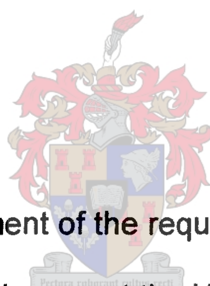
MOLECULAR-GENETIC ANALYSIS OF HIRSCHSPRUNG'S DISEASE IN SOUTH AFRICA

MONIQUE G. JULIES



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Medical Sciences at the University of Stellenbosch.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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Summary

Hirschsprung's disease, or aganglionic megacolon, is a common cause of intestinal obstruction in neonates and is associated with the congenital absence of intrinsic ganglion cells in the myenteric and submucosal plexuses of the gastrointestinal tract. The affected area is usually restricted to the distal part of the colon (short segment disease), but total colonic or intestinal involvement occurs in some patients (long segment disease).

DNA analysis was performed on samples from 53 unrelated sporadic HSCR patients to search for mutations in *RET proto-oncogene*, *endothelin-B receptor* (*EDNRB)* and *endothelin-3* (*EDN3) genes*. The patients were from different ethnic groups in South Africa, including 29 coloured, 14 white (Caucasian) and 9 black individuals. The origin of 1 patient was unknown. PCR HEX-SSCP analysis of the *RET proto-oncogene* revealed one previously described (P973L) and five novel mutations (V202M, E480K, IVS10-2A/G, D771N, IVS19-9C/T), likely to cause or contribute to the HSCR phenotype. Nine polymorphisms were also identified in the *RET proto-oncogene*, of which four were novel (IVS6+56delG, IVS13-29C/T, IVS16-38delG, X1159) and five previously described (A45, A432, L769, S904, R982). All the mobility shifts detected in the *EDNRB gene* represented polymorphisms (A60T, S184, I187, V234, L277, IVS3-6C/T, IVS4+3A/G). No sequence variants were identified in the *EDN3 gene*. The majority of mutations in the *RET proto-oncogene* (28.6%) were identified in coloured patients while no mutations were identified in black patients. A mutation in *RET* was identified in two of 14 patients (14%) presenting with HSCR and Down's syndrome compared to 6 mutations identified in 9 of 39 patients (23%) with

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only HSCR. The fact that Down's syndrome patients have a high chance of developing HSCR, implies the involvement of modifier gene(s) in a HSCR/Down's syndrome phenotype.

This study demonstrated that, within the South African HSCR patient population, the *RET proto-oncogene* is the major susceptibility gene, whereas *EDNRB* and *EDN3* may contribute only to a minority of cases. In 81% of patients no disease-causing mutation could be identified, which is in keeping with the heterogeneous nature of HSCR. The identification of mutations in HSCR patients would in future lead to improved and accurate counselling of South African HSCR patients and their families.

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Opsomming

Hirschsprung se siekte (HSCR), ook bekend as aganglionosis megakolon, is 'n algemene oorsaak van intestinale obstruksie in pasgeborenes en word geassosieer met die kongenitale afwesigheid van intrinsieke ganglion selle, in die miënteries en submukosa pleksus van die gastrointestinale kanaal. Alhoewel die aangetaste deel hoofsaaklik by die distale area van die kolon geleë is (kort segment siekte), kom totale koloniese of intestinale betrokkenheid ook in sommige pasiënte voor (lang segment tipe).

Molekulêre DNS analise van 53 nie-verwante Suid Afrikaanse sporadiese HSCR pasiënte (29 kleurlinge, 14 blankes, 9 swartes en 1 individu van onbekende oorsprong) is uitgevoer in die *RET proto-onkogeen, endoteel-B reseptor (EDNRB) en endoteel-3 (EDN3)* gene. Heterodupleks-enkel string konformasie polimorfisme (HEX-SSCP) analise van polimerase ketting reaksie (PKR) geamplifiseerde produkte van die *RET proto-onkogeen* het gelei tot die identifikasie van vyf nuwe mutasies (V202M, E480K, IVS10-2A/G, D771N, IVS19-9C/T) en een bekende mutasie (P973L). Vier nuwe polimorfismes (IVS6+56delG, IVS13-29C/T, IVS16-38delG, X1159) en vyf bekende polimorfismes (A45, A432, L769, S904, R982) is ook aangetoon. Sewe polimorfismes (A60T, S184, I187, V234, L277, IVS3-6C/T, IVS4+3A/G) is in die *EDNRB* geen geïdentifiseer. Geen veranderinge is in die *EDN3* geen waargeneem nie. Die meerderheid mutasies waargeneem in die *RET proto-onkogeen* is in die kleurling populasie (28.6%) waargeneem, terwyl geen mutasies in die swart populasie geïdentifiseer is nie. 'n *RET* mutasie is in twee van 14 (14%) pasiënte met 'n HSCR en Down's sindroom fenotipe waargeneem, in vergelyking met

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6 mutasies geïdentifiseer in 9 van 39 pasiënte (23%) met slegs HSCR. Die algemene voorkoms van Down's sindroom met HSCR, impliseer die rol van ander gene in die HSCR/Down's sindroom fenotipe.

Die meerderheid mutasies wat aanleiding gee tot die HSCR fenotipe kom voor in die *RET proto-onkogeen* (19%), terwyl slegs polimorfismes in die *EDNRB* geen waargeneem is. Geen HEX-SSCP bandpatroon veranderinge is in die *EDN3* geen waargeneem nie. Ongeveer 81% van die Suid Afrikaanse HSCR pasiënte was mutasie-negatief wat dui op die heterogene aard van die siekte. In die toekoms sal analise van siekte-verwante mutasies in die *RET* geen lei tot akkurate diagnose en verbeterde genetiese voorligting van HSCR in die Suid-Afrikaanse populasie.

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To my mother…

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LIST OF ABBREVIATIONS

^{o}C	degrees Celcius
µl	microlitre
µg/ml	microgram per millilitre
U/µl	units per microlitre
A (Ala)	alanine
ACHN	renal adenocarcinoma cell-line
AECE	adenocarcinoma endothelin converting enzyme
APS	ammonium persulphate
ARM	anorectal malformation
bp	base-pair
C (Cys)	cysteine
Ca^{2+}	calcium
C/A	colonic aganglionosis
CCHS	congenital central hypoventilation syndrome
Cd	cadherin-like sequence
cDNA	complementary deoxyribonucleic acid
cm	centimeter
COOH	carboxy-terminus
Cys	cysteine-rich region
D (Asp)	aspartic acid
ddH_2O	double distilled water
dH_2O	distilled water
DMSO	dimethyl sulfoxide

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DNA	deoxyribonucleic acid
dNTP	2'-deoxy-nucleoside-5'-triphosphate
Dom	dominant megacolon
E (Glu)	glutamic acid
e	extracellular loop
ECE1	*endothelin-converting enzyme 1*
ECE1CS	endothelin-converting enzyme 1 cleavage site
EDN1	*endothelin-1 gene*
EDN2	*endothelin-2 gene*
EDN3	*endothelin-3 gene*
EDNRA	*endothelin-A receptor gene*
EDNRB	*endothelin-B receptor gene*
EDTA	ethylenediaminetetraacetic acid
ENS	enteric nervous system
ET1	endothelin-1
ET2	endothelin-2
ET3	endothelin-3
EtOH	ethanol
F (Phe)	phenylalanine
F	forward primer
FCS	furin cleavage site
FMTC	Familial medullary thyroid carcinoma
G (Gly)	glycine
g	gram
GDNF	*glial cell line-derived neurotrophic factor gene*

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GDNFR-alpha	glial cell line-derived receptor alpha
H (His)	histidine
HEX-SSCP	heteroduplex single-strand conformation polymorphism analysis
HMG	high mobility group
HSCR	Hirschsprung's Disease
I (Ile)	isoleucine
i	intracellular loop
IBD	identity-by-descent
K (Lys)	lysine
kb	kilobase
kD	kilo Dalton
$KHCO_3$	potassium hydrogen carbonate
L (Leu)	leucine
M (Met)	methionine
M	moles per litre
MEN 2A	Multiple endocrine neoplasia type 2A
MEN 2B	Multiple endocrine neoplasia type 2B
mg	milligram
$MgCl_2$	magnesium chloride
mg/ml	milligram per millilitre
min	minutes
MITF	microphthalmia associated transcription factor
ml	millilitre
mM	milli-moles per litre

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mRNA	messenger ribonucleic acid
MTC	medullary thyroid carcinoma
N (Asn)	asparagine
NaCl	sodium chloride
$NaClO_4$	sodium perchloride
NH_4Cl_2	ammonium chloride
NH_2	amino-terminus
NIH3T3	NIH (USA) cell line 3T3
nm	nano meter
P (Pro)	proline
p	short arm of chromosome
PAA	polyacrylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
pmol/µl	picomole per microlitre
Q (Glu)	glutamine
q	long arm of chromosome
R (Arg)	arginine
R	reverse primer
RET	*REarranged during transfection proto-oncogene*
RET9	9 amino acid C-terminal isoform
RET43	43 amino acid C-terminal isoform
RET51	51 amino acid C-terminal isoform
rpm	revolutions per minute

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R/S	rectosigmoidal aganglionosis
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
S (Ser)	serine
S	signal sequence
SDS	sodium dodecyl sulphate
SH2	src homology 2 domain
SOX10	*sex-dependent Y factor-like homeobox 10*
SP	signal peptide
SRY	sex-dependant Y factor
SSCP	single-strand conformation polymorphism
T (Thr)	threonine
Taq	*thermus aquaticus*
TBE	tris-borate/EDTA
TCA	total colonic aganglionosis
TE	tris-EDTA
TEMED	N, N, N' N',-tetramethylethylenediamine
TK	split tyrosine kinase domain
Tm	transmembrane domain
U	units
UTR	untranslated region
V (Val)	valine
V	volts
v/v	volume per volume
Val	valine

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W (Trp)	tryptophan
w/v	weight per volume
X (Unk)	unknown
Y (Tyr)	tyrosine

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CHAPTER 1

INTRODUCTION

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1.1. BACKGROUND

Hirschsprung's disease (HSCR) was described by Harald Hirschsprung in 1888 as congenital megacolon, but only recently, following research, have the pathogenesis and pathophysiology become more clear. The disease is characterised by the congenital absence of intrinsic ganglion cells in the myenteric and submucosal plexuses of the gastrointestinal tract, thereby the name aganglionic megacolon. The main cause of clinical presentation is due to partial or complete intestinal obstruction during infancy because of a malfunction of the involved segment and adults usually present with severe constipation. The aganglionic segment is restricted to the distal part of the colon (rectosigmoidal area) in 75% of cases (short segment disease), to the sigmoid, splenic flexure or transverse colon in 17% of cases, and to the total colon plus a short segment of the latter part of the ileum in the remaining 8% of cases. In the rarest form, total aganglionosis extends from the duodenum to the rectum. Patients usually present with one or more of the following additional clinical symptoms: failure to pass meconium, secondary electrolyte disturbances, megacolon, colonic distension, severe constipation and sepsis due to enterocolitis. The most common chromosomal abnormality associated with HSCR is Down's syndrome (Bodian *et al* 1951), but other chromosomal abberations such as mosaic trisomy 18 (Passarge 1973), partial trisomy of region 21q22 and 11q23 (Beedgen *et al* 1986) and interstitial deletions observed on 13q (Kiss and Osztovics 1989, Bottani *et al* 1991) and 10q (Martucciello *et al* 1992, Luo *et al* 1993) are also associated with the disease. Other syndromes associated with HSCR include metaphyseal chondrodysplasia, McKusick type (McKusick *et al* 1965), Waardenburg syndrome

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(Omenn and McKusick 1979), Smith-Lemli-Opitz syndrome type II (Curry *et al* 1987), multiple endocrine neoplasia type 2A and 2B, and medullary thyroid carcinomas (Eng *et al* 1994). At present, the disease can be diagnosed using a combination of tests, including rectal manometry, radiological examinations and histological, histochemical or immunohistochemical analysis of the affected segment (Huntley *et al* 1982, Molenaar *et al* 1989). The affected segment is usually removed by surgery by one of the following procedures: Swenson procedure (Swenson 1950), Duhamel procedure (Duhamel 1960), Soave procedure (Soave 1960) or the Rehbein procedure (Rehbein *et al* 1960). Modifications of these procedures included the Martin procedure (Martin 1968), Stringel operation (Stringel 1973), Kimura procedure (Kimura *et al* 1981), Shandling operation (Shandling 1984) and the Boley operation (Boley 1984).

The enteric nervous system originates from neural crest precursor cells which migrate and colonise the wall of the intestinal tract during embryogenesis. The first neuroblasts are detected on the 24th gestational day of embryogenesis in the proximal foregut (Hoar and Monic 1981). The craniocaudal migration occurs during the 5th to 12th week of gestation (Okamoto and Ueda 1967, Sullivan 1996). Extracellular matrix proteins, neurotrophic factors and cell adhesion molecules are important factors required for the developing, differentiation, migration and survival of neurons in the peripheral and central nervous system (Brauer and Markwald 1987, Crossin *et al* 1990, Theonen 1991). The absence of ganglion cells was postulated to be due to a failure of migration of the neural crest cells and the earlier the arrest, the longer the aganglionic segment (Okamoto and Ueda 1967). It has therefore been hypothesised that micro-environmental changes, eg an altered extracellular matrix, causes failure of cell differentiation and results in aganglionosis (Le Douarin and

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Teillet 1974, Gershon *et al* 1980, Greenberg *et al* 1981). However, recent studies using mouse models, indicate that the ganglion cells reach the correct position, but fail to develop or survive, resulting in aganglionosis (Puri *et al* 1998).

1.2. GENETIC RISK

The involvement of genetic factors in HSCR was suggested due to an increased risk to siblings and the frequent association of the disease with chromosomal abnormalities such as Down's syndrome and Waardenburg syndrome. HSCR has an estimated population incidence of 1 in 5000 (0.02%) live births (Passarge 1967) with an overall risk to siblings of 4% - 9% (Badner *et al* 1990). Males are more likely to be affected than females, with a risk 3.5 – 7.8 fold higher than that of females (Badner *et al* 1990). As the aganglionic segment becomes more extensive, the risk to siblings increases and the sex ratio decreases (1.5 to 2.1, respectively, for males and females with long segment disease) (Puri 1997). HSCR mostly occurs sporadically (approximately 90% of cases), with an incidence of 3.6% to 7.8% for familial cases reported (Ikeda and Goto 1984, Puri 1997) with total colonic aganglionosis occuring in 15% to 21% of these familial cases (Kleinhaus *et al* 1979). An earlier age of onset in the patients is usually associated with a higher risk of disease occurrence in the siblings (Angrist *et al* 1993).

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1.3. BASIS OF INHERITANCE

The suspected basis of HSCR was initially believed to be a sex-modified multifactorial trait (Passarge 1967). The finding that males are more likely to be affected than females, suggested that the disease is stimulated by an X-linked recessive trait if transmission is maternal. Consequently, multifactorial inheritance, with the involvement of multiple genes and environmental factors in the development of the clinical phenotype, was suggested (Passarge 1967, Garver *et al* 1985). However, the observation of multiple chromosome aberrations in HSCR patients, supported genetic heterogeneity, with autosomal dominant, autosomal recessive and polygenic forms of inheritance. The occurrence of the disease with chromosomal abnormalities and congenital anomalies, strengthened this school of thought, implicating the involvement of a large number of genes, each with a small cummulative effect (Badner *et al* 1990). In cases with aganglionosis beyond the sigmoid colon and in some large pedigrees, the pattern of inheritance was most compatible with an incomplete penetrant dominant gene (Badner *et al* 1990).

