ELECTRONIC LETTER

Chinese patients with sporadic Hirschsprung's disease are predominantly represented by a single *RET* haplotype

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Hirschsprung's disease is a developmental disorder characterised by the absence of ganglion cells in the nerve plexuses of the lower digestive tract. The Hirschsprung phenotype is variable and can be classified into two groups: SSA, or short segment aganglionosis, which includes patients with aganglionosis as far as the rectosigmoid junction; and LSA, or long segment aganglionosis, which includes patients with aganglionosis beyond the rectosigmoid junction. The condition presents in the neonatal period as a failure to pass meconium, chronic severe constipation, colonic distension, secondary electrolyte disturbances, and sometimes enterocolitis and bowel perforation.

The estimated population incidence is 1/5000 live births, although this is a representative value. The highest incidence is in Asian populations (2.8 per 10 000 life births) and the lowest in Hispanics (1 per 10 000 life births).[1] The male to female (M:F) ratio is approximately 4:1 for SSA patients and approximately 1:1 for LSA patients.[1] Approximately 20% of cases are familial. The recurrence risks for siblings of SSA variant probands ranges between 1.5% and 3.3%, while the risks for those of LSA variant probands varies from 2.9% to 17.6%.[1] Hirschsprung's disease is often associated with chromosomal abnormalities, with other neurodevelopmental disorders such as Waardenburg syndrome type 4, and with a variety of additional isolated anomalies and syndromes.[1][2]

The disease has a complex genetic aetiology, and many studies indicate the receptor tyrosine kinase gene (*RET*) as the major susceptibility gene for Hirschsprung's disease.[1–14] *RET* mutations are also associated with multiple endocrine neoplasia type 2 (MEN2),[15] and medullary thyroid carcinoma.[16] Papillary thyroid carcinoma is associated with *RET* somatic rearrangements.[17]

Mutations in the *RET* gene account for up to 50% of the familial cases and anywhere between 7% and 35% of the sporadic cases, and they lack genotype–phenotype correlation.[5–14] Other Hirschsprung genes identified so far mainly code for protein members of interrelated signalling pathways involved in the development of enteric ganglia: RET, endothelin receptor B (EDNRB),[18–24] and the transcriptional regulator SOX10 signalling pathways.[25][26] Mutations in the *SIP1* gene have been found to cause syndromic Hirschsprung's disease.[27] These genes account for a small proportion of patients with this disorder (7%). Approximately 50% of affected individuals do not have mutations in the Hirschsprung genes.[1] However, genetic linkage analyses in both LSA and SSA variant families repeatedly implicate the *RET* gene as a major susceptibility locus,[28][29] even though the evidence of those linkage analyses is not reflected in the *RET* mutation status of many patients in whom *RET* coding region mutations were not found.

There are several possible explanations for the complex genetic aetiology of Hirschsprung's disease:

- mutations in as yet unidentified genes;

Key points

- Hirschsprung's disease has a complex genetic aetiology, with *RET* being the major gene. This study was initiated to evaluate the *RET* haplotypes associated with the disease in the Chinese population. It was hypothesised that if the Hirschsprung phenotype is also related to the particular combinations of *RET* single nucleotide polymorphisms (SNPs), and if the incidence of those SNPs differs among populations, identification of those population specific alleles and haplotypes contributing to disease would help elucidate the molecular basis underlying Hirschsprung's disease.

- The transmission disequilibrium test (TDT) and standard case–control statistics were used for comparison of *RET* haplotypes. Haplotypes were also compared between patients with different Hirschsprung's disease phenotypes and *RET* mutation status.

- One main haplotype—allele A of c135G>A(A45A), allele G of c1296G>A(A432A), and allele G of c2307T>G(L769L)—represented 66% of the patients with Hirschsprung's disease (A-G-G, $\chi^2 = 22.23$; $p = 0.000002$). Allele A of c1296G>A and haplotypes comprising it were associated with the more severe manifestations of the disease ($p = 0.000074$), regardless of the *RET* mutation status.

