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<th><strong>Title</strong></th>
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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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M Garcia-Barceló, M H Sham, V C H Lui, B L S Chen, J Ott and P K H Tam

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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 (DEL 2609). Statistically significant differences were found for A→G1364. Genotypes comprising allele G were underrepresented in patients (19% v 36%; $\chi^2=9.30$; p=0.005) and 22% v 36%; $\chi^2=7.38$; 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.1 Males are 3.5–4.0 times more likely to be affected than females.1 HSCR may occur with other neurodevelopmental disorders such as Waardenburg syndrome type 4 (WS4),2 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 lower gastrointestinal tract during embryonic development. The extent of the aganglionic segment is variable, and its boundaries and bowel perforation. The estimated population incidence is 1/5000 live births.1 Males are 3.5–4.0 times more likely to be affected than females.1 HSCR may occur with other neurodevelopmental disorders such as Waardenburg syndrome type 4 (WS4),2 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%.

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Hirschsprung's disease

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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, and that differentiation is allowed. The EH program, which assumes 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 \( \chi^2 \) contingency tables and the estimating haplotype frequencies (EH) program.20 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 (H). The EH program also provides log likelihood, \( \chi^2 \), and the number of degrees of freedom under hypotheses H 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 (lnL<sub>case</sub>, lnL<sub>control</sub>, and lnL<sub>combined</sub>) were used to calculate the relevant statistic test \( T = [\ln(L_{\text{case}}) + \ln(L_{\text{control}}) - \ln(L_{\text{combined}})] / 2 \). Twice this value gives an approximate \( t \) 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→G<sub>C</sub>) in intron 2, 100 bp away from the intron/exon boundary. We investigated the possibility of A→G<sub>C</sub> 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→C<sub>G</sub>) in codon 253 of the PHOX2B exon 3.

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 (two 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 and HSCR.

Association analysis was therefore performed on PHOX2B three coding regions (including their 5′ flanking regions, including intron-exon boundaries, were derived from the PHOX2B gene sequence available in GenBank (accession No AB015671). The primers were as follows in size of amplified DNA is in parentheses; forward primer is shown first in each set): exon 1: GACCTCAGAGGCCATCTCA and AATTAC- CCCCCTGCGATAC (586 bp); exon 2: CGCGCTATGACCT- GACCTT and ACAGCCAACACAAATCCAGT (442 bp); and exon 3: ACCCTAAACGGTTGCITTCCT and ACAATAGCCTT- GGGCTACCC (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 \( \chi^2 \) tests. \( \chi^2 \) tests were also performed to determine whether each polymorphism was in Hardy-Weinberg equilibrium within each group.

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A→C2607 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 DEL2609) in exon 3, starting from codon 254 and just 2 bp downstream of the A→C2607 polymorphism (fig 1). This 15 bp deletion occurs in one of the two polyalanine stretches in the C terminus, resulting in the loss of five alanine residues (codons 254-258). The polyalanine 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), and at position 2118 of the gene (intron 2 and 3, respectively). (fig 1). The Genbank accession numbers for PHOX2B (AB015671 and AF117979) record T and G, respectively. These may represent sequencing errors in the GenBank entries.

In addition to the three polymorphisms described in this study, there are three other PHOX2B SNPs recorded in GenBank. Polymorphism ss3136952 is an A→C1364 transition at position 2118 of the PHOX2B gene (intron 2); polymorphism ss1551093 is a T→A1364 transversion at position 4457 (end of 3′ untranslated region); and polymorphisms ss1551094 is a C→A1364 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.

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→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 deletions 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

<table>
<thead>
<tr>
<th>Polymorphism</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→G1364 AA</td>
<td>0.81 (n=73)</td>
<td>0.77 (n=56)</td>
<td>0.64 (n=46)</td>
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<tr>
<td></td>
<td>0.16 (n=15)</td>
<td>0.19 (n=14)</td>
<td>0.36 (n=25)</td>
</tr>
<tr>
<td></td>
<td>0.03 (n=3)</td>
<td>0.03 (n=3)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td></td>
<td>χ²=9.30*; p=0.0095*; χ²=7.38 †; p=0.024 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A→C2607 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>0.10 (n=9)</td>
<td>0.12 (n=9)</td>
<td>0.17 (n=12)</td>
</tr>
<tr>
<td></td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td></td>
<td>χ²=1.74*; p=0.23*; χ²=0.71 †; p=0.39 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEL2609 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>0.08 (n=7)</td>
<td>0.10 (n=7)</td>
<td>0.13 (n=9)</td>
</tr>
<tr>
<td></td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
<td>0.00 (n=0)</td>
</tr>
<tr>
<td></td>
<td>χ²=1.11*; p=0.29*; χ²=0.41 †; p=0.52 †</td>
<td></td>
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</tr>
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</table>