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1.4. CANDIDATE GENES FOR HSCR

Several genes are involved in the pathogenesis of HSCR, including the *REarranged during transfection proto-oncogene (RET)* (Takahashi *et al* 1985), *endothelin-B receptor gene* (*EDNRB*) (Puffenberger *et al* 1994b), *endothelin-3 gene* (*EDN3*) (Edery *et al* 1996, Hofstra *et al* 1996) *glial cell line-derived neurotrophic factor gene* (*GDNF*) (Treanor *et al* 1996, Jing *et al* 1996), *endothelin-converting enzyme 1* (*ECE1*) (Hofstra *et al* 1999), the *sex dependent Y factor-like homeobox 10 gene* (*SOX10*) (Pingualt *et al* 1997) and *neurturin* (*NTN)* (Doray *et al* 1998). The genes that have been implicated in HSCR are important for the migration of cells originating from the neural crest and their subsequent development as enteric ganglia, possibly acting in the signalling pathway which differentiates pre-ganglion cells into maturity.

1.4.1. *RET proto-oncogene*

1.4.1.1. Structure and function of the *RET proto-oncogene*

The *RET* gene was originally identified by Takahashi and colleagues during a classic NIH 3T3 transformation assay, cloned as a chimeric oncogene (Takahashi *et al* 1985). *RET* is expressed in the brain, thymus, lung, heart, spleen, testis and small intestine of adults, and in embryos, the level of expression is 20-50 fold higher than that observed in adults (Tahira *et al* 1988, Höppener and Lips 1996). These high embryonic levels reduce to adult levels by day 14 of gestation. The *RET proto-oncogene* is a cell-surface molecule that transduces signals for cell growth and

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differentiation and encodes a putative transmembrane receptor tyrosine kinase protein.

Five different mRNA species of *RET*, due to alternative splicing and polyadenylation, were identified in a human neuroectodermal cell line (Tahira *et al* 1990). These mRNAs were 7.0 kb, 6.0 kb, 4.6 kb, 4.5 kb and 3.9 kb in size. Two isoforms of *RET* were identified: a *RET* protein consisting of 1072 amino acids (mRNA – 7.0 kb, 4.5 kb and 3.9 kb) and a protein consisting of 1114 amino acids (mRNA – 6.0 kb and 4.6 kb). The first 1063 amino acids are identical in both isoforms and two tyrosine residues that represent additional autophosphorylation and substrate binding sites, are included in the larger isoform at the 51 amino acid (*RET*51) C-terminal. These 51 amino acids are replaced with 9 alternative amino acids (*RET*9) at the C-terminal in the shorter isoform to generate a 1072 amino acid protein, resulting from differential splicing at the 3' end of exon 19 (Tahira *et al* 1988,1990, Takahashi *et al* 1988, Kwok *et al* 1993). The 20 exons of *RET* were shown, by restriction mapping and Northern analysis, to be encoded by the 51 amino acid C-terminal isoform, but various lengths of the 3' untranslated region (UTR) and alternative splicing occur in this area. No splicing occurs in the 9 amino acid *RET* isoform, but it includes 9 codons which lie within intron 19, directly downstream of exon 19 (Tahira *et al* 1990).

In the characterisation of the 3' region of *RET*, using cloned cDNA and reverse transcriptase PCR (RT-PCR), a protein with an alternative 43 amino acid C-terminal (*RET*43) was identified (Myers *et al* 1995). This resulted from splicing of exon 19 to a previously unrecognised coding exon, exon 21. Alternative splicing was also shown at the 5' end of the gene which resulted in transcripts lacking exon 3, exons 3 and 4

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or exons 3, 4 and 5. Two mature *RET* proteins of 170 kD and 175 kD were generated when fully glycosylated and were localised to the cell membrane, functioning as cell-surface receptors. Smaller *RET* proteins of 150 kD and 155 kD were generated when partially glycosylated and were localised to the cytoplasm, implicating that they cannot bind extracellular ligands (Takahashi *et al* 1991,1993, Bunone *et al* 1995). The gene therefore comprises 21 exons (Myers *et al* 1995) in a genomic region spanning ± 50 kb with exons varying in size from 60 bp to 287 bp. The *RET* proto-oncogene can be divided into different domains as illustrated in Figure 1.1: an extracellular cadherin-like domain which is encoded by exons 1 to 10, a transmembrane domain encoded by exon 11 and an intracellular (split tyrosine kinase) domain encoded by exons 12 to 21. The protein is localised to the cell membrane and functions as a cell-surface receptor.

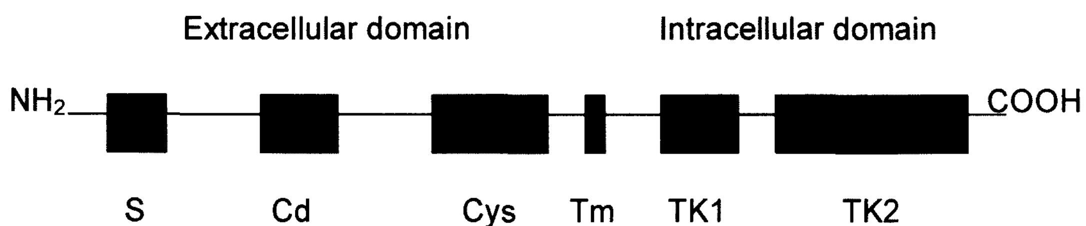

Figure 1.1. Schematic representation of the structure of the *RET proto-oncogene.* Abbreviations: S – signal sequence, Cd – Cadherin-like sequence, Cys – Cysteine-rich region, Tm – Transmembrane domain, TK1 and TK2 – Split tyrosine kinase domain; NH_2 – amino-terminus; COOH – carboxy-terminus

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The hydrophobic signal sequence NH_2 -terminus targets the molecule to the cell surface (Höppener and Lips 1996). The extracellular domain shows homology to the cadherin family and consists of highly conserved cysteine residues located close to the cell membrane. The cysteine residues play a role in ligand binding and dimerisation. Cadherins are known to be Ca^{2+} - dependent adhesion molecules which are involved in cell to cell interactions, but it is not yet known whether they have similar functions in the *RET* protein. The hydrophobic Tm anchors the molecule in the plasma membrane. The intracellular domain consists of the tyrosine kinase regions 1 and 2, which is the most highly conserved area of the gene. The tyrosine kinase protein regulates the proliferation, migration, differentiation and survival of particular neural crest cells.

1.4.1.2. HSCR and the *RET proto-oncogene*

RET plays an important role in the development of neural crest derivatives during the early stages of embryogenesis. The identification of an interstitial deletion of chromosome 10 in a patient with long segment disease as well as the identification of similar deletions in other patients with HSCR implicated chromosome 10 in the pathogenesis of the disease (Martucciello *et al* 1992, Luo *et al* 1993). Families displaying dominantly inherited HSCR with incomplete penetrance were used to localise the gene to chromosome 10q11.2 by linkage analysis (Luo *et al* 1993, Lyonnet *et al* 1993, Angrist *et al* 1993). With the cloning of *RET* (Takahashi *et al* 1985) and the availability of the DNA sequence of the exon-intron boundaries (Kwok *et al* 1993, Ceccherini *et al* 1993), the identification of mutations affecting different

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domains of the *RET* gene was facilitated. The involvement of the *RET* gene in HSCR pathogenesis was confirmed by the identification of many different variants, including missense, nonsense, deletion and insertion mutations. These mutations account for approximately 25% of HSCR cases and are not restricted to a particular area of the gene as depicted in Figure 1.2 (Edery *et al* 1994a, Romeo *et al* 1994, Luo *et al* 1994, Attie *et al* 1995a, Angrist *et al* 1995, Seri *et al* 1997).Most HSCR cases occur sporadically and the frequency of *RET* gene mutations is higher in familial cases (28.4%) than in sporadic cases (17.6%). More mutations occur in patients with long segment disease, even in sporadic cases (see figure 1.2 for details of *RET* mutations in HSCR). *RET* mutations are also associated with diseases such as multiple endocrine neoplasia type 2A and 2B (MEN 2A and 2B), familial medullary thyroid carcinoma (FMTC), sporadic medullary thyroid carcinomas and pheochromocytoma and papillary thyroid carcinoma (Eng and Mulligan 1997).

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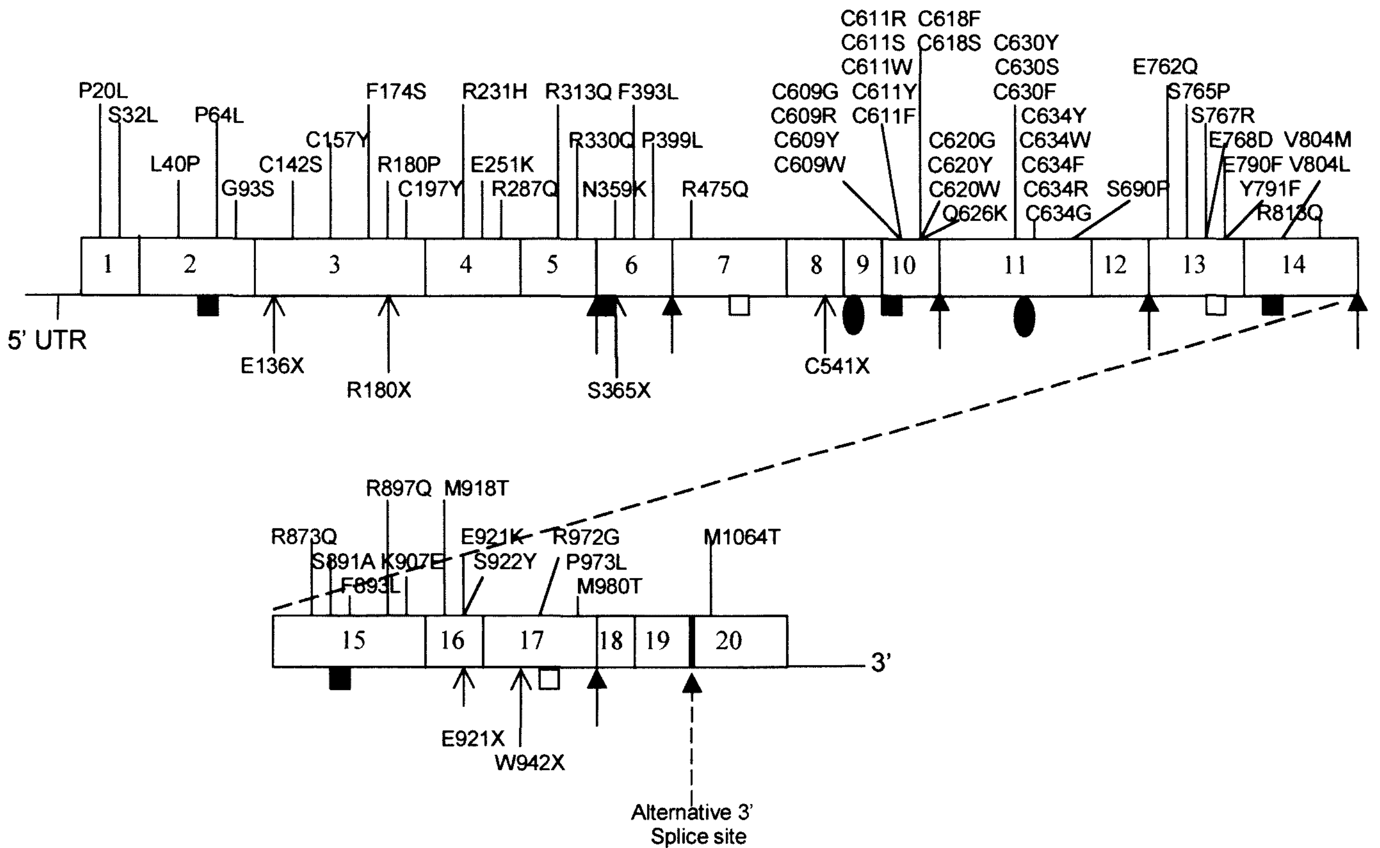

5' UTR – 5' untranslated region

——— - point mutations

⟶ - point mutations resulting in a termination codon

⟶ - splice site mutation

■ - deletion mutation

□ - insertion mutation

⬬ - complex event involving duplication, insertion and deletion resulting in stop codon

Figure 1.2. Spectrum of documented mutations in the *RET proto-oncogene* (from Eng and Mulligan 1997)

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1.4.2. *Endothelin-B Receptor gene*

1.4.2.1. Structure and function of the *EDNRB* gene

Endothelins belong to a family of potent vasoactive peptides consisting of 3 isopeptides, ET1, ET2 and ET3 (Yanagisawa *et al* 1988). The *EDNRB* gene has a genomic span of 24 kb and encodes a 442 amino acid heptahelic receptor that equally binds *EDN-1*, 2 and 3 (Arai *et al* 1993). The gene consists of seven exons divided by six introns that vary in size from 109 bp to 2855 bp and 0.2 to 14.5 kb, respectively. The gene is relatively conserved between different species.

Each exon encodes several structural units; exon 1 encodes the entire 5'- noncoding region and the coding region through the second transmembrane domain, exon 2 encodes the first extracellular loop and the third transmembrane domain, exon 3 encodes the second cytoplasmic loop, the fourth transmembrane domain and the second extracellular loop, exon 4 encodes the fifth transmembrane domain and the third cytoplasmic loop, exon 5 encodes the sixth transmembrane domain and the third extracellular loop, exon 6 encodes the seventh transmembrane domain and exon 7 encodes the cytoplasmic carboxyl tail and the entire 3' – noncoding region (Figure 1.3.).

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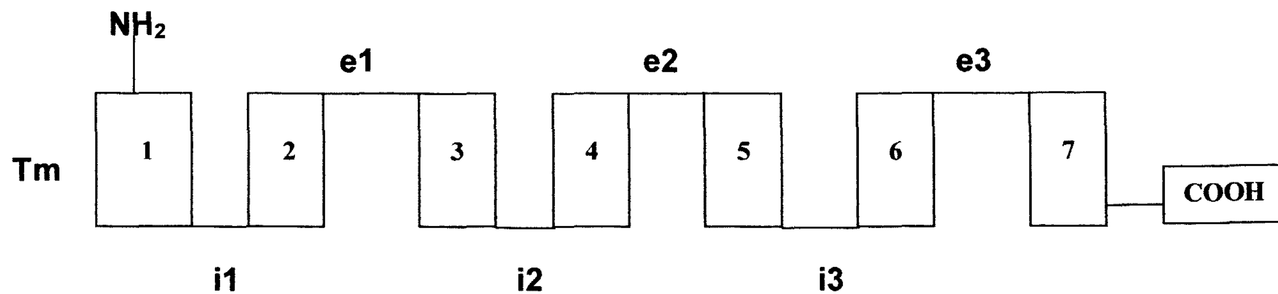

Figure 1.3. Schematic representation of the structure of the *EDNRB* gene

Abbreviations: Tm – transmembrane domains (1-7), e – extracellular loop, i – intracellular loop; NH_2 – amino-terminus; COOH – carboxy-terminus.

Each intron lies in close proximity to the putative transmembrane domains which implies that each exon encodes a functional unit. The fifth transmembrane domain is important for the ligand-binding characteristics and signal transduction (Becker *et al* 1994). It has been demonstrated that mutant receptors lead to dysfunctional signalling pathways, altered processing pathways and faulty translocation (Arai *et al* 1993).

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1.4.2.2 HSCR and the *EDNRB* gene

Interstitial deletions of chromosome 13 in patients with HSCR, suggested a role for chromosome 13 in the pathogenesis of the disease (Sparkes *et al* 1984, Lamont *et al* 1989, Kiss and Osztovics 1989, Bottani *et al* 1991). In 1994 the *EDNRB* gene was mapped to the region 13q22 by using identity-by-descent (IBD) and linkage disequilibrium mapping of a large inbred (Mennonite) kindred with HSCR, demonstrating autosomal recessive inheritance (Puffenberger *et al* 1994b). A missense mutation identified in this gene (Trp276Cys) segregated with HSCR in the Mennonite kindred (Puffenberger *et al* 1994a) and was associated with incomplete penetrance of the phenotype. *EDNRB*, identified as the second gene for HSCR, was subsequently cloned, characterised and the exon-intron boundaries, 5'-flanking region and the 3'-flanking region were determined (Arai *et al* 1993). Targeted disruption of the mouse *Ednrb* gene resulted in aganglionic megacolon (Hosoda *et al* 1994, Gariepy *et al* 1996). These findings established *EDNRB* as an important component in the normal development of the enteric ganglion neurons and epidermal melanocytes (neural crest-derived cell lineages).