- The finding of A-G-G in 66% of Chinese patients with Hirschsprung's disease indicates that they share the same genetic aetiology.

- These findings are relevant to Hirschsprung's disease disease risk and to gene mapping, particularly for genome-wide linkage disequilibrium testing through the use of SNP markers.

- mutations in *RET* non-coding region that remain to be found;

- *RET* could be under epistatic regulation, and the interaction of several unlinked genes is required to produce the phenotype[23][24][28][29];

- *RET* specific SNPs, or a combination of these, could act as low susceptibility alleles or as factors modifying the phenotypic expression, or even be in linkage disequilibrium with an unknown susceptibility locus.[30–33]

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Abbreviations: HWE, Hardy-Weinberg equilibrium; LSA, long segment aganglionosis; NT, chromosomes not transmitted; SNP, single nucleotide polymorphism; SSA, short segment aganglionosis; TDT, transmission disequilibrium test; TR, chromosomes transmitted

Genetic differences in risk factors among human populations derive mainly from gradations in allele frequencies rather than from distinctive ''diagnostic'' genotypes. Thus the structure of human populations is relevant in various epidemiological contexts, and to our knowledge, *RET* polymorphism studies have only been conducted in white populations. *RET* SNP profiling, together with the investigation of *RET* alleles that could act as susceptibility factors in our population, will help to provide a better understanding of the mechanism of Hirschsprung's disease. In this paper, we present the first study on the *RET* polymorphisms found in Chinese patients with Hirschsprung's disease, together with their genotype and haplotype distributions.

METHODS
Patients and controls
Eighty seven ethnic Chinese patients diagnosed with sporadic Hirschsprung's disease between January 1984 and April 2003 were included in the study. The diagnosis was based on histological examination of either biopsy or surgical resection material for the absence of enteric ganglia. Using polymerase chain reaction (PCR) amplification and direct sequencing, we screened the 21 exons of the *RET* gene, including intron/exon boundaries, from 87 patients, 52 available parents, and 100 ethnically matched controls. All individuals assented to the molecular analyses. The primers and PCR conditions used were as previously described.[34] For exon 21, we generated the following pair of primers: 5'-AAAGGGAGTTTTGCCAAGGCC-3' (forward) and 5'-TTTAAGTCTGAAGAGCAGGC-3' (reverse), which yielded a 157 base pair (bp) product.

Statistical analysis
The allele frequencies in patients and controls were calculated from the genotype frequencies and compared by the χ^2 test or Fisher's exact test when an expected cell value was less than 5. Haplotype frequencies were estimated, using the EM algorithm as implemented in the EH program.[35] EH is a linkage utility program to test and estimate linkage disequilibrium between different markers or between a disease locus and markers. To test whether haplotype frequencies were significantly different between cases and controls, we did three separate analyses using the EH program: on the case subjects alone, on the control subjects alone, and on the combination of case and controls subjects. The three log-likelihoods obtained ($\ln L_{case}$, $\ln L_{control}$, and $\ln L_{combined}$) were used to calculate the relevant statistical test:

Table 1 Classification of the patients according to the length of the aganglionic segment (phenotype) and the *RET* mutation status

Phenotype	With mutation	Without mutation
LSA (n = 14)	6 (37.5%)	8 (11.3%)
SSA (n = 73)	10 (62.5%)	63 (88.7%)
	(n = 16)	(n = 71)

LSA, long segment aganglionosis; SSA, short segment aganglionosis.

$T = [\ln(L_{case}) + \ln(L_{control}) - \ln(L_{combined})]$. 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 linkage disequilibrium between polymorphisms. GENEHUNTER (version 2.1) was used to undertake a transmission disequilibrium test (TDT) for marker haplotypes. Probability (p) values were considered suggestive at a level of <0.05, and significant at a level of <0.005, to allow for the testing of 13 partially correlated markers.

RESULTS
RET mutations and polymorphisms
Sequencing analyses showed that 16 patients (18.4%) had potentially disease causing mutations in the *RET* gene (Garcia-Barceló M-M *et al*, unpublished data). Classification of the patients according to the length of the aganglionic segment (phenotype) and the *RET* mutation status is shown in table 1.

