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Novel *RET* mutations in Hirschsprung's disease patients from the diverse South African population

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Hirschsprung's disease (HSCR) is a common cause of intestinal obstruction in neonates with an incidence of one in 5000 live births. The disease occurs due to the absence of parasympathetic neuronal ganglia in the hindgut, resulting in irregular or sustained contraction of the affected segment. DNA samples of 40 unrelated subjects with HSCR were subjected to mutation screening of the *RET* (REarranged during Transfection) proto-oncogene, the major susceptibility gene for HSCR. Five novel (V202M, E480K, IVS10-2A/G, D771N, IVS19-9C/T) and one previously described mutation (P973L) were identified. Only two of the mutation-positive patients (from different ethnic groups) displayed total colonic aganglionosis, and both were heterozygous for mutation D771N. The potential disease-causing mutations occurred in 20% of individuals, with more males (22.5% representing seven of 31 males) affected than females (12.5% representing one of eight females). This study represents the first comprehensive genetic analysis of this disease in the diverse South African population. *European Journal of Human Genetics* (2001) **9**, 419–423.

Introduction

Hirschsprung's disease (HSCR), or aganglionic megacolon, occurs due to the absence of parasympathetic neuronal ganglia in the hindgut and results in a malfunction of the affected segment. The condition therefore presents as a common cause of intestinal obstruction in neonates, with an incidence of one in 5000 (0.02%) live births and an overall risk to siblings of 4%.[1–3] Aganglionosis can involve the rectosigmoid area (75%), transverse colon (17%) or total colon (8%) and may extend to the small bowel. Males are more likely to be affected than females (3.5–7.8-fold higher risk) and as the aganglionic segment becomes more extensive, the risk to siblings increases and the sex ratio decreases.[4–6] A higher risk of disease occurrence has been reported in siblings of patients demonstrating an earlier age of onset.

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HSCR was believed to be a sex-modified multifactorial trait because males are more likely to be affected than females.[1] Thereafter, inheritance was suggested to be multifactorial with the involvement of multiple genes and environmental factors in the development of the clinical phenotype. However, the occurrence of the disease in individuals displaying chromosomal abnormalities and sometimes multiple chromosome aberrations, suggested genetic heterogeneity.[4] Chromosomal abnormalities such as Down's syndrome are frequently (2–15%) associated with HSCR.[7,8] The relatively constant association of Down's syndrome with HSCR disease suggests the involvement of a modifier gene on chromosome 21 in the pathogenesis of the disease.[1,9]

The first gene implicated in HSCR was the RET (REarranged during Transfection) proto-oncogene. This was demonstrated by the detection of an interstitial deletion of 10q occurring in a patient with long segment HSCR, as well as extensive segregation analysis of this area in large HSCR families demonstrating dominant inheritance.[2,10,11] *RET* involvement was substantiated by the identification of mutations associated with both familial and sporadic cases in 25% of all HSCR cases.[10,12–16] *RET* mutations in sporadic

HSCR occurs in approximately 40% of patients with long segment disease and in 8% of patients with short segment disease.[6] In both familial and sporadic cases of HSCR, *RET* mutations are not restricted to a particular area of the gene. In this study we performed mutation analysis of the *RET* gene in 40 unrelated HSCR patients from the diverse South African population.

Materials and methods
Subjects
In a retrospective study, colonic tissue samples from frozen tissue were obtained from 40 apparently unrelated HSCR patients and 30 control subjects (17 paraffin embedded and 13 frozen colonic tissue samples), including six patients with anorectal malformations. DNA samples, extracted from whole blood, of an additional 56 subjects were included as controls. Inclusion of HSCR patients were based on histopathological evaluation and confirmation of absence of ganglia in the biopsies. HSCR occurred sporadically in all of the patients, except in one individual with a family history of the disease. Total colonic aganglionosis was observed in eight of these patients and the remaining patients all had aganglionosis restricted to the rectosigmoid area.

The study population were from different ethnic groups of South Africa, including 51 subjects of the Coloured population (22 HSCR patients – 55%; 29 controls – 34%), 39 Blacks (nine HSCR patients – 22.5%; 30 controls – 35%;) and 33 Whites (eight HSCR patients – 20%, 25 controls – 29%). The ethnic origin of one HSCR patient (2.5%) and two controls (2%) were unknown. In this study the 'White' population was of European descent, mainly of British origin, 'Coloured' refers to an individual of mixed ancestry with descendants from San, Khoi, African Negro, Madagascar, Javanese and European origin;[17] 'Black' refers to native Black populations of South Africa.