The gene encodes a heptahelical receptor protein and the signaling pathway through this receptor is important for the establishment of the enteric nervous system. Southern blot analysis indicated that only one copy of the gene exists in the human genome and the size of the gene corresponds with other G protein-coupled receptor genes (Arai *et al* 1993). Mutations identified in the *EDNRB* gene indicated that males had a greater risk of being affected than females (Puffenberger *et al* 1994a). *EDNRB* mutations account for 5-6% of HSCR cases and the patients with such a mutation

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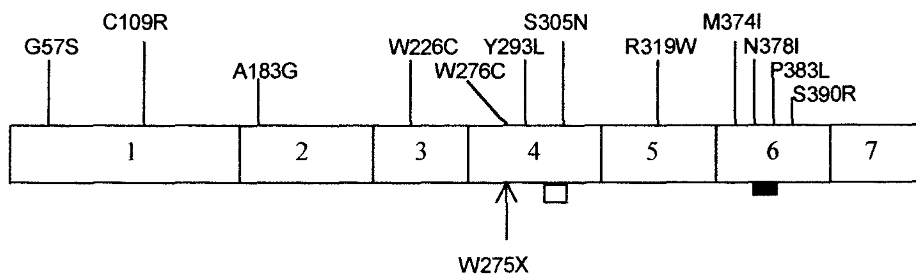
mutation have aganglionosis restricted to the distal part of the colon (Chakravarti 1996). Figure 1.4 is a schematic representation of the distribution of *EDNRB* mutations identified to date. Homozygous mutations of *EDNRB* have been shown to contribute to a HSCR-Waardenburg syndrome phenotype, whereas heterozygous mutations contribute to non-syndromic HSCR with incomplete penetrance (Amiel *et al* 1996). This suggested that *EDNRB* mutations could be dosage sensitive and that one or more modifier genes are involved in HSCR patients with heterozygous mutations. Puffenberger *et al.* (1994a) indicated that homozygotes and heterozygotes for the *EDNRB* mutation identified in the Mennonite family, had a 74% and 21% risk, respectively, of developing HSCR. In certain studies a loss-of-function or dysfunction of the *EDNRB* gene was indicated in isolated HSCR cases (Kusafuka *et al* 1996, Auricchio *et al* 1996, Svensson *et al* 1999).



—— - point mutation

⟶ - point mutation resulting in termination codon

■ - deletion mutation

□ - insertion mutation

Figure 1.4. Spectrum of documented mutations in the *EDNRB* gene

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1.4.3. *Endothelin-3 gene*

1.4.3.1. Structure and function of the *EDN3* gene

The endothelins are encoded in prepropolypeptide precursors and are produced from the precursor through its intermediate. The precursors are first cleaved by furin at tetrapeptide recognition sites (Barr 1991, Seidah and Chretien 1992). The cleavage produces big endothelin, a biologically inactive intermediate, which contains 41 amino acids in humans (Arinami *et al* 1991, Kusafuka and Puri 1998). Big endothelin is then further cleaved at the Trp-21-Val/Ile-22 site by endothelin-converting enzyme 1 (*ECE1*), a metalloprotease. This produces the mature form of *EDN3*. A second cleavage site for furin also occurs after the big *EDN3* sequence.

The mRNA of *EDN3* encodes a 230 amino acid precursor that includes *EDN3* and a 15 amino acid homologous segment called the *EDN3*-like sequence. The *preproendothelin-3 gene* comprises 5 exons and 4 introns. The regions encoding big *EDN3* and *EDN3*-like peptides show 50% to 60% homology to *EDN1* and *EDN2*. Exon 2 encodes the mature endothelin portion and the tail portion of big *EDN3* is encoded by exons 2 and 3 (Arinami *et al* 1991). Strong conservation of the nucleotide sequence that encodes the mature endothelin-3 gene (21 amino acids) is observed. Exon 3 encodes the *EDN3*-like peptides in which the relative positions of the four cysteine residues are perfectly conserved. Figure 1.5 provides a representation of the *EDN3* gene.

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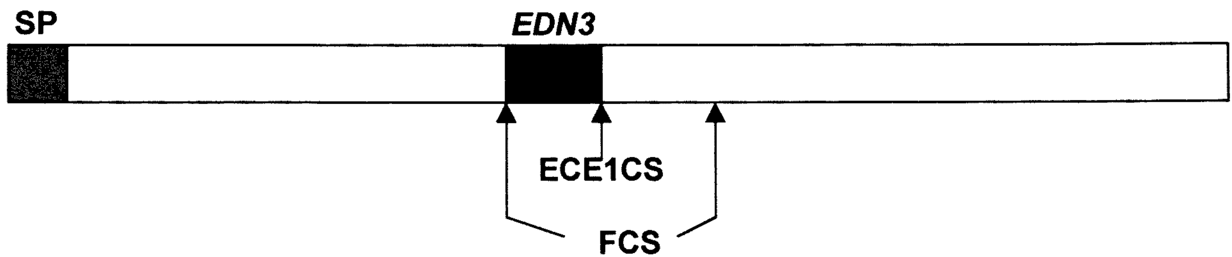

Figure 1.5. Schematic representation of the preproendothelin-3 protein. Abbreviations: SP – signal peptide, *EDN3* – mature *EDN3*, FCS – furin cleavage site, ECE1CS – endothelin-converting enzyme 1 cleavage site

When *EDN3* activates *EDNRB* (a G protein-coupled receptor), it induces the flow of calcium into the cell (Puffenberger *et al* 1994a). Both genes were identified on enteric neurons and gut mesenchyme cells of human fetuses, which suggested that the function of *EDN3* and *EDNRB* could be the regulation of interactions between neural crest cells and gut mesenchyme cells that are required for normal neural crest migration (Robertson *et al* 1997).

1.4.3.2 HSCR and the *EDN3* gene

Endothelins (potent vasoactive peptides) act on G protein-coupled heptahelical receptors and consists of 21 amino acid peptides. Three mammalian endothelins have been identified, *EDN1*, *EDN2* and *EDN3*, and are encoded by separate genes (Rubanyi and Polokoff 1994). Endothelin-A receptor (*EDNRA*) and endothelin-B

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receptor (*EDNRB*) are two subtypes which act as receptors for the endothelins (Arai *et al* 1990, Sakurai *et al* 1990, Sakamoto *et al* 1993). *EDN1* has a high affinity for *EDNRA*, whereas *EDNRB* binds equally to these endothelins (Yanagisawa 1994).

A targeted disruption of the murine *endothelin-3 ligand gene* showed a recessive phenotype of megacolon and coat colour spotting, as observed in mouse-models of the *Ednrb* gene (Baynash *et al* 1994). No abnormalities were observed in mice with heterozygous mutations of the *EDN3* gene. These studies indicated that *EDN3* plays an important role in the normal development of epidermal and choroidal melanocytes and enteric ganglion cells, and that the signal conveyed by *EDN3* through *EDNRB* is required for the development of these cells. Although *EDNRB* binds equally to *EDN1* and *EDN2*, the study indicated normal levels of these endothelins in homozygous knockout *Edn3/Edn3* mice. This implies that these endothelins cannot compensate for the function of *EDN3* and therefore *EDN1* and *EDN2* do apparently not play a role in the development of the two cell lineages.

EDN3 mRNA is mainly expressed in the jejenum and adrenal gland, and to a lesser extent in the brain, spleen and renal medulla (Arinami *et al* 1991). The *EDN3* locus was assigned to chromosome 20 by analysing genomic DNA from human-mouse somatic cell hybrids (Bloch *et al* 1989a, 1989b). The gene was further localised to chromosome region 20q13.2-q13.3 by *in situ* hybridisation (Gopal Rao *et al* 1991, Arinami *et al* 1991). Mutations identified in the *EDN-3* gene in HSCR patients have contributed to elucidating the involvement of the gene in disease pathogenesis (Edery *et al* 1996, Hofstra *et al* 1996). Thus far, only homozygous mutations have been identified in patients with syndromic HSCR (Shar-Waardenburg syndrome)

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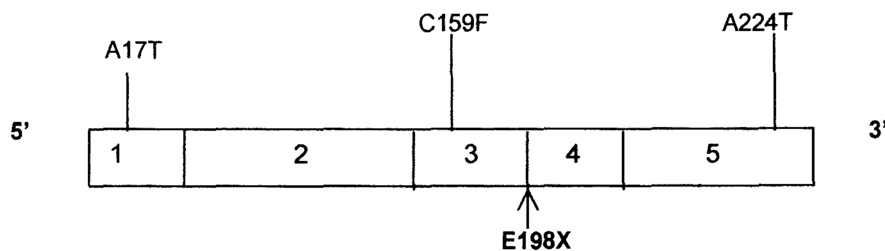
(Hofstra *et al* 1996, Kusafuka and Puri 1998, Edery *et al* 1996). Figure 1.6 provides a summary of mutations identified in the *EDN3* gene. No heterozygous mutations have been identified in HSCR and non-syndromic HSCR patients. Relatives of individuals with homozygous *EDN3* mutations, show heterozygous mutations with no phenotype of either HSCR or Waardenburg syndrome (Svennson *et al* 1999). Similar heterozygous mutations have however been found in individuals with isolated HSCR or congenital central hypoventilation syndrome (CCHS) (Bolk *et al* 1996a). Svennson and colleagues (1999) postulated that the difference in phenotypic expression could be due to reduced penetrance of some *EDN3* mutations and/or accessory mutations in modifier genes. To date no mutations in other HSCR genes (*RET* and *EDNRB*) have been reported in combination with *EDN3* mutations.



——- point mutations

——>- point mutation resulting in a termination codon

Figure 1.6. Spectrum of documented mutations in the *EDN3* gene

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1.4.4. OTHER GENES ASSOCIATED WITH HSCR

1.4.4.1. *Glial cell line-derived neurotrophic factor* (*GDNF*)

GDNF was identified in 1996 as one of the ligands of the *RET* proto-oncogene, which encodes a tyrosine kinase receptor (Treanor *et al* 1996, Jing *et al* 1996). The gene, an extracellular neurotrophic molecule localised to chromosome 5p12-p13.1 (Schindelhauer *et al* 1995), acts as a ligand for a multisubunit receptor in which the glycosylphosphatidylinositol-linked protein *GDNFR-alpha* provides ligand-binding and the signaling components are provided by *RET*. Previous studies have shown that in the absence of *GDNF* or *GDNFR-alpha*, *RET* signalling is reduced or absent (Treanor *et al* 1996, Robertson *et al* 1997). Total intestinal obstruction was also observed in mice carrying two null alleles for *Gdnf* (Sanchez *et al* 1996). These studies suggested that neurons of the enteric nervous system (ENS), in the absence of *GDNF*, die during late embryonic development. Receptor tyrosine kinase converts extracellular information into chemical signals that can be transduced inside the cell (Martucciello *et al* 1995), including binding of *GDNF* as a dimer in which *GDNF*R-alpha is involved, receptor dimerisation, activation of tyrosine kinase and transphosphorylation of intracellular protein targets. Alterations in this signaling pathway have been postulated to result in a HSCR phenotype. The *GDNF* mature protein consists of 134 amino acids and is encoded by two exons consisting of 151 bp and 485 bp, respectively. *GDNF* mutations identified in HSCR patients range in frequency from 0.9% to 5.5% (Angrist *et al* 1996, Salomon *et al* 1996, Ivanchuk *et al* 1996). Studies performed by Angrist *et al* (1996) and Salomon *et al* (1996) have suggested that GDNF mutations are not sufficient to cause HSCR *per se*, but their

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interaction with other susceptibility loci, such as *RET*, could contribute to HSCR susceptibility. Overall, *GDNF* contributes to only a minority of HSCR cases and its function can be compensated for by other neurotrophic factors through other pathways (Angrist *et al* 1996, Salomon *et al* 1996).

1.4.4.2. *Endothelin-converting enzyme 1* (*ECE1*)

Mice with *Ece1* deficiencies lack enteric neurons and epidermal/choriodal melanocytes, reproducing the phenotype of *endothelin-3* and *Ednra* knockout mice (Yanagisawa *et al* 1998). The *ECE1* gene locus was mapped to chromosome 1p36.1 by isotopic *in situ* hybridisation, and comprises 19 exons in a genomic region of 68 kb (Valdenaire *et al* 1995, Matsuoka *et al* 1996, Albertin *et al* 1996). The enzyme is a zinc-chelating metalloprotease containing the typical HEXXH (HELTH) motif (Schmidt *et al* 1994). The complete cDNA of human *ECE* was isolated by RT-PCR and cDNA screening. Human *ECE* cDNA was also obtained by screening an ACHN human renal adenocarcinoma library, referred to as AECE, encoding a 770-codon open reading frame (Yorimitsu *et al* 1995). This sequence differs at the amino end from the sequence isolated by Schmidt *et al* (1994) with a similarity of 96% between the amino acids of rat ECE and human AECE. Endothelin 1, -2 and –3, encoded by the genes *EDN1, EDN2* and *EDN3,* respectively, are catalyzed by ECE1 enzyme from their inactive form to their biologically active peptides, ET1, ET2 and ET3. The involvement of the *ECE1* gene in Hirschsprung's disease was indicated by the presence of a C to T transition, resulting in the substitution of cysteine for arginine at position 742 (Hofstra *et al* 1999) in a single individual. This mutation is in close

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proximity to the active site of *ECE1* (Valdenaire *et al* 1995) and appears to be responsible or at least contributed to the patient's phenotype (Hofstra *et al* 1999).

1.4.4.3. *Sex dependent Y factor-like homeobox 10 gene* (*SOX 10*)

Research on mice, performed at the Jackson Laboratory (Lane and Liu 1984) demonstrated that *Dom*/+ heterozygous mice (*Dom* – dominant megacolon) had regional deficiencies of neural crest – derived enteric ganglia in the distal colon and that *Dom/Dom* homozygous mice were embryonic lethal. The *Dom* gene in mice is located in the mid-terminal region of mouse chromosome 15 and synteny was shown between the *Dom* locus and human chromosome 22q12-q13 (Pingualt *et al* 1997). By positional cloning, *SOX 10* was considered a candidate gene for the *DOM* locus (Southard-Smith *et al* 1998). Futher studies demonstrated that *SOX 10* is essential for the proper development of the peripheral nervous system in mice (Herbarth *et al* 1998). The *SOX gene* family consists of genes related to SRY, the testis-determining gene, with more than 60% sequence identity with SRY HMG (high mobility group) box. The *Sox 10* gene of rats was cloned and expression was demonstrated mainly in the glial cells of the nervous system (Kuhlbrodt *et al* 1998). The gene comprises 5 exons and spans a region of 14 kb of genomic DNA. A role for *SOX 10* in conferring cell specifity to the function of other transcription factors in developing and mature glia was proposed by Kuhlbrodt *et al* (1998). *SOX 10* was therefore considered a likely candidate gene for HSCR, especially since it is associated with features of Waardenburg syndrome (Southard-Smith *et al* 1998, Herbarth *et al* 1998). Recently, mutations of the *SOX 10* gene have been identified in four families with Shah-

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Waardenburg syndrome, which resulted in haploinsufficiency of the *SOX 10* product (Pingualt *et al* 1998, Kuhlbrodt *et al* 1998).