Thirteen single nucleotide polymorphisms (SNPs) were found, of which five were new. The frequencies of these five new polymorphisms were <5% in both patients and controls, and are likely to be private to our population, thus indicating ethnic differences in the *RET*-SNP profile. Except for c135G>A(A45A), the rest of the loci were in Hardy-Weinberg equilibrium (HWE) when checked for the total population; c135G>A(A45A) deviated slightly from HWE in the total population but HWE was conserved in the control population. The frequencies of the SNPs and their comparison with those reported for white subjects are shown in table 2.[36] [37] The alleles associated (p<0.05) with

Table 2 Allele frequencies of *RET* single nucleotide polymorphisms in 87 sporadic Chinese cases of Hirschsprung's disease and 100 ethnically matched controls

Exon	Nucleotide change	Codon	Frequency of the variant allele (%) Controls (100)	Patients (87)	Statistic χ^2	p Value	Spanish* Controls v patients (χ^2; p value)	German† Controls v patients (χ^2; p value)
–	IVS1-17 T>G (new)	–	0	1.1	1.15	0.28	ND	ND
2	c129 C>G (new)	D43E	1	0	0.87	0.34	ND	ND
2	c135 G>A	A45A	41	73	38.62	<0.0001	16 v 59 (62.5; <0.0001)	23 v 73.4 (93.1; <0.001)
2	c200 G>A (new)	R67H	2.5	0.5	2.18	0.14	ND	ND
–	IVS2+9 G>A	–	10.5	5.1	3.60	0.06	ND	ND
3	c375 C>A	V125V	0.5	0.5	0.01	0.92	3 v 7 (F; 0.15)	1.9 v 2.4 (0.12; 0.7)
7	c1296 G>A	A432A	21	12	5.30	0.02	30 v 21.8 (F; 0.14)	27.6 v 25.8 (0.14; 0.7)
7	c1465 G>A (new)	D489N	2	3.4	0.75	0.39	ND	ND
11	c2071 G>A	G691S	10.5	4.5	4.53	0.03	23.5 v 12.5 (5.4; 0.02)	20.2 v 10.5 (5.8; 0.016)
11	c2037 C>T (new)	P679P	0.5	0.5	0.01	0.92	ND	ND
13	c2307 T>G	L769L	49	75.2	28.05	<0.0001	14 v 30.5 (12; <0.0001)	23.7 v 42.7 (15.5; <0.001)
14	c2508 C>T	S836S	0	0	–	–	4 vs. 2.3 (F; 0.54)	3.6 v 0 (4.8; 0.032)
15	c2712 C>G	S904S	10.5	4.5	4.53	0.03	23.5 v 12.5 (5.4; 0.02)	20 v 11.3 (4.5; 0.03)

*Borrego *et al*, 1999; †Fitze *et al*, 1999.
F, Fisher's exact test; ND, not described;

Table 3 Transmission disequilibrium test (TDT) by individual polymorphic locus

Codon	SNP (allele)	TDT			
		TR	NT	χ^2	p Value
A45A	c135G>A (A)	49	12	22.44	0.000002
A432A	c1296G>A (A)	9	24	6.82	0.009
L769L	c2307T>G (G)	53	15	21.24	0.000004

NT, chromosomes not transmitted; SNP, single nucleotide polymorphism; TR, chromosomes transmitted

Hirschsprung's disease in our population were allele A of c135G>A(A45A), allele A of c1296G>A(A432A), allele G of c2307T>G(L769L), allele G of c2712C>G(S904S), and allele A of c2071G>A(G691S). Association of allele A of c135G>A(A45A), allele A of c1296G>A(A432A), and allele G of c2307T>G(L769L) was independently tested by TDT (table 3). The allele A of c1296G>A(A432A) was significantly underrepresented in Chinese patients.

