

# Chasing the ubiquitous *RET* proto-oncogene in South African MEN2 families – implications for the surgeon

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## Summary

The *RET* proto-oncogene (REarranged during Transfection; *RET*) plays an important role in the causation of many thyroid tumours. Germline *RET* proto-oncogene missense mutations have been clearly linked to medullary thyroid carcinoma (MTC) and the inherited cancer syndrome multiple endocrine neoplasia type 2 (MEN2A, MEN2B).

**Methods.** We investigated a cohort of MEN2-related patients referred to Tygerberg Hospital, W Cape (2003 - 2009). The study cohort was divided into three groups based on pathology (viz. MEN/MTC, pheochromocytoma, and a miscellaneous group of MEN pathologies). Families with identified high-risk factors were recalled. Serum calcitonin levels were monitored where indicated.

DNA was extracted from whole blood by standard techniques and polymerase chain reaction (PCR) products screened for *RET* gene variations by heteroduplex single-strand duplication techniques (heteroduplex single-strand conformation polymorphism analysis) being validated with automated sequencing techniques showing conformational variants in acrylamide gel.

**Results.** We screened 40 persons, male/female ratio 1:1.5. Three ethnic groups were represented (white (12), black (11) and mixed race (17)). Nine were index MTC cases, 5 pheochromocytoma, 3 Hirschsprung's disease-MEN associations and 2 miscellaneous (1 neuroblastoma, 1 intestinal neuronal dysplasia), while 1 fell into the MEN2B category. The remaining 19 were unaffected relatives screened for carrier status, among whom a familial recurrence was observed in 7.

On genetic testing, an *RET* point mutation at the high-risk 634 cysteine allele was identified in 11 cases. A further cysteine radical mutation at the 620 position was related to MEN2 in 3 families plus 1 other family referred from elsewhere. Other less-recognised gene variations were

detected throughout the *RET* gene in 70% of cases and included the 691 position on codon 11 (11 cases); the 432 position (4 cases, 1 homozygous) intronic mutations on exon 4 (1 case); and an IVS19-37G/C and a D1017N variation in exon 19 in 2 MEN families.

Fifteen MTC patients have had thyroidectomies, of which 2 were prophylactic (C-cell hyperplasia; early occult MTC). A further 3 are awaiting prophylactic surgery.

**Conclusion.** *RET* gene mutation carries a risk of MEN2 and MTC in all ethnic groups in South Africa. Prophylactic surgery may prevent MTC, so genetic screening is important to identify and treat high-risk patients.

Multiple endocrine neoplasia type 2 (MEN2) syndromes are autosomally dominant clinical associations characterised by a number of tumours, including medullary thyroid carcinoma (MTC), pheochromocytoma, thyroid C-cell hyperplasia (CCH), parathyroid tumours (MEN2A) and ganglioneuroma of the gastrointestinal tract (MEN2B). The common factor in the MEN2 syndromes is MTC, a poorly differentiated thyroid malignancy that represents 3 - 10% of thyroid tumours<sup>1,2</sup> but is hereditary in 20 - 30% of cases.

MTC (and other thyroid tumours) have been shown to be genetically linked to mutations in the *RET* (REarranged during Transfection) proto-oncogene (situated at 10q22<sup>3,4</sup>), which plays a pivotal role in at least four clinical syndromes (MEN2A and MEN2B, familial medullary thyroid carcinoma (FMTC) and Hirschsprung's disease (HSCR)) in a unique 'switch on, switch off' manner.<sup>5</sup> *RET* gene variations are present in more than 50% of MTC cases as well as a significant number of papillary thyroid carcinomas (chromosomal inversions or translocations (*RET/PTC*)).<sup>6</sup>

Inheritance is important, and the risk to family members appears to be particularly high in MEN2. Patients with genetic mutations involving the cysteine radicals of the gene account for 92 - 95% of those who later develop MTC.<sup>7,8</sup> Children of families with MTC and the MEN2 syndromes have been reported to

develop early metastatic tumours even before the age of 5 years.<sup>9</sup> In tumours associated with mutations at the 918 allele (MEN2B), early aggressive behaviour with distant metastatic spread is typical<sup>10</sup> and cases of inherited MTC have been reported as early as 2 months of age.<sup>11</sup> These patients may develop the premalignant CCH even earlier,<sup>12</sup> and early lymph node metastases may occur.<sup>13</sup>

MTC is a particularly chemo/radio-resistant tumour (spreading mostly via the lymphatic system) often resulting in death, so prophylactic removal of the target organ before the onset of malignancy remains a surgical goal.<sup>6</sup> Research over the past decade has clearly shown that *RET* gene evaluation is of great value in familial screening and evaluating risk in familial cases, thus determining the timing of prophylactic surgical intervention.<sup>14</sup>

As there is some evidence that oncogenic *RET* mutations may vary between specific ethnic groups,<sup>15,16</sup> evaluation of the diverse South African population is of specific local interest.