1.5. THE ASSOCIATION OF DOWN'S SYNDROME WITH HSCR

HSCR has been found to be associated with Down's syndrome (mongolism) (Bodian *et al* 1951), which occurs in 2% to 15% of all HSCR cases (Puri 1993, Polly and Coran 1993). These patients present with symptoms including constipation, neonatal intestinal obstruction, abdominal distension, enterocolitis and meconium plug syndrome (Moore and Johnson 1998). The mean maternal and paternal age of 33.5 and 37.4 years, respectively are significantly higher in patients with both HSCR and Down's syndrome compared with patients with only HSCR and control patients (mean maternal age = 26.6 years, mean paternal age = 29.2 years) (Garver *et al* 1985). The consistent association of HSCR with Down's syndrome suggests a role of chromosome 21 in the pathogenesis of the disease (Passarge 1967). Emanuel *et al* (1965) postulated that the gene involved in HSCR could be located on chromosome 21 or that abnormalities associated with Down's syndrome could, in the embryonic period, provide an alternative mechanism which results in HSCR. Studies performed using identity-by-descent (IBD) analysis and linkage disequilibrium in a Mennonite kindred revealed preliminary evidence of a genetic modifier for HSCR on chromosome 21q22 (Puffenberger *et al* 1994b). In another instance a case of leukocyte adhesion deficiency (LAD) resulting from a deletion on chromosome 21 was associated with a HSCR-like presentation (Rivera-Matos *et al* 1995). LAD has been mapped to the region encoding CD18 on chromosome 21 and localised to

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21q22.1-qter and q22.3 (Petersen *et al* 1991). This coincides with the same area that was suggested by Puffenberger *et al* (1994b) for a modifier gene that could be involved in HSCR.

1.6. OBJECTIVES

The aim of this study was to screen for *RET*-, *EDNRB*- and *EDN3* gene mutations in HSCR and Down's syndrome patients (with HSCR). The identification of novel mutations in these genes may broaden our understanding of their biological role in differentiation of cells of neural crest origin. Patients with a family history of HSCR could thereafter be selected for mutation screening. This would lead to improved counseling of families and individuals at risk of developing HSCR.

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CHAPTER 2

SUBJECTS AND METHODS

Detailed experimental procedures

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2.1. SUBJECTS

The study cohort included subjects from different ethnic groups in South Africa: coloureds, blacks and whites. In this study "white" refers to an individual of European descent, mainly Dutch, French, German and British stock; "coloured" refers to an individual of mixed ancestry, including San, Khoi, African Negro, Madagascar, Javanese and European origin; "black" refers to South Africans of central African descent. Colonic tissue samples were obtained from 53 clinically diagnosed HSCR patients, including 41 frozen tissue samples and 12 formalin-fixed paraffin embedded samples (Table 2.1). HSCR occurred sporadically in these patients. Three of these patients have total colonic aganglionosis (6%), four have colonic aganglionosis (7%) and the remaining forty-six patients (87%) have short segment disease. Fourteen of the patients with short segment disease were also clinically diagnosed with Down's syndrome. Both the ganglionic and aganglionic tissue samples for four of these patients were also included. The control samples comprised 6 anorectal malformations and 24 normal colonic tissue samples, including 17 formalin-fixed paraffin embedded samples and 13 frozen tissue samples, and 56 randomly chosen blood samples (Table 2.2).

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Table 2.1. Classification of HSCR patients

ID	Sex	Race	Extent of aganglionosis	Additional Clinical Features	SECTION OBTAINED
H1	M	C	R/S		Rectosigmoid colon
H4	M	W	R/S		Rectosigmoid colon
H5	M	W	C/A		Colon
H7	M	C	R/S		Rectosigmoid colon
H9	M	B	C/A		Colon
H10	M	B	R/S		Rectosigmoid colon
H11	M	C	TCA		Colon
H13	M	C	R/S		Upper Rectum
H14	M	C	R/S		Rectosigmoid colon
H15	M	C	R/S		Rectosigmoid colon
H17	F	C	R/S		Rectosigmoid colon
H18	M	C	R/S		Rectosigmoid colon
H19	M	B	R/S		Rectosigmoid colon
H20	U	U	R/S		Rectosigmoid colon
H21	M	C	R/S	15cm aganglionosis	Rectal Biopsy
H23	M	W	R/S	Down Syndrome	Rectosigmoid colon
H25	M	B	R/S		Rectosigmoid colon
H26	F	C	R/S		Rectosigmoid colon
H27	M	B	R/S		Rectosigmoid colon
H28	M	W	TCA		Colon
H29	M	C	R/S		Rectosigmoid colon
H33	M	B	C/A		Colon
H34	M	W	R/S		Rectal Biopsy
H37	M	C	R/S		Rectosigmoid colon
H38	M	C	R/S		Rectal Biopsy
H39	M	C	R/S		Rectosigmoid colon
H40	M	C	R/S		Rectosigmoid colon
H41	F	C	R/S		Rectosigmoid colon
H42	F	C	C/A		Colon
H44	F	C	R/S	Down Syndrome	Rectosigmoid colon
H63	M	C	R/S		Rectosigmoid colon
H64	M	W	R/S		Rectosigmoid colon
H65	M	B	TCA		Colon
H66	F	C	R/S		Rectosigmoid colon
H67	M	B	R/S		Rectosigmoid colon
H68	M	C	R/S		Rectosigmoid colon
H69	M	W	R/S		Rectosigmoid colon
H70	F	C	R/S		Rectosigmoid colon
H71	M	B	R/S		Rectosigmoid colon

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H72	F	W	R/S		Rectosigmoid colon
H73	M	W	R/S		Rectosigmoid colon
HP45	F	W	R/S	Down Syndrome	Distal Bowel
HP46	M	W	R/S	Down Syndrome	Colon
HP47	F	C	R/S	Down Syndrome	Colon
HP48*	M	C	R/S	Down Syndrome	Rectosigmoid colon
HP49*	M	C	R/S	Down Syndrome	Rectosigmoid colon
HP50	M	C	R/S	Down Syndrome	Colon
HP51	F	W	R/S	Down Syndrome	Rectum
HP52^	F	C	R/S	Down Syndrome	Rectosigmoid colon
HP53^	F	C	R/S	Down Syndrome	Rectosigmoid colon
HP54^	F	C	R/S	Down Syndrome	Rectosigmoid colon
HP56^	F	C	R/S	Down Syndrome	Sigmoid colon
HP55#	F	W	R/S	Down Syndrome	Rectal biopsy
HP59#	F	W	R/S	Down Syndrome	Colon
HP57$	F	C	R/S	Down Syndrome	Rectum
HP58$	F	C	R/S	Down Syndrome	Rectum
HP60	F	C	R/S	Down Syndrome	Rectal Biopsy
HP61	F	C	R/S	Down Syndrome	Colostomy end
HP62	M	W	R/S	Down Syndrome	Rectosigmoid colon

Abbreviations: R/S – rectosigmoidal aganglionosis; C/A – colonic aganglionosis; TCA – total colonic aganglionosis; M – male; F – female; B – black; W – white; C – coloured; U – unknown; *^#$ Different sections obtained from these four patients; H – frozen tissue samples; HP – formalin-fixed paraffin embedded samples.

Table 2.2. Classification of control patients

ID	Sex	Race	Additional Clinical Features	SECTION OBTAINED
KS2	F	C		Colon
KS3	M	C		Colon
KS6	M	C		Colon
KS8	U	U		Colon
KS12	F	C	ARM	Colon
KS16	M	C	ARM	Colon
KS22	F	C	ARM	Colon
KS24	M	B	ARM	Colon
KS30	M	B	MECONIUM PLUG	Colon
KS31	F	W	ARM	Colon
KS32	U	U		Colon
KS35	M	W		Colon
KS43	M	W		Colon
KP1	F	C	Adenocarcinoma	Colon

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KP2	F	C	Adenocarcinoma	Colon
KP3	F	C	Adenocarcinoma	Colon
KP4	M	C	Adenocarcinoma	Colon
KP5	F	C	Adenocarcinoma	Colon
KP6	M	C	Adenocarcinoma	Colon
KP7	F	W	Adenocarcinoma	Colon
KP8	F	W	Adenocarcinoma	Colon
KP9	M	B	Adenocarcinoma	Colon
KP10	M	W	Adenocarcinoma	Colon
KP11	F	C	Adenocarcinoma	Colon
KP12	F	C	Adenocarcinoma	Colon
KP13	F	C	Adenocarcinoma	Colon
KP14	F	C	Adenocarcinoma	Colon
KP15	M	W	Adenocarcinoma	Colon
KP16	F	W	Adenocarcinoma	Colon
KP17	M	C	Adenocarcinoma	Colon
KB1	F	W		Blood
KB2	F	W		Blood
KB3	F	W		Blood
KB4	F	W		Blood
KB5	M	C		Blood
KB6	M	W		Blood
KB7	F	W		Blood
KB8	F	C		Blood
KB9	F	W		Blood
KB10	F	C		Blood
KB11	F	W		Blood
KB12	F	W		Blood
KB13	F	W		Blood
KB14	F	W		Blood
KB15	F	W		Blood
KB16	F	W		Blood
KB17	F	W		Blood
KB18	F	W		Blood
KB19	F	W		Blood
KB20	M	W		Blood
KB21	F	B		Blood
KB22	F	B		Blood
KB23	F	B		Blood
KB24	F	B		Blood
KB25	F	B		Blood
KB26	F	B		Blood
KB27	F	B		Blood
KB28	F	B		Blood
KB29	F	B		Blood

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KB30	F	B		Blood
KB31	F	B		Blood
KB32	F	B		Blood
KB33	F	B		Blood
KB34	F	B		Blood
KB35	F	B		Blood
KB36	F	B		Blood
KB37	F	B		Blood
KB38	F	B		Blood
KB39	F	B		Blood
KB40	F	B		Blood
KB41	F	B		Blood
KB42	F	B		Blood
KB43	F	B		Blood
KB44	F	B		Blood
KB45	F	B		Blood
KB46	F	B		Blood
KB47	F	B		Blood
KB48	M	C		Blood
KB49	M	C		Blood
KB50	M	C		Blood
KB51	M	C		Blood
KB52	F	C		Blood
KB53	F	C		Blood
KB54	F	C		Blood
KB55	F	C		Blood
KB56	F	C		Blood

Abbreviations: M-male; F-female; W-white; C-coloured; B-black; ARM – anorectal malformation; KS – frozen tissue samples; KP – formalin-fixed paraffin embedded samples; KB – blood samples

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2.2. METHODS

2.2.1. DNA extraction procedures

2.2.1.1. DNA extraction from formalin-fixed paraffin-embedded tissue

DNA was extracted from 20 solid colonic tissue samples fixed in formalin and embedded in paraffin blocks by a technique adapted from Min *et al* (1991). Briefly, 10X5 µ sections were cut from the paraffin blocks and placed into a 1.5 ml microcentrifuge tube. Xylene (800 µl) was added to the paraffin sections in the tubes and was vortexed until the paraffin was dissolved. After the solution was left for 10 min at room temperature, 400 µl of EtOH (99%-) was added to the solution, vortexed and centrifuged for 5 min at 14 000 rpm. The supernatant was removed and the pellet re-suspended in 800 µl EtOH (99%-). The solution was again vortexed and centrifuged at 14 000 rpm for 5 min. Afterwards, the supernatant was removed and the pellet air-dried. The dry pellet was resuspended in 800 µl of extraction buffer and incubated overnight at 37°C. The extraction buffer was adapted from that published by Albert and Fenyo (1990) as follows: 10 mM Tris (pH 8.3), 1mM EDTA, 0.5% Triton X–100, 0.001% SDS (w/v), 500 µg/ml Proteinase K. The enzyme was inactivated at 94°C for 15 min and the tube sample quenched on ice. The DNA was stored at 4°C.

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2.2.1.2. DNA extraction from solid tissue – method 1

DNA was extracted from 11 frozen colonic solid samples by a modification of the procedure by Blin and Stafford (1976). Frozen solid tissue samples were thawed at room temperature. 10-20 mg of tissue was mixed with liquid nitrogen in a porcelain dish and crushed to a powder. Ice cold, 1 ml phosphate buffered saline (PBS) was added to the crushed powder and the suspension was placed in a 50 ml polypropylene Falcon tube and left on ice. The dish was washed with 1 ml of PBS and added to the solution in the Falcon tube. Recovery of the cells was performed by centrifugation at 1500 rpm for 10 min at 4°C. The supernatant was removed and the pellet resuspended in 10Xvolume of ice-cold PBS, followed by centrifugation at 1500 rpm for 10 min. Following removal of the supernatant, the cells were resuspended in 1 ml 1xTE (pH 8.0). Extraction buffer (10 ml)(10 mM Tris (pH 8.0), 0.1 M EDTA (pH 8.0) and 20 µg/ml pancreatic RNAse, 0.5% SDS) was added to the cell mix and the solution incubated for 1 hour at 37°C. After incubation, 150 µl of proteinase K (100 µg/ml) was added and the solution was incubated overnight at 55°C.

Following overnight incubation, the solution was cooled to room temperature and an equal volume of ice-cold phenol-chloroform was added to the solution. The two phases were gently mixed for 10 min. After centrifugation, the phases were separated by centrifugation at 1500 rpm for 10 min and the viscous aqueous phase was transferred to a clean Falcon tube. Two additional phenol-chloroform steps were performed. After the third extraction with phenol-chloroform, the aqueous phase was transferred to a clean Falcon tube and 1/10 of the volume of a 5 M $NaClO_4$ and 2 times the volume of ice-cold EtOH (99%-) was added and the solution centrifuged for

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30 min at 40 000 rpm. Following a 70% EtOH wash, the supernatant was removed and the DNA left to air dry. The DNA was dissolved in 500 µl of sterile distilled H_2O (ddH_2O) and left overnight at room temperature to dissolve. This DNA was transferred to a 1.5 ml microcentrifuge tube and stored at 4°C.

2.2.1.3. DNA extraction from solid tissue – method 2

DNA was extracted from 44 solid colonic tissue samples (frozen tissue) with a commercially available extraction kit (GenomicPrep™ Cells and Tissue DNA Isolation Kit - Pharmacia Biotech). Briefly, a 1.5 ml tube containing 600 µl of **cell lysis solution** was kept on ice until the solution turned cloudy. The frozen tissue (10-20 mg) was added to the solution, removed from the ice and homogenised by using a microfuge tube pestle. Thereafter, 6 µl of proteinase K (10 mg/ml stock) was added to the solution and incubated overnight at 55°C.

Following overnight incubation, 3 µl of **Rnase A solution** was added to the solution and mixed by inverting the tube 25 times, followed by incubation at 37°C for 60 min. After incubation, the solution was cooled to room temperature and 200 µl of **protein precipitation solution** was added to the Rnase treated solution. The solution was vortexed vigorously for 20 min followed by centrifugation at 13 000 rpm for 3 min. The supernatant was transferred to a fresh 1.5 ml tube containing 600 µl of 100% isopropanol and gently mixed until the DNA threads were visible. The solution was vortexed at 13 000 rpm for 1 min, the supernatant removed and the pellet was washed with 70% EtOH followed by centrifugation at 13 000 rpm for 1 min. The supernatant was removed and the pellet left on the bench to air-dry. **DNA hydration**

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solution (100 µl) was added to the dry pellet and left overnight at room temperature to rehydrate. The DNA was stored at 4°C. All the components shown in boldface type were provided as part of the kit.

2.2.1.4. DNA extraction from whole blood

DNA was extracted from 25 whole blood samples by a modification of the technique of Miller *et al* (1988). Cold lysis buffer (40 ml) (0.155 M NH_4Cl_2, 0.01 M $KHCO_3$, 0.0001 M EDTA – pH 7.4) was added to each whole blood sample in a 50 ml polypropylene Falcon tube. The solution was kept on ice until the red blood cells had undergone lysis and the cell suspension was then centrifuged at 1500 rpm for 10 min. After the supernatant was removed, the pellet was washed twice with 5 ml cold PBS. The intact pellet was resuspended in 3 ml nucleic lysis buffer (0.01M Tris-HCl, 0.4 M NaCl, 0.002 M EDTA - pH 8.2), 0.3 ml 10% SDS and 0.5 ml proteinase K, and incubated overnight in a water bath at 55°C.