RET genotype distribution

Forty two different genotypes encompassing the 13 RET SNPs found were observed among cases and controls (not shown). The overall genotype distribution differed significantly between cases and controls ($\chi^2 = 79.08$; 41 df; p = 0.000327). One to one genotype comparisons showed that four main genotypes (of 42) showed a significant difference between patients and controls (genotypes 1 to 4 in table 4). The most common disease associated RET genotype (50.6%; genotype 1) was that homozygous for both allele A of c135G>A(A45A) and allele G of c2307T>G(L769L), and wild-type for the rest of polymorphisms found. Genotypes heterozygous for these two polymorphisms (genotypes 2 and 8) were instead overrepresented in the control population.

Identification of the main RET haplotype in Chinese patients with Hirschsprung's disease

We constructed the haplotypes comprising the three most significantly disease associated RET SNPs in our population (c135G>A(A45A), c1296G>A(A432A), and c2307T>G(L769L)) and on the basis of published reports.[36][37] Significant differences were observed in the global haplotype distribution between patients and controls ($\chi^2 = 42.46$; p<0.00001 for haplotypes; $\chi^2 = 57.52$; p<0.00001 for genotypes). One to one

comparison revealed significant differences between patients and controls for haplotypes A-G-G, G-G-T, and G-A-T. A TDT test done in 52 parent–proband trios confirmed the results (table 5). A-G-G was the most common case haplotype in our population, with an estimated frequency of 66%.

RET-SNPs and RET haplotypes in different Hirschsprung phenotypes

To investigate whether RET-SNPs are associated with a particular Hirschsprung phenotype, we compared the allelic distribution of the polymorphisms described above between patients with long/total segment aganglionosis (LSA) and patients with short segment aganglionosis (SSA), and also between patients harbouring mutations in RET or not. Only allele A of c1296G>A(A432A) was significantly underrepresented in SSA patients (table 6). TDT, done in 45 parent–proband trios with SSA, showed that allele G was the most frequently transmitted allele (TR:NT 22:6; $\chi^2 = 9.14$; p = 0.002). None of the SNPs revealed statistically significant differences in frequencies between patients with RET mutation and those without (table 6).

Three-loci haplotype

To test the previously observed association of allele A of c1296G>A(A432A) with LSA, we investigated whether the haplotype distribution differed between LSA and SSA patients. Allele A had already been found significantly underrepresented when all patients with Hirschsprung's disease were compared with controls, and from these observations a protective role for this allele seemed plausible. However, the frequency of allele ''A'' in patients affected with LSA, although not statistically significant, was higher than for the same allele in controls, which is at odds with a

Table 4 Frequency of RET genotypes in patients with Hirschsprung's disease and controls

Genotype‡§	A45A c135 G>A	V125V c375 C>A	A432A c1296 G>A	G691S c2071 G>A	L769L c2307 T>G	Chinese patients (n = 87) (n)	(%)	Chinese controls (n = 100) (%)	χ^2	p Value	Spanish patients (n = 62)*† (%)	Spanish controls (n = 65) (%)
1	**AA**	**CC**	**GG**	**CC**	**GG**	**44¶**	**50.6**	**12**	**29.81**	**<0.0001**	**4.8 (CC)**	**1.5**
2	**GA**	**CC**	**GG**	**CC**	**TG**	**5**	**5.7**	**26††**	**11.83**	**<0.0001**	**3.2 (AC)**	**0.0**
3	**GG**	**CC**	**GA**	**CC**	**TT**	**0**	**0.0**	**5**	**4.47**	**0.03**	**4.8 (AD)**	**18.5**
4	**GG**	**CC**	**GG**	**CC**	**TG**	**1**	**1.1**	**8**	**4.77**	**0.02**	**0.0 (AL)**	**1.5**
5	AA	CC	GG	CC	TT	0	0	0	–	–	11.3 (BB)	0.0
6	AA	CC	GG	CC	TG	3	3.5	1	1.33	0.24	9.7 (BC)	3.1
7	GA	CC	GG	CC	GG	4	4.6	2	1.01	0.31	0.0 (CL)	0.0
8	GA	CC	GA	CC	TG	3**	3.5	9	2.39	0.12	11.3 (AF/CD)	0.0
9	GA	CA	GG	CC	TT	0	0	0	–	–	9.7 (BH)	0.0
10	GA	CC	GA	CC	TT	0	0	2	1.76	0.18	6.5 (BD)	0.0
11	GG	CC	GG	CC	TT	5	5.7	4	0.31	0.57	0.0 (AA)	12.3
Rest of genotypes combined						22	25.3	31	5.9	0.01	38.7	63.1