The aim of this study was to evaluate the profile of *RET* in MEN2 families with special attention to the risk to children, and to assess risk and determine treatment protocols for their management.

## Methods

### Subjects

We investigated a cohort of MEN2-related patients referred to Tygerberg Hospital (2003 - 2009). The study cohort was divided into three groups based on pathology (viz. MEN/MTC, pheochromocytoma, and a miscellaneous group of MEN pathologies). Families with identified high-risk factors were recalled.

Unaffected family members of patients with high-risk gene variations were recalled for genetic testing and genetic counselling.

Serum calcitonin and other biochemical variables were analysed where possible.

Patients with HSCR-MEN co-segregation were identified as part of a separate study of *RET* in HSCR.<sup>17</sup>

### DNA analysis

DNA extraction was performed on colonic tissue samples and whole blood using standard techniques. Polymerase chain reaction (PCR) amplification of the 21 exons of the *RET* gene was performed using intronic primers.<sup>18</sup> The PCR products were subjected to heteroduplex single-strand conformation polymorphism analysis<sup>19</sup> and resolved in a polyacrylamide (PAA) gel supplemented with 7.5% urea (consisting of 4.5 g urea, 18 ml 5XTBE, 24 ml dH<sub>2</sub>O, 18 ml PAA (1% C of a 40% stock), 600 µl APS (10%) and 60 µl TEMED) at 4°C (350 V) for 18 hours. The DNA fragments were also electrophoresed in a 10% PAA gel supplemented with 5% glycerol (consisting of 3 ml glycerol, 6 ml 5XTBE, 36 ml dH<sub>2</sub>O, 15 ml PAA (1% C of a 40% stock), 800 µl APS (10%) and 80 µl TEMED) at room temperature (300 V). The DNA fragments were stained in ethidium bromide and visualised by ultraviolet light transillumination. Semi-automated DNA sequencing (ABI 310 PRISM) was performed on PCR products demonstrating mobility or conformational variants on the PAA gels.

### Controls

An additional group of unaffected controls (*N*=60) were recruited from the normal population for comparison of the genetic profile

of the ethnic population groups represented in the study (20 patients per ethnic group, i.e. 20 white, 20 black and 20 coloured patients). Unaffected family members of patients with high-risk gene variations were recalled for genetic testing and genetic counselling.

### Ethical permission

The research project was approved by the research committee at Stellenbosch University (2001/C019) and conducted according to the accepted ethical codes and guidelines, as outlined in the declaration of Helsinki

## Results

Forty persons were screened (male/female ratio 1:1.5). Their ages ranged from newborn to 54 years, and 7 were children aged <13 years. All ethnic groups were represented (white 12, black 11, coloured 17). There were 9 index MTC cases, 5 pheochromocytoma, 3 HSCR-MEN associations and 2 miscellaneous (1 neuroblastoma, 1 intestinal neuronal dysplasia); 1 patient fell into the MEN2B category. The remaining 19 were unaffected relatives screened for carrier status, among whom a familial recurrence was observed in 7 (37%). Serum calcitonin levels were raised in 19 of the 21 affected patients. In 2 patients early MTC was not associated with raised calcitonin levels.

On genetic testing, an *RET* point mutation was identified in 21 of the 40 patients (52.5%) (Table I). The genetic variation occurred at the high-risk 634 cysteine allele in 11 of these (52%). A further cysteine radical mutation at the 620 position was related to MEN2 in 3 families, plus 1 other unique family referred from elsewhere, in which MEN and HSCR co-existed.<sup>20</sup> Other less-recognised gene variations were detected throughout the *RET* gene in 70% of cases and included the 691 position on codon 11 (11 cases); the 432 position (4 cases, 1 homozygous); intronic mutations on exon 4 (1 case); and an IVS19-37G/C and a D1017N variation in exon 19 in 2 MEN families (Table I). Additional polymorphisms of the gene occurred in both patients and controls (Table II).

