polymorphisms.

RESULTS

Polymorphic 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→Gmut) in intron 2, 100 bp away from the intron/exon boundary. We investigated the possibility of A→Gmut being part of PHOX2B intronic splice regulation sites. The PHOX2B intron 2 sequence was screened for branch sites (additional intronic splice signals), but sequence analysis did not indicate such a possibility.

The second polymorphism identified was an A→C change (referred as A→Cmut) in codon 253 of the PHOX2B exon 3.
A→G<sub>1364</sub> is a silent transition that does not alter the amino acid sequence (GCA→GCC).

The third sequence variant was a 15 bp deletion (referred as DEL<sub>2609</sub>) in exon 3, starting from codon 254 and just 2 bp downstream of the A→G<sub>1364</sub> polymorphism (fig 1). This 15 bp deletion occurs in one of the two polyanaline stretches in the C terminus, resulting in the loss of five alanine residues (codons 254–258). The polyanaline region in the normal PHOX2B protein (codons 245–264) may form α-helical structures and serve as a flexible linker. The functional consequence of this deletion of five alanine residues is unclear at present. Interestingly, among the 71 normal individuals studied, nine had this deletion in one of their PHOX2B alleles. We ruled out the possibility of this observed deletion being an experimental artefact. Specific PCR amplification enzymes were used to resolve the high GC sequence content of exon 3 (see methods). In addition, those samples with the deletion were reamplified and resequenced 5–10 times.

Finally, in every sample analysed, we found an A at both positions 243 and 1418 of the PHOX2B gene (intron 2); polymorphism G<sub>1364</sub>→A<sub>1364</sub> (fig 1). The Genbank accession numbers for PHOX2B (AB015671 and AF117979) record T and G, respectively. These may represent sequencing errors in the GenBank. Polymorphism ss3136952 is a A→C<sub>3136952</sub> transversion at position 4517 (end of 3′ untranslated region); and polymorphisms ss1551094 is a C→A<sub>1551094</sub> transversion at position 4517 (end of 3′ untranslated region). These polymorphisms were not examined in our study as they are outside the coding regions and splice sites.

![Figure 1 Polymorphisms identified in the PHOX2B gene. The three PHOX2B exons are represented by rectangles. Black boxes indicate polyanaline stretches (from codon 162 to 169 and from codon 245 to 264). Downward arrows show the relative location of the polymorphisms.](image-url)

### Allelic 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→G<sub>1364</sub> χ<sup>2</sup>=2.41; p=0.12; A→C<sub>1364</sub> χ<sup>2</sup>=1.62; p=0.20; DEL<sub>2609</sub> χ<sup>2</sup>=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→G<sub>1364</sub> (χ<sup>2</sup>=9.30; p=0.0095). An association was also found when 75 patients with no mutations in RET were compared (χ<sup>2</sup>=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 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→G&lt;sub&gt;1364&lt;/sub&gt;</td>
<td>A→C&lt;sub&gt;1364&lt;/sub&gt;</td>
<td>DEL&lt;sub&gt;2609&lt;/sub&gt;</td>
<td>Patients (n=91)</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>
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<td>A</td>
<td>C</td>
<td>D</td>
<td>0.000</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>ND</td>
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<tr>
<td>G</td>
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<td>D</td>
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<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>