*Borrego et al, 2000.
†Capital letters in brackets represent genotypes as designated in Borrego et al, 2000.
‡Only those genotypes with significant differences in frequencies between both populations are represented.
§In bold, genotypes with statistically significant differences between Chinese patients and controls.
¶One individual was TG for IVS1-17 T>G and another individual was GA for c1465 G>A D489N.
**Two individuals were GA for IVS2+9 G>A.
††One individual was GA for IVS2+9 G>A and another individual was GA for c1465 G>A D489N.

Table 5 Comparison of haplotype frequencies between patients with Hirschsprung's disease and controls, and results of haplotype analysis using the transmission disequilibrium test

A45A c135G>A	A432A c1296G>A	L769L C2307T>G	Frequencies estimated by EH			TDT			
			Controls (n = 100)	All patients (n = 87)	p Value	TR	NT	χ^2	p Value
A	G	G	0.372	0.660	0.009	43	9	22.23	0.000002
G	A	T	0.164	0.052	0.01	3	16	8.89	0.0028
G	G	T	0.322	0.147	0.03	7	27	11.76	0.0006
A	A	G	0.009	0.021	>0.05	3	2	0.20	0.65
A	A	T	0.004	0.011	>0.05	0	1	1.00	0.32
A	G	T	0.024	0.037	>0.05	1	2	0.33	0.56
G	A	G	0.032	0.036	>0.05	4	4	0.00	1.00
G	G	G	0.071	0.035	>0.05	4	4	0.00	1.00

EH, linkage utility program; NT, chromosomes not transmitted; TDT, transmission disequilibrium test; TR, chromosomes transmitted.

Table 6 Allele frequencies (%) of the most frequent *RET* polymorphisms in SSA and LSA patients and in patients with and without mutation in *RET*

Codon	SNP (allele)	Sporadic cases of Hirschsprung's disease	
		LSA (n = 14) v SSA (n = 73)	Without mutation (n = 71) v with mutation (n = 16)
A45A	c135G>A (A)	67.8 v 73.9 ($\chi^2 = 0.45$, p = 0.50)	70.4 v 84.3 ($\chi^2 = 2.6$, p = 0.10)
A432A	c1296G>A (A)	25 v 9.5 ($\chi^2 = 5.26$, p = 0.02)	12.6 v 15.6 ($\chi^2 = 0.47$, p = 0.50)
L769L	c2307T>G (G)	78.5 v 74.6 ($\chi^2 = 0.19$, p = 0.66)	87.5 v 64.8 ($\chi^2 = 2.9$, p = 0.08)

LSA, long segment aganglionosis; SNP, single nucleotide polymorphism; SSA, short segment aganglionosis.

protective role. This suggested that the association of allele A with LSA was in combination with other polymorphisms within *RET*.

Global haplotype and genotype distributions showed statistically significant differences between SSA and LSA patients ($\chi^2 = 20.84$; p = 0.004 for haplotypes, and $\chi^2 = 35.9$; p = 0.0006 for genotypes). Haplotype A-A-G was more common in LSA patients (14% v 0% in SSA; Fisher's two tailed exact test, p = 0.000075) and haplotype A-G-G in SSA (69.7% v 46.4% in LSA; $\chi^2 = 16.3$; p = 0.000041). TDT confirmed the preferable transmission of the haplotype A-G-G in the SSA group (table 7).

Although parents were only available for seven LSA patients, TDT results showed a statistical trend towards the transmission of A-A-G in this group. It is likely that the TDT values did not reach statistical significance in LSA patients owing to the small sample size analysed. However, as our patients and controls are ethnically matched, there is little distortion of the data obtained by EH, and the significance reached by case–control standard statistics accurately reflects the trends observed.