DNA analysis
DNA extraction was performed on the colonic tissue samples and whole blood using standard techniques. Polymerase chain reaction (PCR) amplification of the 21 exons of *RET* gene was performed using intronic primers.[18] The PCR products were subjected to heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis,[19] resolved in a polyacrylamide (PAA) gel supplemented with 7.5% urea (consisting of 4.5 g urea, 18 ml 5 × TBE, 24 ml dH$_2$O, 18 ml PAA (1%C of a 40% stock), 600 μl APS (10%) and 60 μl TEMED) at 4°C (350 V) for 18 h. The DNA fragments were also electrophoresed in a 10% PAA gel supplemented with 5% glycerol (consisting of 3 ml glycerol, 6 ml 5 × TBE, 36 ml dH$_2$O, 15 ml PAA (1%C of a 40% stock), 800 μl APS (10%) and 80 μl TEMED) at room temperature (300 V). The DNA fragments were stained in ethidium bromide and visualised by ultraviolet light transillumination. Semi-automated DNA sequencing (ABI 310 PRISM) was performed on

PCR products demonstrating mobility or conformational variants on the PAA gels.

Statistical analysis
Allele frequencies were determined and testing for significant association of alleles among patient and control groups were based on the chi-square (χ^2) and Fisher's exact tests. A *P* value smaller than 0.05 was regarded as statistically significant.

Results
Mutation analysis of the *RET* proto-oncogene revealed six potential disease-related mutations in eight HSCR patients (Table 1). Nine polymorphims, of which five have been described previously[14,18,20] were also detected (Table 2). HEX-SSCP analysis showed no aberrant banding pattern in exons 1, 4, 5, 8, 9, 10, 12 and 16 of the *RET* gene. Missense mutations and splice variants were identified in exons 3, 7, 13 and 17, and introns 10 and 19, respectively. The *RET* mutational spectrum defined in the South African population is illustrated in Figure 1.

All the patients were heterozygous for the respective mutations identified. The missense mutations (4/6, 66.6%) are not located to a specific hot-spot area of the *RET* proto-oncogene. None of the potential disease-associated mutations were detected in any of the 86 control individuals tested. Both mutations V202M and E480K, detected in the extracellular domain of the *RET* proto-oncogene, were observed in men with aganglionosis restricted to the rectosigmoidal area. E480K was detected only in the Coloured population (2/22, 9%), however owing to the small numbers of the other two HSCR populations, the occurrence of this mutation in these populations cannot be excluded.

A mutation altering the splice site in intron 10 (IVS10-2A/G) was identified in a male with rectosigmoid aganglionosis. Mutation D771N was identified in two patients, from different ethnic backgrounds (patient 11, a Coloured male and patient 28, a White male), both with total colonic aganglionosis. A rare variant was identified in intron 16 (IVS16-38delG) in the same individual with missense mutation P973L. Only one patient with rectosigmoid aganglionosis presented with the IVS19-9C/T variant in intron 19.

Codon numbers were used for designation of the polymorphisms, because discrepancies were observed between the intronic sequences published by Ceccherini *et al*[18] and Munnes *et al*[21] (Blast #AJ243297). Statistically significant differences in allele frequencies of the polymorphism identified in exon 15 of the *RET* gene was observed between the patient and control groups from the Black (*P* < 0.05) and White (*P* < 0.01) populations, respectively (Table 2). The genotypes of the White and Coloured patients with mutation D771N were compatible with an identical chromosomal background for this mutation, whilst mutation E480K was associated with two different haplotypes.

Table 1 Potential disease-causing mutations identified in the *RET proto-oncogene*

Patient no.	Exon/Intron	Mutation	Nucleotide change	Effect on coding sequence	Gender/ethnic group	Extent of aganglionosis
7	3	V202M	GTG→ATG	Missense	M/C	R/S
18	7	E480K	GAA→AAA	Missense	M/C	R/S
29	7	E480K	GAA→AAA	Missense	M/C	R/S
21	10	IVS10-2A/G		Splice acceptor	M/C	R/S
11	13	D771N	GAC→AAC	Missense	M/C	TCA
28	13	D771N	GAC→AAC	Missense	M/W	TCA
36	17	P973L[a]	CCA→ATA	Missense	F/C	R/S
4	19	IVS19-9C/T		Splice acceptor	M/W	R/S

[a]Previously reported by Yin *et al*.[26] M, male; F, female; W, White; C, Coloured; TCA, total colonic anganglionosis; R/S, rectosigmoidal aganglionosis; IVS, intervening sequence.