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

### Table 2

<p>| Table 2 Estimated haplotype frequencies in patients and controls determined by estimating haplotype frequencies |
|---------------------------------|---------------------------------|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Intron 2</th>
<th>Exon 3</th>
<th>Frequencies</th>
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<tbody>
<tr>
<td>A→G1364</td>
<td>A→C2607</td>
<td>DEL2609</td>
<td>Patients (n=91)</td>
</tr>
<tr>
<td>A A</td>
<td>A A</td>
<td>ND</td>
<td>0.855</td>
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<tr>
<td>A A</td>
<td>A D</td>
<td>D</td>
<td>0.029</td>
</tr>
<tr>
<td>A C</td>
<td>C ND</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td>A C</td>
<td>C D</td>
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<tr>
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<td>G ND</td>
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</tr>
<tr>
<td>G A</td>
<td>A D</td>
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<td>0.009</td>
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<tr>
<td>G C</td>
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<tr>
<td>G C</td>
<td>C D</td>
<td>D</td>
<td>0.000</td>
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</table>

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sexes. 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 $A\rightarrow G_{1364}, A\rightarrow C_{2607}$ with the combination $A\rightarrow G_{1364}, +DEL_{2609}$, statistically significant differences between patients and controls were found for both haplotype combinations ($\chi^2 = 12.41; p = 0.014$ and $\chi^2 = 9.71; p = 0.04$, respectively). Contributions to $\chi^2$ were dissected and demonstrated that the major contribution was that of genotypes of the polymorphic site $A\rightarrow G_{1364}$. In contrast, no statistically significant difference in frequencies was found for the combination $A\rightarrow G_{1364}, + DEL_{2609}$ ($\chi^2 = 3.26; p = 0.19$). When haplotypes comprising the three polymorphisms described in this study were analysed for differences in frequencies between patients and controls, no association was found ($\chi^2 = 11; p = 0.08$, 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 $A\rightarrow G_{1364}$.

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 $A\rightarrow G_{1364}$ polymorphism was in LD with $A\rightarrow C_{1364}$ in both patients and controls, either analysed independently or combined. Surprisingly, no LD was found between $A\rightarrow G_{1364}$ and $DEL_{2609}$ nor between $A\rightarrow C_{2607}$ and $DEL_{2609}$ 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 $A\rightarrow G_{1364}$ 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 manifestations of HSCR disease. We identified two new SNPs ($A\rightarrow G_{1364}, A\rightarrow C_{2607}$) and one deletion ($DEL_{2609}$). $A\rightarrow C_{2607}$ 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, DEL$_{2609}$ showed no association with HSCR despite causing the loss of five alanine residues in the PHOX2B protein. In both patients and controls, DEL$_{2609}$ 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 DEL$_{2609}$, any functional consequence caused by the DEL$_{2609}$ polymorphism may be compensated by the normal allele.

For $A\rightarrow G_{1364}$, we found statistically significant differences in genotype distribution when patients were compared with controls, indicating an association of the $A\rightarrow G_{1364}$ polymorphism with HSCR. The association found could be due to a direct contribution of $A\rightarrow G_{1364}$ to the HSCR phenotype or that $A\rightarrow G_{1364}$ is in LD with another susceptibility locus. Direct involvement of $A\rightarrow G_{1364}$ in HSCR disease would be through alteration of intronic 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 $A\rightarrow G_{1364}$ 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 $A\rightarrow G_{1364}$ polymorphism could directly affect PHOX2B gene expression. It is possible that either the PHOX2B $A\rightarrow G_{1364}$ 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.$^{3,4}$ The association found when patients with no mutations in RET coding regions were examined indicate that $A\rightarrow G_{1364}$ or a locus in LD could directly contribute to the HSCR phenotype. Nevertheless, $A\rightarrow G_{1364}$ 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

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**Table 3** Association testing based on the haplotype frequencies of $A\rightarrow G_{1364}, A\rightarrow C_{2607}$, and $DEL_{2609}$

<table>
<thead>
<tr>
<th>Group</th>
<th>Patients (n=91)</th>
<th>Controls (n=71)</th>
<th>All subjects (n=162)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n^*$</td>
<td>$\ln(l)$</td>
<td>$z^2$</td>
</tr>
<tr>
<td>Patients</td>
<td>91</td>
<td>-90.84</td>
<td>36.34</td>
</tr>
<tr>
<td>Controls</td>
<td>71</td>
<td>-97.86</td>
<td>21.99</td>
</tr>
<tr>
<td>All subjects</td>
<td>162</td>
<td>-192.24</td>
<td>56.49</td>
</tr>
</tbody>
</table>

*Number of individuals.
†The relevant test statistic is $T = \ln(l)$ (cases) $- \ln(l)$ (controls) $- \ln(l)$ (all subjects).

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**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>$A\rightarrow G_{1364}$, $A\rightarrow C_{1364}$</td>
<td>34.67</td>
<td>0.00</td>
<td>15.07</td>
</tr>
<tr>
<td>$A\rightarrow G_{1364}$, $DEL_{2609}$</td>
<td>0.12</td>
<td>0.90</td>
<td>1.86</td>
</tr>
<tr>
<td>$A\rightarrow C_{2607}$, $DEL_{2609}$</td>
<td>0.72</td>
<td>0.86</td>
<td>1.64</td>
</tr>
</tbody>
</table>
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→G1364 polymorphism may indicate that the transition A→G1364 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→G1364 versus DEL2609 and between A→C2607 versus DEL1_2609, which are only two base pairs apart. This indicates that the DEL1_2609 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 CCG 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→G1364 polymorphism. These other possible susceptibility loci would not be in LD with the polymorphism (DEL1_2609) found in the recombination hot spot that defines the breakpoint of a LD block. Therefore, the DEL1_2609 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.

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