Fifteen patients underwent surgical removal of the thyroid gland (total thyroidectomy), including 3 prophylactic thyroidectomies in gene-positive family members. In one 6-year-old, CCH (a premalignant stage<sup>12</sup>) (Fig. 1) was identified along with a twice-normal serum calcitonin level. In another 2, early occult MTC was detected (serum calcitonin 3 times normal in 1) (Fig. 2). A further 2 are awaiting prophylactic surgery.

A cysteine radical mutation (C620R and C620W) was related to MEN2 in 3 families, with proven MTC in recurrent patients in 2 and suspected in the other. In addition, it was a major factor in a unique family referred from elsewhere in which MEN and HSCR co-existed.

## Discussion

This study confirms the association of *RET* gene mutation with cancer risk in MEN2 and MTC families in all ethnic groups in the diverse South African population. As MTC is a particularly chemo/radio-resistant tumour, spreading mostly via the lymphatic system and often resulting in death, the major benefit of *RET* genetic testing lies in the early preclinical identification of carriers of the *RET* mutation and timely prophylactic thyroidectomy,<sup>6</sup> as carried out in this study.

Activating mutations of *RET* appear to be of the order of

**TABLE I. POTENTIAL DISEASE-CAUSING MUTATIONS IDENTIFIED IN THE *RET* PROTO-ONCOGENE IN MEN2 PATIENTS (N=21/40)**

Exon/Intron	Mutation	Nucleotide change	Effect on coding sequence	No. of patients	Gender/ethnic group	Diagnosis
4	IVS4-18G/A		Splice acceptor	1	WM	MEN2B
10	*C620W	TGC→TGG	Missense	2	CM/CF	HSCR-MEN2
11	*C634S	TGC→AGC	Missense	8	BF/BF/BF	MEN2A
11	*C634Y	TGC→TAC	Missense	2	WF	MEN2A
11	*C634R	TGC→CGC	Missense	2	CF/CF	MEN2A
12	A750P	GCA→CCA	Missense	2	CF/CF	MEN2A
13	D771N	GAC→AAC	Missense	1	WF	FMTC
18	M1009V	ATG→GTG	Missense	1	WF	FMTC
19	D1017N	GAC→AAC	Missense	1	CF	FMTC
19	V1041G	GTG→GGG	Missense	1	BF	MEN2A
20	IVS19-37G/C		Splice acceptor	1	CM	MTC carrier

\*Related to cysteine radicals on RET gene.

Ethnic grouping: W (M/F) = white; C (M/F) = coloured (mixed ancestry); B (M/F) = black African.

**TABLE II. POLYMORPHISMS IDENTIFIED IN THE *RET* PROTO-ONCOGENE IN MEN2 PATIENTS AND CONTROLS**

Exon/intron	Codon	Nucleotide change	Allele frequency*			MTC patients
			BC (N=60)	CC (N=58)	WC (N=50)	
2	A45	GCG→GCA	0.06	0.31	0.5 (25)	0.12
7	A432	GCG→GCA	0.0	0.12	0.06	0.1
13	L769	CTT→CTG	0.0	0.20	0.18	-
15	S904	TCC→TCG	0.25	0.41	0.06	-

\*Allele frequency of polymorphic allele denoted.

BC = black control group; CC = coloured control group; WC = white control group.

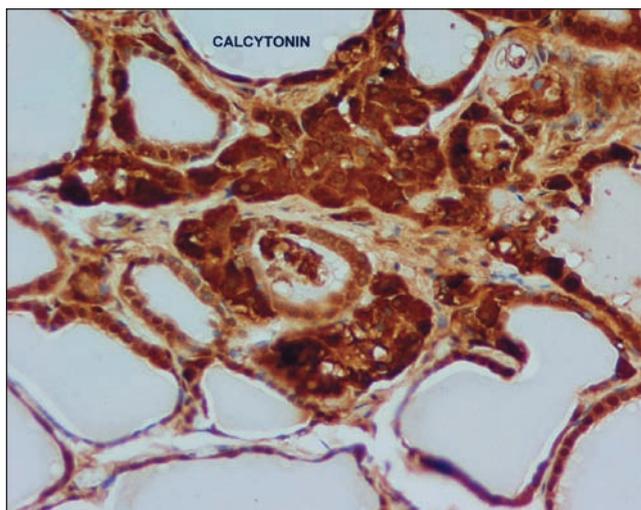


Fig. 1. Calcitonin immunostain of prophylactically excised thyroid gland of a 6-year-old 634 carrier, showing increased calcitonin activity and C-cell hyperplasia (×160).