Following overnight incubation, 1 ml of saturated 6 M NaCl was added and the solution shaken vigorously for 1 min, followed by centrifugation at 3000 rpm for 15 min. The supernatant containing the DNA was transferred to a clean Falcon tube. Three times the volume ice-cold EtOH (99%-) was added and the solution was left at room temperature for 30 min. The precipitated DNA was transferred to a fresh 1.5 ml tube and was washed twice with 1 ml of 70% ice-cold EtOH to remove any excess salt. After centrifugation at 3 000 rpm for 15 min, the excess EtOH was carefully

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removed and the DNA was left to air dry at room temperature. The DNA pellet was dissolved in 500 µl ddH_2O overnight at room temperature and the DNA stored at 4°C.

2.2.1.5. DNA purification and concentration of paraffin-embedded DNA samples

Ethanol precipitation was performed on DNA from paraffin-embedded samples that did not yield a PCR product after a second round of PCR amplification. A 400 µl volume of DNA was mixed with 1/10 the volume of sodium acetate (4 M). Ice-cold 2 times the volume EtOH (99%-) was added and the solution left for 20 min at –80°C. The solution was centrifuged for 1 hour at 4°C (13 000 rpm). Afterwards, the supernatant was removed and the pellet washed with 70% EtOH. The suspension was centrifuged for 5 min at 30 000 rpm. The supernatant was discarded, the DNA pellet left to air dry and the DNA pellet subsequently dissolved in 100 µl of ddH_2O (SABAX).

2.2.2. Polymerase Chain Reaction (PCR)

2.2.2.1. Oligonucleotide Primers

The PCR primers used in this study are listed in Tables 2.3., 2.4. and 2.5.

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Table 2.3. Oligonucleotide primers used for DNA amplification of the *RET proto-oncogene* (primers previously described by Ceccherini *et al* 1994)

EXON	PRIMERS	SEQUENCE 5' 3'
1	R1A(F)	GGG CGG CCA GAC TGA GCG C
	R1B(R)	CTT CGC CCT GGC CCT GCG G
2	R2A(F)	AGT GGC ATT GGG CCT CTA C
	R2A(R)	TGC GGA CAC TGA GCT TCT C
	R2B(F)	GAA GCC ATA TTC TCA CCA TC
	R2B(R)	TCT CCC AGG AGC TAT GGT CC
3	R3A(F)	GCT CCT GCC TCC TCC CAT TCC
	R3B(R)	CAG AGC AAG ACC AGC AGT AG
4	R4A(F)	CGA GGA AAG CGG CTG GCC CG
	R4B(R)	ACC GAG AAA CGA ACT GTG GCC G
5	R5A(F)	CCT AAG GTC TCT GGT TTT GG
	R5B(R)	AAG AGC GAG CAC CTC ATT TC
6	R6A(F)	CAT GAG GAA GCA GCC AGA GC
	R6B(R)	AGT GTC ACC TGC CTC CCT GTG
7	R7A(F)	TCT CTA CCC TCA GGC CAT
	R7B(R)	ACC CTC CCT CCC TGG AGC
8	R8A(F)	GGC CCA GGC CAG CCC CCT GT
	R8B(R)	GCC ATC GCC CCT GCA GGC CT
9	R9A(F)	GGA GGT GGT GGG GGC GTG TG
	R9B(R)	GCT GAA GTG CCT GTG GGA TC
10	R10A(F)	GGG GCA GCA TTG TTG GGG GA
	R10B(R)	GTT GGG ACC TCA GAT GTG C
11	R11A(F)	TGA GGC AGA GCA TAC GCA GC
	R11B(R)	GGA GTC CAG CGA GGG CCG GC
12	R12A(F)	GCC TTC TTC CTC CCC TGT CAT C
	R12B(R)	GAG ACT CCC CCA GGG GCA CTG TG
13	R13A(F)	AAC TTG GGC AAG GCG ATG CAG
	R13B(R)	AGA ACA GGG CTG TAT GGA GC
14	R14A(F)	CTG GAA GAC CCA AGC TGC CT
	R14B(R)	GCT GGC TGG GTG CAG AGC C
15	R15A(F)	GAC CGC TGC TGC CTG GCC ATG
	R15B(R)	GCT TC CAA GGA CTG CCT GC
16	R16A(F)	AGG GAT AGG GCC TGG GCT TC
	R16B(R)	TAA CCT CCA CCC CAA GAG AG
17	R17A(F)	GGA GGG CTC TGT GAG GGC AG
	R17B(R)	TCC CCT CCC TTC CCA AGT G
18	R18A(F)	GGC TGT CCT TCT GAG ACC TG

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	R18B(R)	TAC TGC CCT GGG GTG AGG C
19	R19A(F)	TAG TTG TGG CAC ATG GCT TG
	R19B(R)	GAG AGG AAG GAT AGT GCA G
20	R20A(F)	GGT TTG AAC ATC AAA GGG AG
	R20B(R)	CCA ATG TGA CGT TCA CAA AG
21	R21A(F)	GAG CCT GTG TGA AAG GCC C
	R21B(R)	CTT GGC CTC ACA AAA TGC CAC

Abbreviations: F – forward primer, R – reverse primer

Table 2.4. Oligonucleotide primers used for DNA amplification of the *endothelin-B receptor* (*EDNRB*) *gene* (primers previously described by Tanaka *et al* 1998)

EXON	PRIMERS	SEQUENCE 5' 3'
1	EB1A(F)	TTG TCT CTA GGC TCT GAA AC
	EB1B(R)	TTA GTG GGT GGC GTC ATT AT
	EB1C(F)	TCC GCT TTT GCA AAC CGC AG
	EB1D(R)	GGA CAC AAC CGT GTT GAT G
	EB1E(F)	ATC GAG ATC AAG GAG ACT TTC
	EB1F(R)	CCC TTT ACC TTG TAG ACA TTG
2	EB2A(F)	TCA ATG CAG CTG CTG GCA G
	EB2B(R)	AAG CTT CTA CCT GTC AAT ACT C
3	EB3A(F)	TAT CTT CAG ATA TCG AGC TG
	EB3B(R)	GAA ATT TAC CTG CAT GAA AGC
4	EB4A(F)	ATC CCT ATA GTT TTA CAA GAC AGC
	EB4B(R)	ATT TTC TTA CCT GCT TTA GGT G
5	EB5A(F)	TTT ATT TCA GAG ACG GGA AG
	EB5B(R)	CCT TTC TTA CCT CAA AAG TTC
6	EB6A(F)	TTT GTT GCA GCT TTC TGT TG
	EB6B(R)	AGT CTC TTA CCT TAA AGC AG
7	EB7A(F)	TTG TAC AGT CAT GCT TAT GC
	EB7B(R)	TGT TTT AAT GAC TTC GGT CC

Abbreviations: F – forward primer, R – reverse primer

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Table 2.5. Oligonucleotide primers used for DNA amplification of the *endothelin-3 gene* (*EDN3*) (primers previously described by Bidaud *et al* 1997)

EXON	PRIMER	SEQUENCE 5' 3'
1	E3-1A(F)	CGC TCT GAA AGT TTA TGA CCG
	E3-1B(R)	CCG CCC TGG GTC CTT TTG TG
2	E3-2A(F)	CCC TCC TCA GGT GTT TGG
	E3-2B(R)	TCG GCC GCC TGC TCC TGC
	E3-2C(F)	TGG CGA GGA GAC TGT GGC
	E3-2D(R)	GAA TGA GCA GAT GTG GGC G
3	E3-3A(F)	GCT CAC CTA ACA TTA CC
	E3-3B(R)	CCG AGG GTT GAT GCA TAT
4	E3-4A(F)	GGT GGG GAA GAG GAA GTC
	E3-4B(R)	CCC GAA GGA ATG ACA CTG C
5	E3-5A(F)	TCA CAG AAC TAC AGA GCT AC
	E3-5B(R)	ACT GTG TGT GAG CAA TGA C

Abbreviations: F – forward primer, R – reverse primer

2.2.2.2. DNA amplification

2.2.2.2.1. General PCR

The DNA samples were amplified on a Hybaid Omnigene Thermal Cycler (Hybaid, Teddington, Middlesex, UK). PCR amplification of the various individual exons were performed in 25 µl reactions, overlaid with light mineral oil (Sigma). Each reaction consisted of 0.2 pmol/µl of each primer, 0.5 U Taq polimerase (Boehringer Mannheim) enzyme, 0.2 mM of each dNTP (dGTP, dCTP, dATP, dTTP) 5 µl buffer with $MgCl_2$ (Boehringer Mannheim) and 10 µl Cresol Red (0.0382 g Cresol Red and 60g sucrose up to a final volume of 100 ml). Modifications for exons 1 and 4 of *RET*

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proto-oncogene included the addition of 10% DMSO and 1% Formamide, respectively, to the PCR reaction.

2.2.2.2.2. *RET proto-oncogene*

The optimal PCR conditions for exons of the *RET proto-oncogene* were as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min and annealing as specified for each exon in Table 2.6. for 2 min. This was followed by an extension step at 72°C for 10 min and a final step at 30°C for 1 min.

Table 2.6. Annealing temperatures for the *RET proto-oncogene*

EXON	ANNEALING TEMPERATURE (°C)
2A	66
3	67
4	64
5	66
6	68
8	70
9	67
12	69
13	67
14	59
15	71
16	69
17	63

The PCR conditions for the remaining exons were as follows: an initial denaturation step for 2 min at 95°C, followed by 10 cycles of denaturation at 95°C for 30 seconds,

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annealing as specified for each exon in Table 2.7. (column 1) for 45 seconds, and an extension at 72°C for 30 seconds. This was followed by 30 cycles of the same conditions but with an annealing temperature as specified in Table 2.7. (column 2), followed by an extension step at 72°C for 5 min and a final step of 1 min at 30°C. The exons with only one annealing temperature (column 3) specified, were amplified for 35 cycles.

Table 2.7. Annealing temperatures for the *RET proto-oncogene*

EXON	ANNEALING TEMPERATURE (°C)		
	column 1	column 2	column 3
1			60
2B	60	58	
7	63	60	
10	65	63	
11	66	63	
18			60
19			60
20	59	56	
21	67	64	

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2.2.2.2.3. *Endothelin-B receptor* gene (*EDNRB*)

The optimal PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min and annealing at 60°C for 2 min. This was followed by an extension step at 72°C for 10 min and a final step at 30°C for 1 min. All seven exons were amplified by the same procedure.

2.2.2.2.4. *Endothelin-3 gene* (*EDN3*)

The optimal PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min and annealing as listed in Table 2.8. This was followed by an extension step at 72°C for 10 min and a final step at 30°C for 1 min.

Table 2.8. Annealing temperatures for the *endothelin-3 gene* (*EDN3*)

EXON	ANNEALING TEMPERATURE (°C)
1	62
2A	67
2B	67
3	60
4	60
5	66

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2.2.3. Agarose Gel Electrophoresis

Successful amplification of PCR products was tested on a horizontal agarose gel (2%). The PCR product (5 µl) was mixed with an equal volume of ficoll-orange G loading buffer (consisting of 0.1 g Orange G, 20 g Ficoll and 0.29 g EDTA up to a final volume of 100 ml (pH 7.0)) and loaded on the gel. The products were resolved at 150 V for 30 min in 1xTBE buffer solution and 0.01% (v/v) of ethidium bromide for staining purposes. The products were visualised by ultraviolet light transillumination assisted by a UVP Image Store 5000 Ultra-Violet transilluminator system and photographed with the Sony Video Graphic Printer (UP 860 LE) system.

2.2.4. Heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis

HEX-SSCP analysis of the PCR fragments of the *RET proto-oncogene*, *endothelin-B receptor* and *endothelin-3 gene*, was performed on a 30 cm vertical gel apparatus. Different gel compositions were used to increase the sensitivity of mutation detection, including a 12% polyacrylamide (PAA) (39.6 g acrylamide and 0.4 g bisacrylamide up to a final volume of 100 ml) gel supplemented with 7.5% urea (consisting of 4.5g Urea, 18 ml 5XTBE, 24 ml dH_2O, 18 ml PAA (1%C of a 40% stock), 600 µl APS (10%) and 60 µl TEMED), and a 10% PAA gel supplemented with 5% glycerol

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(consisting of 3 ml glycerol, 6 ml 5XTBE, 36 ml dH_2O, 15 ml PAA, 800 µl APS (10%) and 80 µl TEMED) (Kotze *et al* 1995).

A 0.75 mm gel was cast and once polymerised, prepared for loading of the PCR products. Gels were placed in a Hoefer electrophoresis tank filled with fresh 0.5 X TBE buffer. The upper buffer chamber was filled with 1.5 X TBE buffer for an urea gel and 0.5 X TBE for a glycerol gel.

The PCR products were heat denatured for 5 min at 95°C and then kept on ice. Approximately 7 µl of the denatured PCR product was loaded on the gel. The PCR products of exon 18 (failed to amplify in the presence of Cresol Red) were mixed with an equal volume of loading buffer (consisting of 22.7 ml Formamide (De-ionised), 0.168 g EDTA (disodiumsalt), 0.0125 g Xylene Cyanol and 0.0125 g Bromophenol blue up to a final volume of 25 ml) before denaturing at 95°C. Approximately 18 µl of this product was loaded on the gel. Electrophoresis was as follows: for 12% urea gels electrophoresis was performed at 300 V at 4°C (cold room) for 20 hours and for 10% glycerol gels at 250 V at room temperature for 18 hours.

SSCP analysis was also performed on a 9 cm vertical gel (Hoefer system) and similar procedures were used for setting up and preparing the PCR products as for HEX-SSCP analysis. A gel consisting of 20% PAA (consisting of 5 ml PAA (1%C of a 40% stock), 1 ml 5XTBE, 4 ml dH_2O, 80 µl APS (10%) and 25 µl TEMED) was used for this system and electrophoresis of the PCR products was performed at 150 V for 20 hours at 4°C.

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Following electrophoresis, the gel was dismantled and stained in 0.01% (v/v) ethidium bromide for 10 min and destained in ddH_2O for 3 min. The DNA fragments were visualised by ultraviolet light transillumination assisted by the UVP Image Store 5000 Ultra-Violet transilluminator system and photographed with the Sony Video Graphic Printer (UP 860 LE) system.

2.2.5. AUTOMATED SEQUENCING

Automated DNA sequencing was performed on PCR products demonstrating HEX-SSCP mobility shifts. The PCR products were pre-treated enzymatically by adding 0.5 µl exonuclease I (1.2 U/µl) (removes residual single strand primers and extraneous DNA products) and 0.5 µl Shrimp Alkaline Phosphatase (6 U/µl) (removes remaining dNTP's) to 5 ul of PCR product and incubating the reaction for 15 min at 37°C, followed by incubation at 80°C for 15 min to inactivate the enzymes. The enzymatically treated product was added to a reaction mix which consisted of 2 µl termination ready reaction mix, 3 µl 5Xdilution buffer, 2.5 pmol primer and 7.5 µl ddH_2O (Applied Biosystems™ automated sequencing kit). This was followed by cycle sequencing on a 2 400 Perkin Elmer PCR cycler. The cycle program was as follows: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 20 sec, annealing at 59°C for 20 sec and an extension at 60°C for 30 sec. This was followed by an extension step at 72°C for 5 min and a final step at 25°C for 10 min.

After cycle sequencing, the products were cleaned by spun-column chromatography using centri-sep columns (Applied Biosystems). After spun-column chromatography,

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20 µl $MgCl_2$ (20 µg/ml) and 55 µl 95% EtOH were added to the products. The solution was left on the bench for 10 – 15 min, followed by centrifugation for 15 – 20 min at 13 000 rpm. The supernatant was removed and the pellet left to air-dry. Template suppressant reagent was added to the dry pellet and the products were loaded on an ABI 310 automated sequencer. Following electrophoresis, analysis of electropherograms were performed by using sequencing analysis software supplied by Applied Biosystems.

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CHAPTER 3

RESULTS

Presented in the form of a full-length manuscript in preparation for future publication in a scientific journal.