The results obtained in this three-loci haplotype analysis suggest that while both allele A of c135G>A(A45A) and allele G of c2307T>G(L769L) are associated with Hirschsprung's disease independently of the severity of the

phenotype, allele A of c1296G>A(A432A) in combination with the variant alleles of c135G>A(A45A) and c2307T>G(L769L) is associated with the more severe form of Hirschsprung's disease.

Two-loci haplotype

To determine whether the association found for c1296G>A(A432A) was driven by the other polymorphic alleles associated with Hirschsprung's disease, three two-loci haplotype distributions—c135G>A (A45A)-c2307T>G(L769L), c135G>A(A45A)-c1296G>A(A432A), and c1296G>A(A432A)-c2307T>G(L769L)—were compared between LSA and SSA patients. As expected, for the combination c135G>A(A45A)-c2307T>G(L769L), no difference between SSA and LSA patients was found. However, significant differences in the global genotype and haplotype distributions between LSA and SSA patients were observed for the combination (c135G>A)A45A-c1296G>A(A432A) ($\chi^2 = 19$; p = 0.0012 for genotypes and $\chi^2 = 12.64$; p = 0.0054 for haplotypes), and the combination c1296G>A(A432A)-c2307T>G(L769L) ($\chi^2 = 18.9$; p = 0.001 for genotypes, and $\chi^2 = 13.08$; p = 0.0044 for haplotypes). In particular, for c135G>A(A45A)-c1296G>A(A432A), haplotype A-G was predominant in SSA patients (73% v 49% in LSA; $\chi^2 = 12.11$; p = 0.0005) and this was confirmed by TDT

Table 7 Results of haplotype analysis on LSA and SSA triads using the transmission disequilibrium test

A45A c135G>A	A432A c1296G>A	L769L C2307T>G	TDT (7 LSA trios)				TDT (45 SSA trios)			
			TR	NT	χ^2	p Value	TR	NT	χ^2	p Value
A	G	G	5	1	2.67	0.10	38	8	19.57	0.00001
A	A	G	3	0	3.00	0.08	0	2	2.00	0.15
A	A	T	0	0	–	–	0	1	1.00	0.31
A	G	T	0	0	–	–	1	2	0.33	0.56
G	A	G	1	1	0.00	1.00	3	3	0.00	1.00
G	G	G	2	0	2.00	0.15	2	4	0.67	0.41
G	A	T	0	2	2.00	0.16	3	14	7.12	0.007
G	G	T	0	7	7.00	0.008	7	20	6.26	0.41

LSA, long segment aganglionosis; NT, chromosomes not transmitted; SSA, short segment aganglionosis; TR, chromosomes transmitted.

done in 45 SSA trios (TR:NT 38:8; $\chi^2 = 19.57$; p = 0.00001). Haplotype A-A was significantly more common in LSA patients (18% v 0.8% in SSA; $\chi^2 = 17.37$; p = 0.000031).

As for pair c1296G>A(A432A)-c2307T>G(L769L), the most dramatic difference between SSA and LSA was that of the haplotype A-G (1.5% in SSA v 24% in LSA; $\chi^2 = 22.8$; p = 0.000002). Haplotype G-G was significantly overrepresented in the SSA group (73% v 53%, $\chi^2 = 8.6$; p = 0.003). TDT done in the SSA group also showed that G-G was preferably transmitted (TR:NT 38:9; $\chi^2 = 17.89$; p = 0.00002).

Two-loci haplotype analysis confirmed that allele A of c1296G>A(A432A) in *cis* combination with either allele A of c135G>A(A45A) or allele G of c2307T>G(L769L) is associated with LSA.

Two- and three-loci haplotype distributions showed no differences between patients with *RET* mutation and patients without (data not shown).

