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Association study of *PHOX2B* as a candidate gene for Hirschsprung's disease

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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 (DEL₂₆₀₉). Statistically significant differences were found for A→G₁₃₆₄. Genotypes comprising allele G were underrepresented in patients (19% v 36%; $\chi^2=9.30$; p=0.0095 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.^{2,3} HSCR may occur with other neurodevelopmental disorders such as Waardenburg syndrome type 4 (WS4),⁴ 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.^{5–11}

It has been postulated that the degree of expression of the receptor tyrosine kinase gene (*RET*) is critical for the HSCR phenotype.⁷ 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 (EDNBR) signalling pathways. Interaction between these two signalling pathways could modify *RET* expression¹¹ and therefore HSCR phenotype.^{7,12} Nevertheless, for almost every HSCR gene described, incomplete penetrance and variable expression of the HSCR phenotype has also been observed.^{13,14} 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.^{15,16}

The human *PHOX2B* gene has been cloned and sequenced.¹⁷ Its coding region consists of 945 base pairs (bp) allocated in three exons, yielding a homeodomain protein of 314 amino acid residues.¹⁸

The compelling evidence of the murine model has made the human *PHOX2B* gene an attractive target for exploration as a

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.

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 previously described.¹⁹ 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 μ l reaction volume containing 100 ng genomic DNA; a mixture of all four nucleotides (final concentration 0.2 mM for each nucleotide); 2.5 μ l of 10 \times reaction buffer; 0.5 μ M 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 μ l reaction volume containing 100 ng genomic DNA; a mixture of all four nucleotides (final concentration 0.2 mM for each nucleotide); 5 μ l of 5 \times GC-RICH PCR reaction buffer; 2.5 μ l of 5 M GC-RICH resolution solution; 0.5 μ M 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: GACCTCAGACAAGGCATCTCA and AATTACCCTCCCTGCAATC (586 bp); exon 2: CTGCCGTATGACCTGACCTT and ACAGCCACACCAAATCCAGT (442 bp); and exon 3: ACCCTAACCGGTGCTTTTCT and ACAATAGCCTTGGGCCTACC (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 μ l 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 χ^2 tests. χ^2 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 χ^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_1) among markers and without (H_0). The EH program also provides log likelihood, χ^2 , and the number of degrees of freedom under hypotheses H_0 and H_1 . 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 ($\ln L_{\text{case}}$, $\ln L_{\text{control}}$ and $\ln L_{\text{combined}}$) were used to calculate the relevant statistical test $T = [\ln(L_{\text{case}}) + \ln(L_{\text{control}}) - \ln(L_{\text{combined}})]$. Twice this value gives an approximate χ^2 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→G₁₃₆₄) in intron 2, 100 bp away from the intron/exon boundary. We investigated the possibility of A→G₁₃₆₄ 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→C₂₆₀₇) in codon 253 of the *PHOX2B* exon 3.

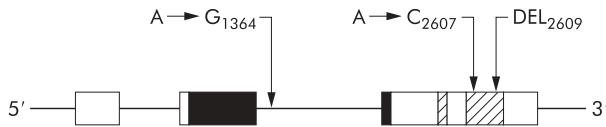


Figure 1 Polymorphisms identified in the *PHOX2B* gene. The three *PHOX2B* exons are represented by rectangles. Black boxes indicate the homeodomain. Hatched boxes indicate polyalanine stretches (from codon 162 to 169 and from codon 245 to 264). Downward arrows show the relative location of the polymorphisms.

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.²¹ 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 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. Alternatively, the Chinese population analysed in this study may have had these SNPs.

In addition to the three polymorphisms described in this study, there are three other *PHOX2B* SNPs recorded in GenBank. Polymorphism ss3136952 is a A→C₂₁₁₈ transition at position 2118 of the *PHOX2B* gene (intron 2); polymorphism ss1551093 is a T→A₄₄₅₇ transversion at position 4457 (end of 3' untranslated region); and polymorphisms ss1551094 is a C→A₄₅₁₇ 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.