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Mutation analysis of the *RET proto-oncogene* (*RET*), *Endothelin-B receptor* (*EDNRB*) gene and *Endothelin-3* (*EDN3*) gene loci in sporadic Hirschsprung's disease in the South African population.

Abstract

Hirschsprung's disease (HSCR) is a common cause of intestinal obstruction in neonates with an incidence of 1 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 53 unrelated subjects with sporadic HSCR were screened for mutations in the *RET proto-oncogene*, a major susceptibility gene for HSCR, as well as the *endothelin-B receptor* (*EDNRB)* and the *endothelin-3 genes* (*EDN3)*. Five novel and one previously described mutation were identified in the *RET proto-oncogene*. Mobility shift aberrations identified in the *EDNRB* gene represented polymorphisms. No sequence variants were identified in the *EDN3* gene. Potential disease-related mutations were identified in the *RET proto-oncogene* in 19% of individuals and more males (24% representing 9 of 37 males) were affected than females (6% representing 1 of 16 females). This study demonstrated that all the potential disease-related mutations identified in South African patients with sporadic HSCR occur in the *RET* gene. A mutation in *RET* was identified in only 2 of 14 patients (14%) presenting with HSCR and Down's syndrome compared to 6 mutations identified in 8 of 39 patients (20.5%) with only HSCR.

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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 1 in 5000 (0.02%) live births and an overall risk to siblings of 4% (Passarge 1967, Angrist *et al* 1993, Lyonnet *et al* 1993). Aganglionosis can involve the rectosigmoid area (75%), transverse colon (17%) or total colon with a short segment of the terminal ileum (8%). Clinical features of HSCR vary from colonic obstruction to colonic dysfunction and the presence of one or more of the following symptoms: severe constipation, megacolon, secondary electrolyte disturbances and sepsis due to enterocolitis. 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 (Badner *et al* 1990, Puri 1997). A higher risk of disease occurrence has been reported in siblings of patients demonstrating an earlier age of onset.

The suspected basis of HSCR was believed to be a sex-modified multifactorial trait because males are more likely to be affected than females (Passarge 1967). 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 (Badner *et al* 1990). Chromosomal abnormalities

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such as Down's syndrome are often (2% - 15%) associated with HSCR (Puri 1993, Polly and Coran 1993). The relatively constant association of Down's syndrome with HSCR disease suggests the involvement of another gene on chromosome 21 in the pathogenesis of the disease (Passarge 1967). Studies performed more recently using identity-by-descent (IBD) and linkage disequilibrium analysis in a Mennonite kindred revealed preliminary evidence of a genetic modifier for HSCR on chromosome 21q22-q23 (Puffenberger *et al* 1994b).

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 (Lyonnet *et al* 1993, Luo *et al* 1993, Angrist *et al* 1995). *RET* involvement was substantiated by the identification of mutations associated with both familial and sporadic cases in 25% of all HSCR cases (Romeo *et al* 1994, Edery *et al* 1994a, Luo *et al* 1994, Attie *et al* 1995a, Angrist *et al* 1995, Seri *et al* 1997). The frequency of *RET* gene mutations is higher in familial cases (28.4%) than in the more common sporadic cases (17.6%). In familial cases, 35.6% of *RET* mutations are associated with long segment disease and 29.6% with short segment disease. *RET* mutations in sporadic HSCR occurs in 42.2% of patients with long segment disease and in 7.9% of patients with short segment disease (Puri 1997). In both familial and sporadic cases of HSCR, *RET* mutations are not restricted to a particular area of the gene.

Involvement of the *endothelin-B receptor gene* (*EDNRB)* and its ligand the *endothelin-3 gene* (*EDN3)* in HSCR was first recognised when targeted disruptions of

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these genes in mice resulted in a similar phenotype in both cases, that of aganglionic megacolon and spotted coat colour (Baynash *et al* 1994). Puffenberger *et al* (1994b) localised *EDNRB*, the second susceptibility gene for HSCR, to chromosome 13q22 by identity-by-descent (IBD) analysis followed by linkage disequilibrium analysis of an inbred Mennonite kindred. A missense mutation (W276C) identified in these studies demonstrated incomplete penetrance, as some unaffected individuals were homozygous for the same mutation, implicating the involvement of other susceptibility genes in HSCR pathogenesis. The *EDN3* gene was localised to chromosome 20q13.2-13.3 by *in situ* hybridisation analysis (Gopal Rao *et al* 1991, Arinami *et al* 1991). Recent studies indicated that heterozygous mutations resulted in isolated HSCR or central hypoventilation syndrome (CCHS) (Bolk *et al* 1996a).

In order to determine the molecular basis of HSCR in the South African population, we performed polymerase chain reaction (PCR) amplification and heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis of *RET*, *EDNRB* and *EDN3* loci in samples from 53 unrelated patients with sporadic HSCR.

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Materials and Methods

Subjects

Colonic tissue samples were obtained from 53 (12 paraffin embedded and 41 frozen tissue samples) 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 (27 blacks, 12 coloureds and 17 whites) were included as controls. Inclusion of HSCR patients were based on histopathological evaluation and confirmation of absence of ganglia in the biopsies. Fourteen of the 53 HSCR patients were also clinically diagnosed with Down's syndrome and different sections of the colon, with and without ganglion cells, were obtained for 4 of these patients. HSCR occurred sporadically in all of the patients. Total colonic aganglionosis was observed in seven 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 58 coloureds (21 HSCR patients – 54%; 8 HSCR/Down's syndrome patients – 57%; 29 controls – 34%), 39 blacks (9 HSCR patients – 23%; 30 controls – 35%;) and 39 whites (8 HSCR patients – 21%; 6 HSCR/Down's syndrome patients – 43%; 25 controls – 29%). The ethnic origin of 1 HSCR patient (1.9%) and 2 controls (2%) were unknown. In this study "white" refers to an individual of European descent, mainly Dutch, French, German and British stock; "coloured" refers to an individual of

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mixed ancestry, including San, Khoi, African Negro, Madagascar, Javanese and European origin; "black" refers to South Africans of central African descent.

Methods

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*, 7 exons of *EDNRB* and 5 exons of *EDN3* was performed using intronic primers (Ceccherini *et al* 1994, Bidaud *et al* 1997, Tanaka *et al* 1998). The PCR products were subjected to heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis (Kotze *et al* 1995). Briefly, seven µl of heat denatured PCR product in Cresol Red loading buffer was resolved on a 12% polyacrylamide (PAA) gel supplemented with 7.5% urea (consisting of 4.5 g urea, 18 ml 5XTBE, 24 ml dH_2O, 18 ml PAA (1%C of a 40% stock), 600 µl APS (10%) and 60 µl TEMED) at 4°C (350 V) for 18 hr in a Hoefer vertical gel-electrophoresis system. The DNA fragments were also resolved in a 10% PAA gel supplemented with 5% glycerol (consisting of 3 ml glycerol, 6 ml 5XTBE, 36 ml dH_2O, 15 ml PAA (1%C of a 40% stock), 800 µl APS (10%) and 80 µl TEMED) at room temperature (300 V). Following adequate electrophoresis time for separation of PCR fragments, the gel was dismantled and stained in 0.01% (v/v) ethidium bromide for 10 minutes and destained in double distilled H_2O for 3 minutes. The DNA fragments were visualised by ultraviolet light transillumination and photographed. Automated DNA sequencing was performed on PCR products demonstrating mobility or conformational variants in the PAA gels, using a custom-made kit from Applied Biosystems™. The products were loaded on an ABI 310 automated sequencer and following electrophoresis, analysis of the

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electropherograms was performed by using sequencing analysis software supplied by Applied Biosystems.

Statistical analysis

Allele frequencies were determined by allele counting. Testing for significance of heterogeneity in mutation frequencies among patient and control groups was 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 6 potential disease-related mutations in 10 HSCR patients (Table 3.1.). Nine polymorphisms, of which five were previously described (Ceccherini *et al* 1994, Edery *et al* 1994b, Romeo *et al* 1994) and four novel, were also detected (Table 3.2.). HEX-SSCP analysis showed no aberrant banding pattern in exons 1, 4, 5, 8, 9, 10, 12 and 16 of the *RET* gene, in any of the HSCR patients. Missense mutations and splice variants were identified in exons 3, 7, 13 and 17, and introns 10 and 19, respectively. These mutations were absent in the control individuals. Although IVS16-38delG was detected only in patients with HSCR and not in control individuals, this apparently rare variant are unlikely to contribute to the disease phenotype, based on the location in the *RET* gene.

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Table 3.1. Potential disease-causing mutations identified in the *RET proto-oncogene*

PATIENT NR	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
46**	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
39	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
44**	17	P973L#	CCA→CTA	Missense	F/C	R/S
4	19	IVS19-9C/T		Splice acceptor	M/W	R/S

#First reported by Yin *et al* 1994
**HSCR/Down's syndrome patients
Abbreviations: M – male, F – female, W – white, C – coloured, TCA – total colonic aganglionosis, R/S – rectosigmoidal aganglionosis; IVS - intervening sequences

Table 3.2. Polymorphisms identified in the *RET proto-oncogene*

EXON/ INTRON	CODON	NUCLEOTIDE CHANGE	ALLELE FREQUENCY					
			B (n=9)	BC (n=30)	W (n=14)	WC (n=25)	C (n=29)	CC (n=29)
2	A45	GCG→GCA	G:0.95 A:0.05	0.94 0.06	0.47 0.53	0.42 0.58	0.48 0.52	0.69 0.31
6	IVS6+56delG		G:1.0 delG:0.0	1.0 0.0	0.97 0.03	1.0 0.0	0.86 0.14	0.90 0.10
7	A432	GCG→GCA	G:1.0 A:0.0	1.0 0.0	1.0 0.0	0.94 0.06	0.83 0.17	0.87 0.13
13	L769	CTT→CTG	T:0.78 G:0.22	1.0 0.0	0.81 0.19	0.70 0.30	0.64 0.36	0.70 0.30
13	IVS13-29C/T		C:0.95 T:0.05	0.98 0.02	1.0 0.0	1.0 0.0	0.97 0.03	1.0 0.0
15	S904	TCC→TCG	C:0.50 G:0.50	0.75 0.25#	0.71 0.29	0.94 0.06*	0.59 0.41	0.58 0.42
16	IVS16-38delG		G:1.0 delG:0.0	1.0 0.0	1.0 0.0	1.0 0.0	0.96 0.04	1.0 0.0
18	R982	CGC→CGT	C:1.0 T:0.0	0.91 0.09	0.90 0.10	0.89 0.11	0.94 0.06	0.97 0.03
21	X1159	TAA→TGA	A:1.0 G:0.0	1.0 0.0	1.0 0.0	1.0 0.0	1.0 0.0	0.98 0.02

#black patients versus black control group: $\chi^2 = 4.06$; $p<0.05$
*white patients versus white control group: $\chi^2 = 7.34$; $p<0.01$
Abbreviations: B – black patients, BC – black control group, W – white patients, WC – white control group, C – coloured patients, CC – coloured control group

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All the patients were heterozygous for the respective mutations identified. The missense mutations (4/6) are scattered throughout the extracellular and intracellular domains of the *RET proto-oncogene*. 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. A mutation altering the splice site in intron 10 (IVS10-2A/G) was identified in a male with rectosigmoid aganglionosis. The mutations in exons 13 (D771N) and 17 (P973L) (Yin *et al* 1994) are located in the intracellular domain. The mutation in exon 13 was identified in two patients, both with total colonic aganglionosis. A rare variant was identified in intron 16 (IVS16-38delG) in the same individual with the missense mutation in exon 17 (P973L). Only one patient with rectosigmoid aganglionosis presented with the mutation identified in intron 19 (IVS19-9C/T).

Codon numbers were used for designation of the polymorphisms, because discrepancies were observed between the intronic sequences published by Ceccherini *et al* (1994) and Munnes *et al* (1999) (Blast #AJ243297). Statistically significant differences in allele frequencies of the polymorphism identified in exon 15 was observed between the patient and control groups from the black and white populations.

HEX-SSCP analysis of the *EDNRB* and *EDN3* genes demonstrated less variation. No sequence changes were observed in the *EDN3* gene, while all the variants detected in the *EDNRB* gene appears to be neutral polymorphisms (Table 3.3). No statistically significant differences in allele frequencies were observed between patient and control groups in the different ethnic groups studied.

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Table 3.3. Polymorphisms identified in the *EDNRB* gene

EXON/ INTRON	CODON	NUCLEOTIDE CHANGE	ALLELE FREQUENCY					
			B (n=9)	BC (n=30)	W (n=14)	WC (n=25)	C (n=29)	CC (n=29)
1	A60T	GCC→ACC	G:0.94 A:0.06	1.0 0.0	0.95 0.05	1.0 0.0	0.96 0.04	0.98 0.02
2	S184	TCC→TCT	C:1.0 T:0.0	0.97 0.03	0.96 0.04	1.0 0.0	0.94 0.06	1.0 0.0
2	I187	ATC→ATT	C:0.94 T:0.06	0.95 0.05	0.89 0.11	0.96 0.04	0.90 0.10	0.98 0.02
3	V234	GTC→GTT	C:1.0 T:0.0	0.93 0.07	0.98 0.02	0.97 0.03	1.0 0.0	0.82 0.18
4	L277	CTG→CTA	G:0.46 A:0.54	0.47 0.53	0.46 0.54	0.45 0.55	0.43 0.57	0.45 0.55
3	IVS4-6C/T		C:0.55 T:0.45	0.54 0.46	0.56 0.44	0.55 0.45	0.57 0.43	0.56 0.44
4	IVS4+3A/G		A:0.69 G:0.31	0.68 0.32	0.72 0.28	0.70 0.30	0.72 0.28	0.71 0.29

Abbreviations: 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*, *EDNRB* and *EDN3* genes in a series of 53 unrelated sporadic HSCR cases, revealed five novel mutations and one previously described mutation in the *RET proto-oncogene* in 10 of 53 (19%) individuals studied. Notably, all the missense mutations identified in the study population involve amino acids which have remained evolutionarily conserved in human, mouse and rat. Eight polymorphisms, of which three were novel and five previously described, were also detected. Seven polymorphisms were identified in the *EDNRB* gene whilst no sequence changes were identified in the *EDN3* gene. This finding was not unexpected since none of the individuals included in the panel of South African HSCR patients presented with a HSCR/Waardenburg syndrome phenotype, frequently associated with these genes (Puffenberger *et al* 1994, Chakraverti *et al* 1996, Edery *et al* 1996, Hofstra *et al* 1996).

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The percentage of individuals presenting with *RET* mutations in this study (19%) is in accordance with the findings by Edery *et al* (1994) and Romeo *et al* (1994), who indicated that *RET* gene mutations account for approximately 25% of HSCR cases. The majority of mutations (80%) were detected in the coloured population, while only two of the 10 *RET* mutations (20%) identified occurred in white patients. No mutations were identified in the black population. The mutations identified to date in RET are scattered throughout the gene (figure 3.1) and a similar picture has emerged in this study.

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. Consensus calcium-binding sequences, also present in the cadherin-like domain of the *RET proto-oncogene*, were shown to mediate calcium binding and calcium-dependent homophilic interactions with cadherin molecules on other cells (Takeichi 1991, Overduin *et al* 1995). Mutations in this area may cause *RET* loss-of-function by a dominant-negative mechanism (Cosma *et al* 1998) whereas mutations in the cystein residues mainly result in tumorigenesis because of the activation of *RET* function in patients with MEN 2A and FMTC (Eng *et al* 1996). Mutations involving the extracellular domain were shown to inhibit transport of the *RET* protein to the plasma membrane, causing a low expression of p170^{ret} at the cell surface.