RET-SNPs and *RET* haplotypes in other populations

Except for allele A of c1296G>A(A432A), those polymorphic alleles associated with Hirschsprung's disease in Chinese patients (in particular allele A of c135G>A(A45A) and allele G of c2307T>G(L769L)) are also associated with the disease in white patients. However, the *RET*-SNP frequencies found in our population (in both patients and controls) differ considerably from those reported for the same SNPs in white subjects (table 2). For instance, the frequency of allele A of c135G>A(A45A) varied greatly between Chinese and white control populations (41% v 16–23%, respectively) but remained rather similar for the two patient populations (73% in Chinese v 59–73.4% in whites). Allele G of c2307T>G(L769L) occurred much more often in Chinese patients (75.2%) than in white patients (30.5%–42.7%). In addition, while the frequencies of the variant alleles of c135G>A(A45A) and c2307T>G(L769L) were similar in the Chinese population (patients and controls), they differed greatly in white subjects (table 2). As to the c2508C>T(S836S) polymorphism, the variant allele T was not found either in patients or controls. In whites, c2508C>T(S836S) is characterised by the low frequency of the allele T in patients with Hirschsprung's disease and its overrepresentation in medullary thyroid carcinoma.[33 38–40]

The ethnic differences observed in the SNP frequencies were reflected in the frequencies of the *RET* genotypes/haplotypes associated with Hirschsprung's disease. Genotypes and haplotypes encompassing the same SNPs were compared with those previously reported in the only *RET* haplotype study done in white (Spanish) patients with Hirschsprung's disease (table 4).[30] While 50.6% of the Chinese patients shared the same genotype—genotype 1; homozygous for both allele A of c135G>A(A45A) and allele G of c2307T>G(L769L) and wild-type for the rest of polymorphisms found—five different genotypes (BB, BC, BD, BH and CD) together accounted for a similar number (48%) of Spanish patients. In addition, those four genotypes were rare in our patient population—that is, the most common Hirschsprung's disease genotype in Spanish people (BB) is not observed in the Chinese. Furthermore, the most common Hirschsprung haplotype seen in Chinese people (A-G-G; 66%; c135G>A(A45A), c1296G>A(A432A), c2307T>G(L769L); table 5) was only seen in 19% of the Spanish patients; vice versa, the most common Hirschsprung haplotype in Spaniards (30.7%; haplotype B: allele A of of c135G>A(A45A) and wild-type for the other sites) was only seen in 3.7% of the Chinese patients. Genotypes and haplotypes comprising both allele A of c135G>A(A45A) and allele G of c2307T>G(L769) are particularly associated with Hirschsprung's disease in Chinese people, while in whites the main genotype and haplotype associated with

Hirschsprung's disease comprise only allele A of c135G>A(A45A).

DISCUSSION

The current data on Hirschsprung's disease indicate that *RET* haplotypes associated with this condition can themselves be functional and lead to a low penetrance loss of function, act as modifiers of the phenotype conferring risk/protection to disease (additive/combinatorial effect of multiple *RET* polymorphisms, combinatory hypothesis), or represent linkage disequilibrium with a low penetrance susceptibility locus. We initiated this study to evaluate the *RET* "risk" haplotypes present in our population, to elucidate the molecular basis of Hirschsprung's disease. We hypothesised that if the Hirschsprung's disease phenotype is related to the particular combinations of *RET*-SNPs, and if the incidence of those polymorphic variants differs among populations, identification of those population specific SNPs and haplotypes contributing to disease would definitely help in the discovery of the molecular basis underlying Hirschsprung's disease.

Our data showed that Chinese patients with Hirschsprung's disease are mainly represented by one *RET* haplotype (comprising variant alleles of c135G>A(A45A) and c2307T>G(L769L)), indicating that if this haplotype was itself functional, 66% of the Chinese patients would share the same genetic aetiology. The existing data on white patients with Hirschsprung's disease show that the haplotype distribution is more diverse and that c135G>A(A45A) alone is driving a strong association between haplotypes containing this polymorphism (allele A) and Hirschsprung's disease. Those differences between populations in the frequencies of the *RET* haplotypes with the best evidence for an association with Hirschsprung's disease suggest that the true *RET* disease susceptibility allele remains to be identified; alternatively they might suggest that different haplotypes in different populations confer risk of or protection against disease, and those "functional haplotypes" would also account for the patients with Hirschsprung's disease with no mutations in any of the Hirschsprung genes.