Table 2 Estimated haplotype frequencies in patients and controls determined by estimating haplotype frequencies

Haplotypes			Frequencies		
Intron 2	Exon 3		Patients (n=91)	Controls (n=71)	All subjects (n=162)
A→G ₁₃₆₄	A→C ₂₆₀₇	DEL ₂₆₀₉			
A	A	ND	0.855	0.777	0.821
A	A	D	0.029	0.031	0.030
A	C	ND	0.000	0.015	0.007
A	C	D	0.000	0.000	0.000
G	A	ND	0.056	0.075	0.064
G	A	D	0.009	0.032	0.019
G	C	ND	0.049	0.069	0.058
G	C	D	0.000	0.000	0.000

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 $\chi^2=2.41$; $p=0.12$; A→C₂₆₀₇ $\chi^2=1.62$; $p=0.20$; DEL₂₆₀₉ $\chi^2=1.06$; $p=0.3$). 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 ($\chi^2=9.30$; $p=0.0095$). An association was also found when 75 patients with no mutations in *RET* were compared ($\chi^2=7.38$; $p=0.024$). 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

Polymorphism	Patients		Controls (n=71)
	HSCR +/-RET mutations (n=91)*	HSCR -RET mutations (n=75) †	
A→G ₁₃₆₄			
AA	0.81 (n=73)	0.77 (n=56)	0.64 (n=46)
AG	0.16 (n=15)	0.19 (n=14)	0.36 (n=25)
GG	0.03 (n=3)	0.03 (n=3)	0.00 (n=0)
	$\chi^2=9.30^*$; $p=0.0095^*$; $\chi^2=7.38$ †; $p=0.024$ †		
A→C ₂₆₀₇			
AA	0.90 (n=82)	0.88 (n=64)	0.83 (n=59)
AC	0.10 (n=9)	0.12 (n=9)	0.17 (n=12)
CC	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
	$\chi^2=1.74^*$; $p=0.23^*$; $\chi^2=0.71$ †; $p=0.39$ †		
DEL ₂₆₀₉			
NN‡	0.92 (n=79)	0.90 (n=66)	0.87 (n=62)
ND§	0.08 (n=7)	0.10 (n=7)	0.13 (n=9)
DD	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
	$\chi^2=1.11^*$; $p=0.29^*$; $\chi^2=0.41$ †; $p=0.52$ †		

*HSCR patients regardless of their *RET* mutation status.

†HSCR patients with no *RET* mutations.

‡N no deletion.

§D deletion.

Table 3 Association testing based on the haplotype frequencies of A→G₁₃₆₄, A→C₂₆₀₇, and DEL₂₆₀₉

Group	n*	ln(L)	χ ²	p†
Patients	91	-90.84	36.54	0.42
Controls	71	-97.86	21.99	
All subjects	162	-192.24	56.49	

*Number of individuals.

†The relevant test statistic is $T = \ln(L, \text{cases}) + \ln(L, \text{controls}) - \ln(L, \text{cases+controls together})$. Twice this value has an approximate χ^2 distribution with a number of degrees of freedom (df) equal to the number of haplotypes estimated. For the above data: $(-90.84) + (-97.86) - (-192.24) = 3.54$; $\chi^2 = 2 \times 3.54 = 7.08$ which on 7 df is associated with an empirical significance level of $p=0.42$.

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→G₁₃₆₄ + A→C₂₆₀₇ with the combination A→G₁₃₆₄ + DEL₂₆₀₉, 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 χ^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→C₂₆₀₇ + DEL₂₆₀₉ ($\chi^2=3.26$; $p=0.19$). When haplotypes comprising the three polymorphisms described in this study were analysed for differences in frequencies between patients and controls, no association was found ($\chi^2=11$; $p=0.08$, 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→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 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→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.^{13 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 4 Pairwise linkage disequilibrium

Pairwise comparison	Patients (n=91)		Controls (n=71)		All subjects (n=162)	
	χ ²	p	χ ²	p	χ ²	p
A→G ₁₃₆₄ v A→C ₂₆₀₇	34.67	0.00	15.07	0.0017	47.60	0.0001
A→G ₁₃₆₄ v DEL ₂₆₀₉	0.12	0.90	1.86	0.15	1.96	0.58
A→C ₂₆₀₇ v DEL ₂₆₀₉	0.72	0.86	1.64	0.65	2.20	0.53

PHOX2B polymorphism. Recently, Gabriel and colleagues²² 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.²³ 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.²⁴ 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 DEL₂₆₀₉ which are only two base pairs apart. This indicates that the DEL₂₆₀₉ 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→G₁₃₆₄ polymorphism. These other possible susceptibility loci would not be in LD with the polymorphism (DEL₂₆₀₉) found in the recombination hot spot that defines the breakpoint of a LD block.²⁵ Therefore, the DEL₂₆₀₉ 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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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://linkage.rockefeller.edu/ott/linkutil.htm>.

Genbank: <http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html> (*PHOX2B* gene sequence accession numbers: AF117979; AB015671).

dbSNP: <http://www.ncbi.nlm.nih.gov/SNP>

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