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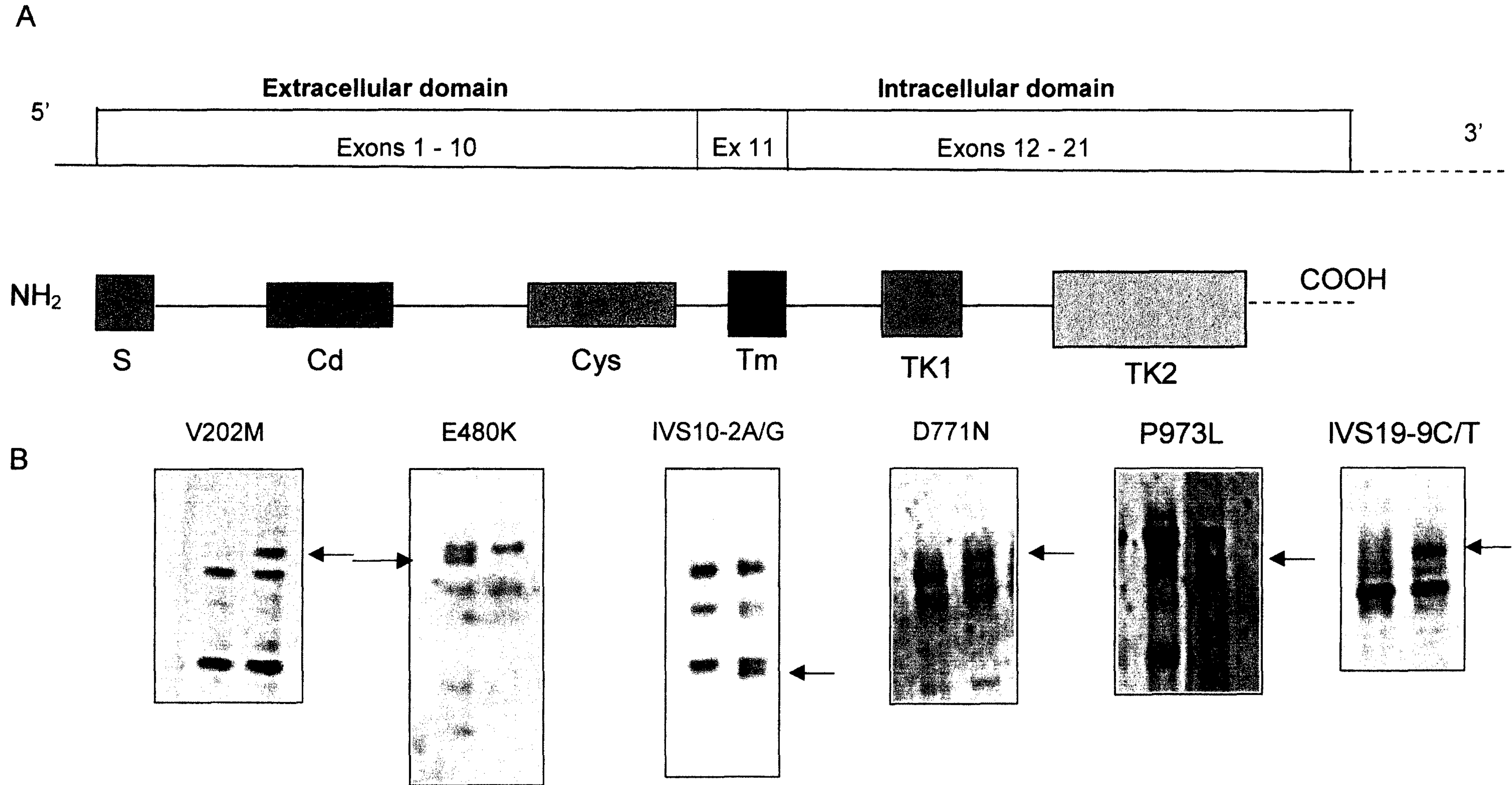

Figure 3.1. Schematic representation of genomic organisation of the *RET proto-oncogene* in relation to three distinct functional domains of the protein. Mutations identified in the South African population are shown. A) Exons encoding for specific domains of the *RET* protein, B) Ethidium bromide stained 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.

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A splice variant (IVS10-2A/G) was identified in intron 10 in an individual with aganglionosis in the rectosigmoid area. Efficient splicing depends on accurate cleavage and rejoining, which appears to be determined by the GT and AG dinucleotides present at the 5' and 3' exon/intron boundaries, respectively. This splice site mutation, altering the AG dinucleotides at the 3' region of the intron, most likely disrupts accurate splicing and interferes with the correct processing of the primary transcript. A sequence variant was also identified in intron 19 (IVS19-9C/T). The consensus sequence reported by Padgett *et al* (1986) showed that sequence changes at position –9 of the 3' splice site, resulting in the substitution of any nucleotide for a pyrimidine, affected the protein structure in 82% of cases. The splice variant can therefore result in abolishing or a reduction in the amount of mRNA. RNA studies might, however, give a more definite answer.

Mutations in exon 13 (D771N) and exon 17 (P973L) involve the intracellular domain, the most highly conserved area of the gene. Missense mutations in the intracellular domain may impair tyrosine kinase activity. The mutation in exon 17 (P973L) was previously described by Yin *et al* (1994) who speculated that this mutation might inactivate *RET* gene function. Subsequent identification of this mutation in a patient with a family history of HSCR, who was not included in this study, reflects the low penetrance of the disease. The variant identified in intron 16 (IVS16-38delG) occurred in the two HSCR patients with mutation P973L, and therefore these sequence changes are probably located on the same chromosome. This variant appears to be a neutral polymorphism that segregates with the mutation, since it was not detected in 86 (172 chromosomes) control subjects.

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The study included fourteen patients who were clinically diagnosed with both HSCR and Down's syndrome. A mutation in *RET* was identified in only two of these fourteen patients (14%), compared with 6 mutations identified in 9 of 39 patients with only HSCR (23%). A gene on chromosome 21q has previously been suggested by Puffenberger *et al* (1994b) for individuals with both HSCR and Down's syndrome. Petersen *et al* (1991) previously localised a possible susceptibility gene to chromosome 21q22.1-22.3.

Different sections of the colon with and without ganglion cells were obtained for four of the HSCR patients who also presented with Down's syndrome. Discrepancies in the occurrence of polymorphic variants in exons 6 (IVS6+56delG), 7 (A432) and 15 (S904) of the *RET proto-oncogene* and exon 2 (S184 and I187) of the *EDNRB gene* were observed in the different tissue samples of these individuals (data not shown). The significance of this finding is currently under investigation but could indicate the occurrence of somatic silent mutations *in situ* in the colon. The role of these polymorphisms in the etiology of HSCR could be postulated to occur at the transcriptional level, thereby influencing the amount of *RET* protein produced and in turn mediating an altered effect in the enteric neural system.

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 coloured and white patients versus the respective control groups. The observed trend of polymorphic allelic frequency variation in the HSCR and HSCR/ Down's syndrome patient groups, consisting mainly of colon tissue, and the mainly blood-derived

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coloured and white patients versus the respective control groups. The observed trend of polymorphic allelic frequency variation in the HSCR and HSCR/ Down's syndrome patient groups, consisting mainly of colon tissue, and the mainly blood-derived control group, could indicate a higher susceptibility of specific tissues to mutational change. In light of the role that *RET* plays in the cellular signalling pathway between different cells, this finding warrants further investigation as recent evidence indicates that silent mutations may possibly influence *RET* transcription and lead to altered protein levels causing phenotypic changes through interaction with other mutated genes (Borrego *et al* 1999, Fitze *et al* 1999). 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. The low mutation detection rate in this study is probably a consequence of the heterogeneous nature of HSCR, but limitations imposed by the HEX-SSCP screening method (Kotze *et al* 1995) used may also be a contributing factor.

Although no direct evidence concerning the causative nature of the novel 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. All the missense mutations have been detected in more than one HSCR patient, while absent in the control groups. Screening for these four potentially founder-type mutations, V202M, E480K, D771N and P973L, may facilitate an improved diagnosis of HSCR in South Africa, particularly in the coloured population where nearly 30% of sporadic HSCR cases were characterised at the molecular level. This study represents the first comprehensive genetic study of HSCR in the diverse South African population.

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CHAPTER 4

DISCUSSION, CONCLUSION and FUTURE PROSPECTS

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4.1. DISCUSSION

The identification of interstitial deletions of chromosome 10 in patients with HSCR implicated the involvement of chromosome 10 in the pathogenesis of the disease. Subsequently, the gene was localised to chromosome 10q11.2, the *RET proto-oncogene* (Martucciello *et al* 1992, Luo *et al* 1993). Targeted disruption of the *Ednrb* and *Edn3* genes in mice, respectively, resulted in aganglionic megacolon and white coat-spotting (Hosoda *et al* 1994, Gariepy *et al* 1996). These findings established *RET, EDNRB* and *EDN3* as important components in the normal development of the enteric ganglion neurons and epidermal melanocytes (neural crest-derived cell lineages) during the early stages of embryogenesis.

Criteria to determine whether a missense mutation contributes to the disease pathogenesis were constructed by Hofstra *et al* (1997), occuring as a single entity or in a combination. These criteria include: 1) a mutation occurring as a *de novo* mutation, 2) the segregation of the mutation with the disease within pedigrees, 3) absense of the mutation in control individuals, 4) a change in the polarity of an amino acid or its size in the encoded peptide, 5) amino acid substitution in an area which is evolutionary conserved between species and/or shared between proteins of the same protein family, 6) effect of the mutation in an animal model or functional in vitro system, 7) mutation included in previously reported disease-specific mutation databases. Points 3, 5 and 7 were considered to assess the likelihood that the novel mutations identified in this study may cause or at least contribute to the HSCR phenotype.

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Several mutations and polymorphisms were identified in the *RET* and *EDNRB genes* after investigating 53 unrelated sporadic HSCR patients from different ethnic groups in South Africa. Mutations identified in the *RET* gene (figure 4.1) may affect different domains of the *RET* protein, resulting in altering expression. The *RET* gene is divided into an extracellular domain (exons 1-10), a transmembrane domain (exon 11) and an intracellular domain (exons 12-21). The novel mutations identified in this study will be discussed in relation to their presumed functions in altering protein expression.

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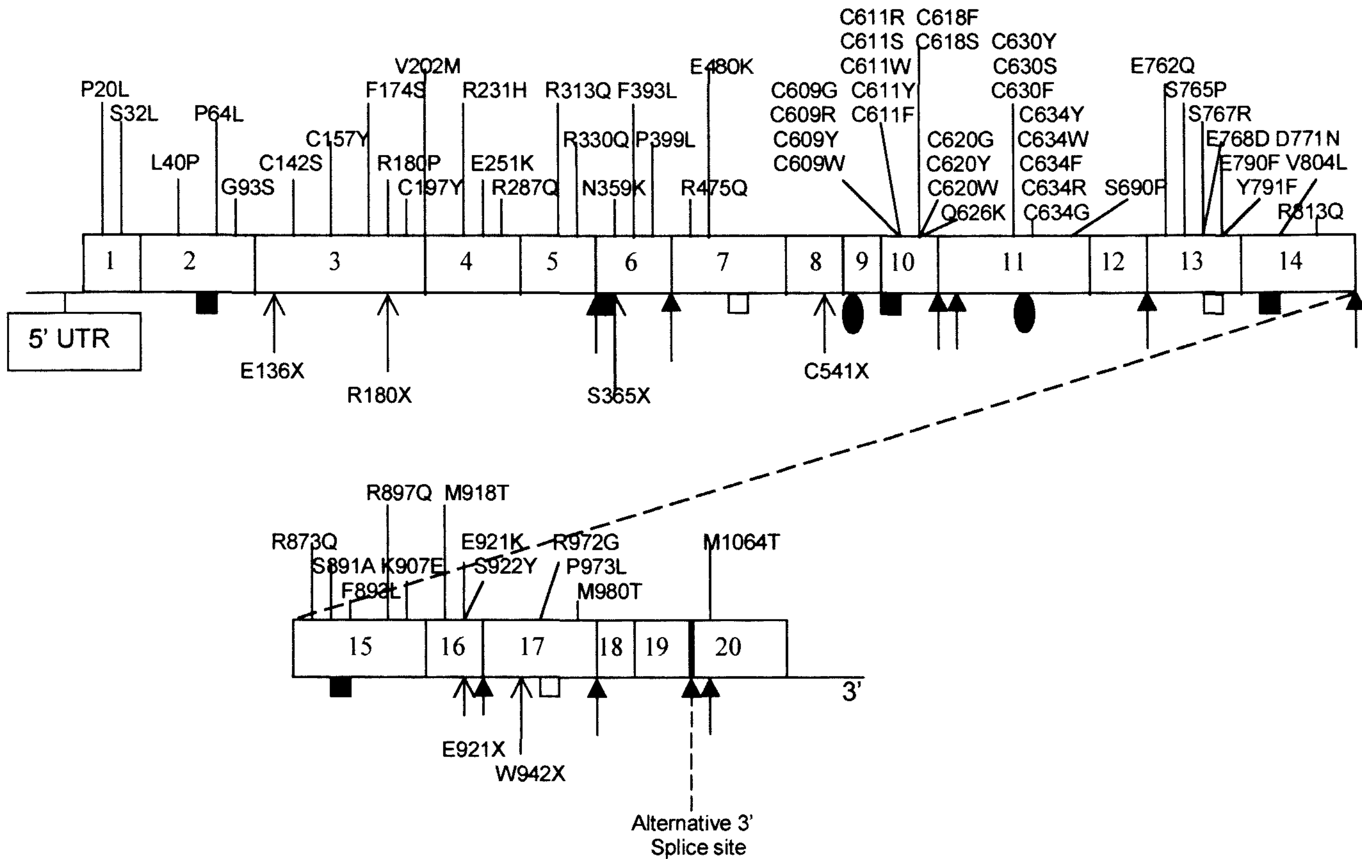

5' UTR – 5' untranslated region

——— - point mutations

⟶ - point mutations resulting in a termination codon

➞ - splice site mutation

■ - deletion mutation

□ - insertion mutation

⬬ - complex event involving duplication, insertion and deletion resulting in stop codon

——— - mutations identified in this study

Figure 4.1. Mutation spectrum of the *RET proto-oncogene* (from Eng and Mulligan 1997)

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4.1.1. *RET PROTO-ONCOGENE*

4.1.1.1. Extracellular domain

The function of the extracellular domain is to bind signalling molecules (for a review see Höppener and Lips 1996). Mutations in the extracellular domain inhibit transport of the *RET* protein to the plasma membrane and result in a low expression of *RET* at the cell surface (Iwashita *et al* 1996). Studies performed by Iwashita *et al* (1996) suggested that sufficient levels of *RET* expression on the cell surface are required for ganglia migration toward the distal part of the colon or for full differentiation. Cosma *et al* (1998) illustrated that mutations in the extracellular domain causes *RET* loss of function by a dominant-negative mechanism. The extracellular domain includes the cadherin-like sequence and the cysteine rich region.

4.1.1.1.1. Cadherin-like sequence (exons 2-6)

The novel G to T transition identified in exon 3 (V202M) resulted in the replacement of a valine for a methionine. This mutation was identified in two males with aganglionosis restricted to the rectosigmoid area. Two polymorphic variants were identified in exons 2 and 6. Several functions for this area have been postulated. *RET* is expressed as a 150-kDa precursor form and a 170-kDa mature form (glycoproteins) in the endoplasmic reticulum and the plasma membrane, respectively. A cadherin-like domain, consisting of 110 amino acids, is present in the extracellular domain of the *RET proto-oncogene*. Cadherins mediate calcium binding

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and calcium-dependent homophilic interactions with cadherin molecules on other cells and in *RET* these consensus calcium-binding sequences are also present (Takeichi 1991). Calcium binding is also essential for the correct folding of cadherin molecules (Overduin *et al* 1995). A study performed by Asai *et al* (1995) demonstrated that a point mutation in the cadherin-like domain resulted in a dramatic reduction of $p170^{ret}$ expression at the plasma membrane, but did not affect the expression of $p150^{ret}$. They hypothesised that mutations in the putative calcium binding site might: 1) interfere with processing of *RET* in the golgi complex, 2) interfere with *RET* transport to the plasma membrane, or 3) full processing and transport of *RET* to the plasma membrane occurs, but it cannot be retained at the plasma membrane in the absence of calcium binding.