Table 2 Polymorphisms identified in the *RET proto-oncogene*

Exon/Intron	Codon	Nucleotide change		B (n=18)	BC (n=60)	Allele frequency W (n=16)	WC (n=50)	C (n=44)	CC (n=58)
2	A45	GCG→GCA	G:	0.95	0.94	0.38	0.5	0.46	0.69
			A:	0.05	0.06	0.62	0.5	0.54	0.31
6	IVS6+56delG		G:	1.0	1.0	0.94	1.0	0.89	0.90
			delG:	0.0	0.0	0.06	0.0	0.11	0.10
7	A432	GCG→GCA	G:	1.0	1.0	1.0	0.94	0.85	0.88
			A:	0.0	0.0	0.0	0.06	0.15	0.12
13	L769	CTT→CTG	T:	0.78	1.0	0.82	0.82	0.62	0.80
			G:	0.22	0.0	0.18	0.18	0.38	0.20
13	IVS13-29C/T		C:	0.95	0.99	1.0	1.0	0.98	1.0
			T:	0.05	0.01	0.0	0.0	0.02	0.0
15	S904	TCC→TCG	C:	0.50	0.75	0.82	0.94	0.57	0.59
			G:	0.50	0.25[a]	0.18	0.06[b]	0.43	0.41
16	IVS16-38delG		G:	1.0	1.0	1.0	1.0	0.98	1.0
			delG:	0.0	0.0	0.0	0.0	0.02	0.0
18	R982	CGC→CGT	C:	1.0	0.92	0.88	0.9	0.91	0.97
			T:	0.0	0.08	0.12	0.1	0.09	0.03
21	X1159	TAA→TGA	A:	1.0	1.0	1.0	1.0	1.0	0.97
			G:	0.0	0.0	0.0	0.0	0.0	0.03

[a]Black patients *vs* Black control group: $\chi^2=4.06$; $P>0.05$. [b]White patients *vs* White control group: $\chi^2=7.34$; $P>0.01$. B, Black patients; BC, Black control group; W, White patients; WC, White control group; C, Coloured patients; CC, Coloured control group.

Discussion

Mutation analysis of the *RET* gene in a series of 40 unrelated HSCR patients, revealed five novel and one previously described mutation in eight of 40 (20%) individuals studied. None of the potential disease causing mutations were identified in a total of 86 control individuals tested. Notably, all the missense mutations identified in the study population involve amino acids that have remained evolutionarily conserved in human, mouse and rat. Nine polymorphisms, of which three were novel, were also detected. Similar to previous studies, the mutations identified in *RET* are scattered throughout the gene. The percentage of individuals presenting with *RET* mutations in this study (20%) is consistent with previous findings of approximately 25% of HSCR cases caused by *RET* mutations.[12,14]

The majority of mutations (66.7%) were detected in the Coloured population which constitutes the largest study group, with mutation E480K in exon 7 occurring in two of 22 Coloured patients (9%). The remainder of the mutations identified (33.3%), occurred in White patients. No mutations were identified in the Black population. Two mutations were identified in the extracellular domain of the gene, encompassing exon 3 (V202M) and exon 7 (E480K). Exons 3 and 7 partly encode the cadherin-like domain and the cysteine-rich area, respectively.[22,23] In this study, no functional analysis was performed on the mutations identified in the *RET* gene, but the likelihood exist that mutations in this area may cause *RET* loss-of-function by a dominant-negative mechanism.[24]

A splice variant (IVS10-2A/G) was identified in intron 10 in an individual with aganglionosis in the rectosigmoid area. A sequence variant identified in the pyrimidine tract of the splice acceptor of intron 19 (IVS19-9C/T), with a pyrimidine to a pyrimidine change is probably not pathologic.[25] Mutations in exon 13 (D771N) and exon 17 (P973L) involve
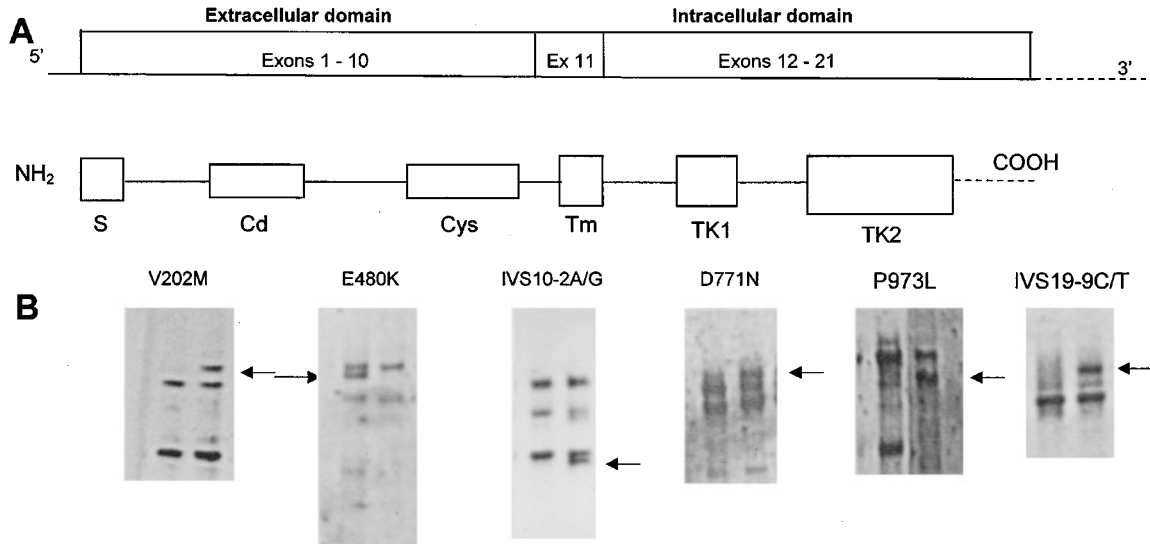
Figure 1 Schematic representation of genomic organisation of the *RET* proto-oncogene in relation to three distinct functional domains of the protein. Mutations identified in the South African population are shown. **A**, Exons encoding for specific domains of the RET protein. **B**, Ethidium bromide stained gel images of PCR-SSCP banding patterns of mutations in 12% polyacrylamide gels supplemented with 7.5% urea. Arrows indicate abnormal bands. S, signal peptide; Cd, cadherin-like sequence; Cys, cysteine-rich region; Tm, transmembrane domain; TK1 and TK2, split tyrosine kinase regions 1 and 2.