### Table 1 Genotype frequency distribution of the PHOX2B gene polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Patients</th>
<th>HSCR +/− RET mutations (n=91)*</th>
<th>HSCR −RET mutations (n=75) †</th>
<th>Controls (n=71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A→G&lt;sub&gt;1364&lt;/sub&gt;</td>
<td>AA</td>
<td>0.81 (n=73)</td>
<td>0.77 (n=56)</td>
<td>0.64 (n=46)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>0.16 (n=15)</td>
<td>0.19 (n=14)</td>
<td>0.36 (n=25)</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>0.03 (n=3)</td>
<td>0.03 (n=3)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>χ&lt;sup&gt;2&lt;/sup&gt;=0.024 †, p=0.9095*; χ&lt;sup&gt;2&lt;/sup&gt;=7.38 †, p=0.024 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A→C&lt;sub&gt;1364&lt;/sub&gt;</td>
<td>AA</td>
<td>0.90 (n=82)</td>
<td>0.88 (n=64)</td>
<td>0.83 (n=59)</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>0.10 (n=9)</td>
<td>0.12 (n=9)</td>
<td>0.17 (n=12)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>χ&lt;sup&gt;2&lt;/sup&gt;=0.23 †, p=0.6407; χ&lt;sup&gt;2&lt;/sup&gt;=0.71 †, p=0.39 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEL&lt;sub&gt;2609&lt;/sub&gt;</td>
<td>NN</td>
<td>0.92 (n=79)</td>
<td>0.90 (n=66)</td>
<td>0.87 (n=62)</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>0.08 (n=7)</td>
<td>0.10 (n=7)</td>
<td>0.13 (n=7)</td>
</tr>
<tr>
<td></td>
<td>DD</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>χ&lt;sup&gt;2&lt;/sup&gt;=0.39 †, p=0.52 †, χ&lt;sup&gt;2&lt;/sup&gt;=0.91 †, p=0.34 †</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HSCR patients regardless of their RET mutation status.
†HSCR patients with no RET mutations.
§No deletion.
\(\$\) Del deletion.
Table 3 Association testing based on the haplotype frequencies of A→G1364, A→C2607, and DEL2609.

<table>
<thead>
<tr>
<th>Group</th>
<th>n*</th>
<th>ln(L)</th>
<th>x²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>91</td>
<td>-90.84</td>
<td>36.34</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>36.49</td>
<td></td>
</tr>
</tbody>
</table>

*Number of individuals.
†The relevant test statistic is \( T = \ln(L, \text{cases}) + \ln(L, \text{controls}) \).

Table 4 Pairwise linkage disequilibrium

<table>
<thead>
<tr>
<th>Pairwise comparison</th>
<th>Patients (n=91)</th>
<th>Controls (n=71)</th>
<th>All subjects (n=162)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( x^2 )</td>
<td>( p )</td>
<td>( x^2 )</td>
</tr>
<tr>
<td>A→G1364 v A→C2607</td>
<td>34.67</td>
<td>0.00</td>
<td>15.07</td>
</tr>
<tr>
<td>A→G1364 v DEL2609</td>
<td>0.12</td>
<td>0.90</td>
<td>1.86</td>
</tr>
<tr>
<td>A→C2607 v DEL2609</td>
<td>0.72</td>
<td>0.86</td>
<td>1.64</td>
</tr>
</tbody>
</table>

discussed in this study were analysed for differences in haplotype frequencies. No statistically significant differences were found between patients and controls were found for both haplotype combinations (\( x^2 = 12.41 \); \( p = 0.014 \) and \( x^2 = 9.71 \); \( p = 0.04 \), respectively). Contributions to \( x^2 \) 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 (\( x^2 = 3.26 \); \( p = 0.19 \)). When haplotypes comprising the three polymorphisms described in this study were analysed for differences in frequencies between patients and controls, no association was found (\( x^2 = 11 \); \( p = 0.08 \), 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→G1364 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→C2607 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.11 12 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
PHOX2B polymorphism. Recently, Gabriel and colleagues described two loci—3p21 and 19q12—as RET dependent modifiers segregating in families affected with short segment aganglionosis. 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 $i$th PHOX2B 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 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 DEL307, which are only two base pairs apart. This indicates that the DEL307 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 foci 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 polymorphism. These other possible susceptibility loci would contribute to HSCR acting either alone or in combination.

As a 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
We extend our gratitude to all subjects who participated in the study. We would like to thank Dr YQ Song for critical review of the manuscript and SK Yung for his valuable technical assistance. This work was supported by research grants from the Hong Kong Research Grants Council (HUK 7538/00M) and from the US National Human Genome Research Institute to JO (HG00008).

Electronic Data Base Information
Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omtnm/ (for HSCR: MIM 142623); RET (MIM 164761); EDBNBR [MIM 121244]; PHOX2B [MIM 603851]; WS4 [MIM 277580]; and GDNF (MIM 600837). EFI software was downloaded from http://linkage.rockefeller.edu/ott/linkutil.htm.

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