Van Weering *et al* (1998) investigated the role of calcium in $p170^{ret}$ expression because of observations that a mutation in the putative calcium-binding domain of *RET* leads to aberrant expression of $p170^{ret}$. Depletion of extracellular calcium resulted in down-regulation of $p170^{ret}$ but did not affect $p150^{ret}$ expression. It was shown that $p150^{ret}$ is the precursor of $p170^{ret}$ and that a mutation of the putative calcium-binding site of *RET* can also inhibit $p150^{ret}$ processing (Asai *et al* 1995). Mutations identified in the extracellular domain resulted in reduced levels of $p170^{ret}$ expression at the plasma membrane and the length of aganglionosis correlates with the degree of impaired $p170^{ret}$ expression (Iwashita *et al* 1996, Carlomagno *et al* 1996). The extracellular domain of *RET* is therefore very sensitive to mutations that alter either *RET* processing or *RET* retention at the plasma membrane and these mutations may cause a defect in proper folding of $p150^{ret}$. The function of the mutation identified in exon3 (V202M) is still unclear, but concluding from previous

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studies, it could lead to inactivating the *RET* protein and thus impairing $p170^{ret}$ expression at the plasma membrane.

4.1.1.1.2. Cystein-rich region (exons 7-10)

A high frequency of mutations in the cystein residues is often associated with diseases such as MEN 2A and FMTC (95% of cases) (Mulligan *et al* 1993, Donis-Keller *et al* 1993). Most mutations giving rise to these phenotypes are located in exon 10 (codons 609, 611, 618, 620 and 634) and include substituting cystein for a single amino acid in the *RET* protein. A novel mutation in exon 7 (E480K), located in the cystein rich area were identified in three males with aganglionosis restricted to the distal part of the colon. This mutation did not, however, alter one of the cystein residues. The mutations identified in MEN 2A, altering cystein residues, were shown to activate *RET* function, resulting in tumorigenesis.

4.1.1.2. Transmembrane domain

The function of the transmembrane domain is to anchor the receptor in the cell membrane and is involved in regulating cell growth and differentiation (Höppener and Lips 1996). A novel splice site mutation in the vicinity of the transmembrane region (IVS10-2A/G) was identified in a male with aganglionosis restricted to the rectosigmoid area. Efficient splicing depends on accurate cleavage and rejoining, which appears to be determined by the GT and AG dinucleotides present at the 5' and 3' exon/intron boundaries, respectively. The splice variant might therefore

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interfere with the correct processing of the primary transcript and impair anchoring of the receptor to the cell membrane.

4.1.1.3. Intracellular domain

The intracellular domain transduces the biological signal from the extracellular domain into a biochemical message to intracellular target proteins (Höppener and Lips 1996). The intracellular domain consists of two tyrosine kinase regions, that is the most highly conserved area of the gene. Mutation analysis of *RET* indicated that mutations in the intracellular domain resulted in impairing tyrosine kinase activity.

4.1.1.3.1. Tyrosine kinase domain 1 and 2

By producing particular tyrosine kinase molecules and transducer molecules, specific biological responses are allowed in different cell types. The tyrosine kinase region plays an important role in tyrosine phosphorylation and substrate binding. Phosphorylation on tyrosine residues is the result of ligand-induced receptor dimerisation by a cross-phosphorylation mechanism. After phosphorylation, cytoplasmic target molecules, containing a so-called src homology 2 (SH2) domain, bind to specific phosphotyrosine-containing peptide sequences of the receptor molecules. The substrate molecules are then phosphorylated on the tyrosine residues, followed by transducing the signal further downstream and causing a cellular response (Illustrated in figure 4.2) (Höppener and Lips 1996).

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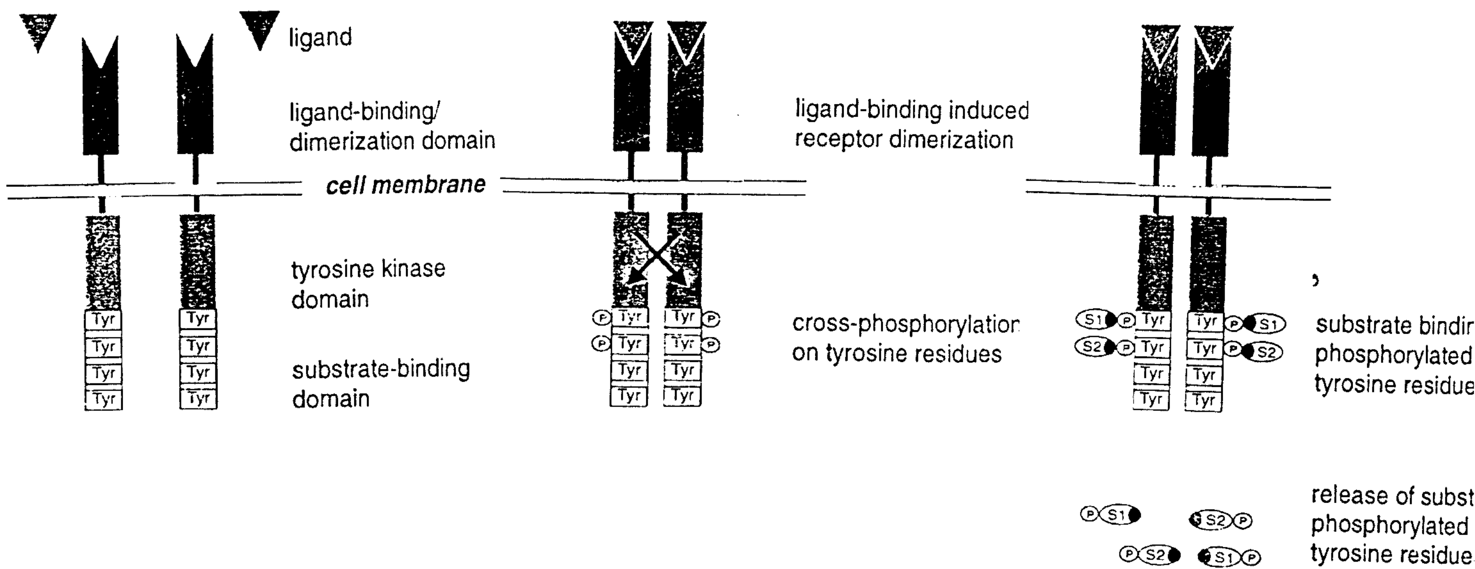
Figure 4.2. Dimerisation model for receptor tyrosine kinase activation (from Höppener and Lips 1996)



The point mutations identified in exon 13 (D771N) and exon 17 (P973L) reside in the split tyrosine kinase region of the *RET proto-oncogene*. The mutation in exon 17, substituting a proline (CCA) for a leucine (CTA), was previously described by Yin *et al* (1994), who speculated that this missense mutation might result in the inactivation of *RET* because of the substitution of amino acids in an area which is highly conserved and these mutations might have a loss-of–function effect. A rare variant identified in intron 16 (IVS16-38delG) was present in the same individual with the mutation in exon 17 (P973L). This variant, although absent in 104 control subjects, is thought to have no effect on the protein, even though it segregates with the mutation, supporting linkage disequilibrium.

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thought to have no effect on the protein, even though it segregates with the mutation, supporting linkage disequilibrium.

A splice variant (IVS19-9C/T) was identified in one individual with aganglionosis restricted to the distal part of the colon. Introns, or the non-coding region of the gene, are removed by a cleavage/ligation reaction before the mRNA is transported to the cytoplasm for translation. This cleavage or ligation reaction is a highly accurate process. Krawczak *et al* (1992) estimated that 15% of all point mutations in an intron result in a mRNA splicing defect, causing human disease. Efficient splicing is dependent on accurate cleavage and rejoining which appears to be determined by the GT and AG dinucleotide present at the 5' and 3' exon/intron boundaries, respectively. However, these sequence motifs are not the only determinants of accurate splicing (Breathnach and Chambon 1981, Mount 1982, Padgett *et al* 1986). Padgett *et al* (1986) derived the following consensus sequences:

5' (donor) splice site consensus sequence

-4	-3	-2	-1		+1	+2	+3	+4	+5	+6
C_{29}/A_{34}	C_{38}/A_{35}	A_{62}	G_{77}	/	G_{100}	T_{100}	A_{60}	A_{74}	G_{84}	T_{50}
		Exon			Intron					

3' (acceptor) splice site consensus sequence

-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4
Y_{78}	Y_{81}	Y_{83}	Y_{89}	Y_{85}	Y_{82}	Y_{81}	Y_{86}	Y_{91}	Y_{87}	N

-3	-2	-1		+1
C_{78}	A_{100}	G_{100}	/	G_{55}
		Intron		Exon

Y=pyrimidine, N=any base, subscript numerals refer to percentage frequency of occurrence.

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Splicing defects are a common cause of human disease resulting in the abolishment or at least a reduction in the amount of mature mRNA generated. These splicing defects could also result in an exon not being recognised (exon skipping) and thereby exclude it from the mature mRNA transcript. The splice variant identified in the intracellular region of the (IVS19-9C/T) might therefore impair or influence kinase activity.

4.1.2. ***ENDOTHELIN-B RECEPTOR*** **AND** ***ENDOTHELIN-3 GENE***

The *EDNRB* and *EDN3* genes are relatively conserved between species and mutations in these genes were shown to contribute mainly to a syndromic HSCR phenotype (HSCR-Waardenburg syndrome). Previous studies performed concluded that *EDNRB* mutations account for approximately 6% of HSCR cases (Chakravarti 1996). Mutations of the *EDN3* gene were previously identified in only a few HSCR cases (Hofstra *et al* 1999) and were mainly associated with Waardenburg syndrome. In this study, no disease-related mutations were identified in either *EDNRB* or *EDN3*. Band aberrations identified in the *EDNRB* gene mutation screen most likely represented neutral polymorphisms. Since none of the South African patients presented with a HSCR-Waardenburg phenotype, the failure to identify mutations in this gene in our panel of HSCR patients was not unexpected.

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4.1.3. GENERAL OBSERVATIONS

Previous studies have demonstrated that *RET* mutations contribute to 25% of all HSCR cases, while mutations identified in this study contributed to 19% of HSCR cases (Edery *et al* 1994a, Romeo *et al* 1994, Angrist *et al* 1995, Eng and Mulligan 1997). Clustering of mutations occurs in exon 10 (95%) in MEN 2A patients (Mulligan *et al* 1995), and in exon 16 (93%) in MEN 2B and FMTC patients in the RET proto-oncogene (Hofstra *et al* 1994), whereas in HSCR, no hotspot area is observed and mutations occur throughout the gene (Edery *et al* 1994a, Romeo *et al* 1994). The *RET* protein appears to play an important role in embryogenesis and tumorigenesis, particularly in tissues of neural crest origin, serving to transduce signals for cell growth and differentiation (Schuchardt *et al* 1994). *RET* regulates proliferation, migration, differentation and survival of enteric ganglion cells or their neural crest precursors and thus an insufficient functional *RET* protein would prevent these processes to be performed (Santoro *et al* 1994, Borrello *et al* 1995). Studies performed by Höppener and Lips (1996) indicated that *RET* gene mutations in MEN 2A, MEN 2B and FMTC result in activating *RET* tyrosine kinase activity. In contrast to these observations, mutations identified in HSCR individuals possibly cause inactivation of *RET* tyrosine kinase activity, resulting in the loss or absence of ganglion cells (Pasini *et al* 1995).

Individuals heterozygous for *RET* mutations may develop HSCR whereas no effect is observed in heterozygous mice, suggesting a gene dosage effect in humans. Mice homozygous for *RET* mutations demonstrate a HSCR phenotype (Schuchardt *et al* 1994). This could be due to other known or novel modifier genes having a greater

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effect in humans than in mice. Mutations identified in *EDNRB* and *EDN3* were previously shown to be involved in the pathogenesis of the disease. These genes, together with *RET*, only explain 20%-25% of HSCR cases. Other genes, that have not yet been identified, are therefore probably involved in the pathogenesis of HSCR.

In this study, a higher frequency of mutations in the *RET proto-oncogene* was identified in males (9 of 10) than in females (1 of 10). No correlation could be made between patients presenting with long segment disease and those with short segment disease due to the lack of long segment disease patients (7 of 53) in our study population. Different sections of the colon, including both aganglionic and ganglionic segments, were obtained from four of the patients presenting with both HSCR and Down's syndrome. HEX-SSCP analysis showed a difference in banding patterns in *RET* and *EDNRB* for the different segments of the same individuals. These abberations represented polymorphisms and when these polymorphisms were identified in the aganglionic segments, they were absent in the ganglionic segments, and vice versa. The presence or absence of these polymorphisms could possibly, together with mutations in other genes, contribute to the disease phenotype.

The potential disease-related mutations in the *RET proto-oncogene* were identified in 28.6% of the coloured patients and in 14% of the white patients. No mutations were identified in the black patients. Population-directed mutation screening may facilitate an improved diagnosis of HSCR in the South African population.

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4.2. CONCLUSION

Potential disease-related mutations were identified in 19% of patients in our study cohort. All the mutations were identified in the *RET proto-oncogene*, being the major susceptibility gene for HSCR. These mutations were not clustered in a particular area of the gene, but occurred throughout the gene. No potential disease-causing mutations were identified in the *EDNRB* and *EDN3* genes and mobility-based aberrations in the *EDNRB* gene represented polymorphisms. These genes are mainly associated with a HSCR/Waardenburg (Shah-Waardenburg syndrome) phenotype, not observed in the South African patients studied.

Further studies have to be performed to elucidate the role of the potential disease-causing mutations identified in South African patients with HSCR. Failure to detect mutations in 81% of patients can be ascribed to the heterogeneous nature of HSCR, or limitations imposed by the HEX-SSCP mutation screening method (Kotze *et al* 1995) used. Although the combination of heteroduplex and SSCP analysis provides a highly efficient method (>90%) to detect small mutations, large rearrangements cannot be ruled out.

A higher frequency of mutations were identified in patients with sporadic rectosigmoidal aganglionosis (17%) compared to previous literature of 7.9% in these patients. Mutation frequency in patients with sporadic total colonic aganglionosis (33.3%) was more or less the same as previously described (42.2%), although only a few patients presented with long segment disease (13%) in this study (Seri *et al*

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1994). Potential disease-related mutations were identified in 28.6% of the coloured patients and in 14% of the white patients, suggesting that screening for these mutations may facilitate improved diagnosis of HSCR in the South African population. Further studies need to be performed to elucidate the genetics of HSCR in patients with no apparent *RET, EDNRB* and *EDN3* disease-related mutations.

4.3. Future prospects

Further studies need to be performed to elucidate the role of the potential disease-related *RET* mutations identified in sporadic HSCR cases in the South African population. Delineation of the effects of these mutations on the protein structure could broaden our understanding of the biological role of the *RET proto-oncogene* in the differentiation of cells of neural crest origin.

Extension of mutation analysis to also include familial cases may lead to the development of a comprehensive DNA test for HSCR in the diverse South African population. Also, including more long segment disease patients could possibly lead to the association of mutations with a specific length of aganglionosis.

Recently, more HSCR loci have been identified in a genome scan, which is estimated to account for all HSCR cases (Bolk *et al* 1998). This data will be incorporated in future studies to possibly explain the HSCR phenotype in patients who could not be characterised at the molecular level. The putative HSCR-associated gene on

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chromosome 21 would be a good candidate for mutation screening in the cohort of patients presenting with both HSCR and Down's syndrome.

The identification of mutations in HSCR patients would in future lead to improved and accurate counselling of individuals with HSCR and their families, and ultimately contribute to optimal therapy targeted at the cause of the disease. The varying penetrance of mutations associated with the HSCR phenotype represents a major challenge in genetic risk assessment. HSCR can be considered a model for the many complex disorders currently under investigation world-wide.

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CHAPTER 5

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