In favour of the first explanation, it has been suggested recently that in Spaniards,[31] *RET* "risk" haplotypes appeared to be in linkage disequilibrium with a putative founding low penetrance susceptibility locus, located upstream of c135G>A(A45A). Thus it could be that the "true" susceptibility locus" is on different haplotype backgrounds because of human population and history and structure, explaining the differences found in the *RET* haplotypes associated with Hirschsprung's disease. The main *RET* haplotype associated with Hirschsprung's disease in Chinese people still belongs to the group of haplotypes in linkage disequilibrium with the low penetrance susceptibility locus upstream of A45A, as previously described in whites.[31] The differences in "risk haplotype" frequencies between Chinese and whites could indicate that recombination events within the region flanked by A45A and L769L may have occurred (after the European–Asian split). This may have originated in two "risk haplotypes," one mainly represented by allele A of A45A, more common in Europeans, and another, more common in the Chinese, represented by the variants alleles of A45A and L769L. Both haplotypes could be in linkage disequilibrium with the low susceptibility locus upstream A45A previously described.[31]

The second explanation is not mutually exclusive with the presence of a putative locus in linkage disequilibrium with *RET* "risk" haplotypes, because particular combinations of SNPs on those haplotypes could also act as modifiers.[30 40]

We also found that allele and haplotype distributions varied with the severity of the Hirschsprung phenotype: allele A of c1296G>A(A432) in combination with the variant

alleles of c135G>A(A45A) and c2307T>G(L769L) was associated with the more severe form of Hirschsprung's disease, while both c135G>A(A45A) and c2307T>G(L769L) polymorphisms were associated with Hirschsprung's disease independently of the severity of the phenotype. However, these data should be viewed with caution until further proof is provided by functional analysis.

Differences in allele frequencies between different Hirschsprung phenotypes have never been reported for c1296G>A(A432A). Instead, in a study of 76 white patients from Germany,[32] differences in the frequency of allele A of c135G>A(A45A) were found between SSA and LSA patients, and also between patients with the RET mutation and those without. It was also shown that c135G>A(A45A) polymorphism can modify the phenotype through a within-gene interaction. In our series c135G>A(A45A) was not associated with any Hirschsprung phenotype or with RET mutations. Instead, c1296G>A(A432A) and haplotypes comprising it were associated with the more severe form of the disease. No differences were found in allele and haplotype distributions when patients harbouring or not harbouring the RET mutation were compared, which could indicate that RET specific haplotypes may influence the Hirschsprung's disease phenotype regardless of the RET mutation status. This influence could be through interaction of the RET-SNPs within RET itself or with other genes.

RET haplotypes containing the allele T of c2508C>T(S836S) have a protective role in the pathogenesis of Hirschsprung's disease.[33 40] This allele has not been found in the Chinese, so there is ethnic variation in the polymorphisms implicated in the phenotype modulation of Hirschsprung's disease and possibly medullary thyroid carcinoma. Interestingly, differences in the distribution of the RET haplotypes have also been detected among patients with papillary thyroid carcinoma from different white populations.[41]

Transmission of mutations in other Hirschsprung genes (EDRNB, GDNF) has been found to be associated with the transmission of RET polymorphisms, proving the genetic interaction underlying Hirschsprung's disease.[24] Combinations of variants in several components of the RET and EDRNB signalling pathways might overall provide an important risk factor for disease susceptibility and also contribute to the necessary response to produce the Hirschsprung phenotype. When a candidate gene contains various polymorphisms, these are usually in linkage disequilibrium because the relatively small genetic distances make it difficult in a single population to dissect out the important polymorphism. Ethnic differences in the haplotypes associated with disease can make it easier to isolate the SNP that actually confers susceptibility to a given disease at a given locus. Thus our data are highly relevant to Hirschsprung's disease risk and to gene mapping, particularly for genome-wide testing for linkage disequilibrium through the use of SNP markers.

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