the intracellular domain, the most highly conserved area of the gene. The mutation in exon 17 (P973L) was previously described by Yin *et al*[26] who speculated that this mutation might inactivate *RET* gene function. The variant identified in intron 16 (IVS16-38delG) in one of the HSCR patients with mutation P973L, might contribute to disease modification and only by functional analysis will the role of this rare association be determined.

Although the numbers were small for statistical analysis, a varying trend was observed upon comparison of the exon 15 polymorphism of the *RET* gene in both the Black and White patients *vs* the respective control groups. Comparison of allelic frequencies of variant S904 in the total HSCR patient group with the total control group, demonstrated a statistically significant difference ($P<0.05$, $\chi^2=5.7$) which is consistent with previous findings of over-representation of the wild type allele in the HSCR population.[27,28] The observed trend of polymorphic allelic frequency variation in the HSCR and the mainly blood-derived control group, could indicate a higher susceptibility of specific tissues to mutational change. Of interest in a recent study conducted in patients presenting with HSCR and Down's syndrome, different *RET* polymorphisms were identified in the same individual when areas of normal ganglionic development were compared to regions of aganglionosis (L du Plessis, unpublished data). Four of the nine variants identified in this study, IVS6+56delG, A432, IVS13-29C/T and IVS16-38delG, may possibly be low penetrant susceptibility alleles. In light of the role that *RET* plays in the cellular signalling pathway between different cells, these findings warrant further

investigation as recent evidence indicates that silent mutations may influence *RET* transcription and lead to altered protein levels causing functional changes in *RET* itself or through interaction with other closely linked mutated genes.[27,28] The likelihood that variant S904 may be in linkage disequilibrium with other disease-causing mutation(s) in the gene should also be considered, particularly in the Black patient group where no mutations could be found. Failure to detect *RET* mutations in Blacks is probably a consequence of the heterogeneous nature of HSCR implying a different genetic basis in this group, but limitations imposed by the HEX-SSCP screening method[19] (mutation detection efficiency ~90%) used may also account for the apparent absence of *RET* mutations in the Blacks with HSCR.

Other genes involved in HSCR includes the *endothelin-B receptor (EDNRB)* gene and its ligand the *endothelin-3 (EDN3)* gene,[9,29] the *glial cell line-derived neurotrophic factor (GDNF)* gene,[30,31] *endothelin-converting enzyme 1 (ECE1)*,[32] the *sex dependent Y factor-like homeobox 10 (SOX10)* gene[33] and *neurturin (NTN)*.[34] Studies are currently underway to screen the *EDNRB* and *EDN3* genes for sequence variation and altered expression in our study population. Future genetic analysis of HSCR in particularly the Coloured population of South Africa, recently defined as a potential candidate of admixture linkage disequilibrium approaches to identify causative genes/mutations,[35] may contribute significantly to a complete understanding of the complex interaction of genes involved in neural crest derived pathologies.

This study represents the first genetic analysis of HSCR in the diverse South African population, and highlights the

importance of the *RET* proto-oncogene in neurocristopathologic disease. Although no direct evidence concerning the causative nature of the novel *RET* mutations is provided, either through loss of activity, enhanced activity or altered specificity of the protein, several lines of evidence support their significance in HSCR. Detection of identical mutations in patients from different ethnic groups (D771N) or associated with possible distinct haplotypes in the same population group (E408K), whilst absent in the control groups, signifies the relative importance of the relevant protein domains in HSCR.

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