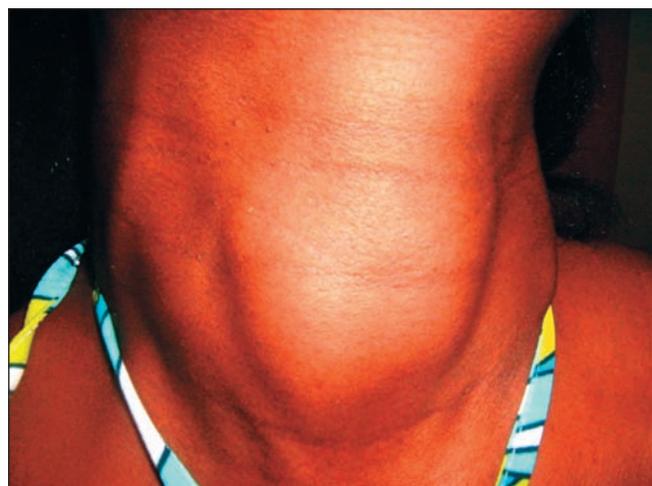


Fig. 2. Medullary thyroid carcinoma in the 36-year-old mother of a Hirschsprung's disease patient with a C620 point mutation.

1:500 000 in the general population.<sup>21</sup> Consistent associations exist between *RET* genotype and phenotype, and the 'gain of function' variations associated with MEN2 can be divided into two main groups (viz. those affecting one of the six cysteine radicals (609, 611, 618, 620, 630 and 634 positions) (MEN2A) and those affecting the tyrosine kinase domain<sup>22</sup> (MEN2B)). MTC in MEN2B is associated with a point mutation (methionine→threonine) in exon 16(M918/T) of the *RET* proto-oncogene in 95% of cases.<sup>23,24</sup> Genetic variants at amino acid positions 634 (MEN2a) and 918 (MEN2b) carry a particularly high-risk profile of MTC in offspring when compared with others.<sup>25,26</sup> Other MEN2 tumours (e.g. pheochromocytoma) may also be related to *RET* but usually occur later in life.<sup>27</sup>

*RET* proto-oncogene testing has been shown to be vastly superior to measurement of calcitonin levels in identifying preclinical MTC cases, with a specificity approaching 95 - 100%.<sup>28</sup>

Genetic screening is extremely sensitive in MEN2 syndromes, and children of MTC families who are carriers of mutant genes are at risk and may be identified by detection of the germline mutation in the *RET* proto-oncogene. These are particularly related to *RET* cysteine mutations in exons 10, 11, 13 and 16, but other *RET* variations also appear to carry risk. In this study, 2 family members with high-risk *RET* mutations and an early MTC detected at thyroidectomy had normal serum calcitonin levels, demonstrating the value of *RET* gene screening in diagnosis.

A further interesting phenomenon is the relatively uncommon co-segregation of HSCR and MEN2 in the same patient,<sup>20,29,30</sup> i.e. involving both gain and loss of gene function. This was encountered in 2 families in South Africa and has been reported elsewhere.<sup>20</sup>

The high frequency with which the C620 *RET* mutation occurs in HSCR-MEN patients suggests the concept of the so-called 'Janus gene' mutation in this position, which like the Roman god of transition can face in two directions (i.e. activation (MEN/MTC) and inactivation (HSCR)).<sup>20</sup>

In 2007 Machens and Dralle<sup>28</sup> stratified MTC risk into three categories according to mutation-related aggressiveness, giving rise to a concept of 'codon-directed' timing of surgery. One long-term follow-up study of 46 *RET* gene carriers categorised the risk to children and young adults (aged 4 - 21 years) into low-risk (level 1) and intermediate- to high-risk (level 2) mutations.<sup>31</sup> Level 1 mutations appeared to be associated with variations in codons 790, 791, 804 and 891 and had a high rate of cure. In contrast, 5 (14%) of the 35 level 2 patients (mutations of codons 618, 620, 630 and 634) had ongoing disease. On long-term follow-up (mean 6.4 years), 2 of those with 634 mutations developed other MEN manifestations (viz. hyperparathyroidism and bilateral pheochromocytoma). This latter risk would probably increase with time, and long-term follow-up is necessary. Level 3 mutations include those at positions 883 and 918.

Patients with phenotypic features resembling MEN2B always require genetic testing even if the family history is negative, because of the high incidence of spontaneous mutations (approximately 50%)<sup>32,33</sup> and the early onset of MTC. Offspring of people with MEN2B-related MTC have a particularly high risk of an early aggressive carcinoma, the youngest reported case being at 2 months of age.<sup>11</sup>

The *RET* proto-oncogene has been shown to have a significant role in cancer treatment and prevention. Although many of the

tumours only occur later in life,<sup>6</sup> surgical decision making is required in the childhood years, which then raises a number of points for discussion. The first of these is the question of timing of codon-directed prophylactic thyroid surgery, the second the timing and extent of genetic screening, and the third the nature of patient follow-up and the subsequent risk of other MEN-related tumours, which has to be accompanied by adequate genetic counselling. It is recommended that testing should be carried out before 1 year of age in children (especially in families with 883/918 codon mutations) and before 5 years in MEN2A families (especially with mutations of codons 611, 618, 620 and 634). The high incidence of early aggressive tumours associated with MEN2B<sup>11,34</sup> warrants an aggressive surgical approach with early prophylactic thyroidectomy in gene carriers (<1 year).<sup>6,13</sup> Total thyroidectomy is recommended, with central lymph node dissection being added in cases of MEN2B.6

In addition to its role in MTC, tyrosine kinase activity upregulation has been demonstrated in other thyroid tumours by the *RET/PTC* fusion gene in papillary thyroid carcinoma.<sup>35</sup> After the Chernobyl disaster (ionising radiation), papillary thyroid tumours in children showed a high prevalence of *RET* fusion gene rearrangements, with at least 11 of these being described (the majority being an *RET/PTC3* rearrangement).<sup>36</sup>

The mechanisms by which the *RET* proto-oncogene variation results in gene activation and thus causes thyroid cancer is not yet fully clear. The 'gain of function' variations in the cysteine-rich extracellular domain of *RET* probably activate *RET* by inducing aberrant disulfide-linked homodimerisation.<sup>24,37,38</sup> In addition, *RET* extracellular domain mutations may result in the unfolding of *RET*. In MEN2B the 918 mutation alters the substrate specificity of *RET* tyrosine kinase and induces a different set of signalling pathways to those in MEN2A. Y1062 (a critical regulator of *Ret9* signalling) appears to be an important location on the gene.<sup>39</sup> The downstream effects of *RET* activation are not fully clear, but *RET* binds to the glial-derived neurotrophic factor (GDNF) co-receptor which then activates various signalling pathways (e.g. RAS/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT (serine/threonine protein kinase AKT pathway), p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways<sup>40</sup>) via its GDNF-family co-receptors (GFR alpha (GDNF-family receptor-alpha), etc.).

Approximately 50% of MTC can therefore be genetically related to an *RET* proto-oncogene mutation,<sup>6</sup> with point mutations mostly in high-risk areas of the gene. Prophylactic surgery appears to prevent the cancer if it is in the CCH pre-cancerous phase; it remains the ultimate surgical goal,<sup>28,41</sup> and should precede the development of lymph node metastases. Pre-operative serum calcitonin measurement is important for follow-up. Although there is some debate as to the extent of the surgery, it is accepted that total thyroidectomy with bilateral cervico-central lymphadenectomy is adequate 'prophylactic' therapy.<sup>42</sup> Revision surgery may entail cervico-lateral lymphadenectomy if the calcitonin levels remain elevated postoperatively, as widespread lymph node involvement may be present.<sup>43</sup>

In addition to identifying patients at cancer risk, study of the *RET* proto-oncogene has more recently led to the development of novel molecular approaches to cancer therapy and clinical trials are currently being evaluated in patients with advanced disease.<sup>44</sup>

## Conclusion

*RET* gene mutation carries a risk of MEN2 and MTC in all ethnic groups in South Africa. Prophylactic surgery may prevent MTC, making genetic screening important in identifying and treating high-risk patients

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