Diagnostic RET genetic testing in 1,058 index patients: A UK centre perspective

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Abstract
Objective: Diagnostic germline RET analysis is offered to all patients with a diagnosis of medullary thyroid carcinoma (MTC), or other conditions associated with multiple endocrine neoplasia type 2 (MEN2) in the United Kingdom. Here, we report the experience of a single centre's germline RET analysis over a 21-year period.

Design: Retrospective case-note review.

Patients: All index patients referred to the Exeter Genomics Laboratory for diagnostic germline RET analysis between 1997 and 2018, and unaffected family members, undergoing predictive testing.

Measurements: The rate and nature of pathogenic variant detection were recorded, as well as the indication for testing.

Results: 1,058 index patients and 551 unaffected family members were tested. The overall rate of pathogenic variant detection was 10.2% amongst index patients and 29% amongst unaffected family members. The commonest indication was isolated MTC, and amongst the 690 patients with isolated MTC, 68 (9.9%) were found to harbour a RET pathogenic variant. Of those with presumed sporadic MTC, 8.5% were found to harbour germline RET pathogenic variants, compared with 36.4% of those with a family history of MEN2-associated conditions. Pathogenic variants were identified in 3.6% and 0% of patients with isolated phaeochromocytoma and primary hyperparathyroidism, respectively.

Conclusions: Although the detection rate of RET germline pathogenic variants in patients with presumed sporadic MTC was significant, the overall detection rate in those with MTC was lower than expected in this series. Advances in RET analysis in response to reports of new variants over the last two decades are likely to have improved the pick-up rate in recent years.

KEYWORDS
c-ret proto-oncogene proteins, genetic testing, medullary carcinoma, multiple endocrine neoplasia type 2a, multiple endocrine neoplasia type 2b, phaeochromocytoma, primary hyperparathyroidism
1 | INTRODUCTION

Medullary thyroid carcinoma (MTC) is a neural crest-derived malignant tumour accounting for up to 2% of thyroid cancers.\(^1\) Approximately 20%-25% of cases are hereditary (hMTC), either as part of the multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) or in isolation (FMTC), however in the majority of cases the disease is sporadic (sMTC).\(^2\)

Whilst the aetiology of sMTC is not fully understood, the underlying cause for hMTC is well established. The REarranged during Transfection (RET) gene on chromosome 10q11.2 was first described in 1985.\(^3\) It encodes a tyrosine kinase involved in the development of the parathyroid glands, adrenal medulla, parafollicular C cells and enteric ganglia. It is primarily these structures that are affected in patients with MEN2, with MEN2A comprising hereditary medullary thyroid carcinoma with or without phaeochromocytoma and primary hyperparathyroidism, and MEN2B comprising hereditary MTC with or without phaeochromocytoma and other characteristic phenotypic features such as mucosal neuromata, marfanoid habitus, corneal nerve thickening and gastrointestinal ganglioneuroma. Shortly after its discovery, germline RET pathogenic variants were found to be present in almost all patients with hMTC.\(^4,\)\(^5\) Despite the lack of heritable germline RET pathogenic variants in the sporadic form of MTC, somatic RET variants are identified in up to 66% of cases.\(^5,\)\(^6\)

A genetic diagnosis of MEN2 is important not only in order to direct investigation for other manifestations of the syndromes, but also to enable genetic counselling and predictive testing for family members. It is particularly important to offer RET testing to all patients with MTC regardless of family history, as around 7% of those with apparently sporadic MTC do indeed harbour germline pathogenic variants,\(^7\) and 75% of MEN2B cases result from de novo RET pathogenic variants.\(^1\) For these reasons, the American Thyroid Association, British Thyroid Association and European Thyroid Association guidelines all recommend RET analysis in any patient with confirmed MTC regardless of family history.\(^1,\)\(^8,\)\(^9\)

In a research setting, germline RET pathogenic variants have been identified in exons 5, 8, 10, 11 and 13-16 in over 95% of patients with MEN2A and FMTC, and a pathogenic variant in codon 918 (M918T) has been identified in over 95% of patients with MEN2B.\(^10\) However, RET pathogenic variant detection rates in the routine clinical setting are less studied. Furthermore, the last two decades have seen an increase in reports of novel germline RET pathogenic variants in hMTC, and this has resulted in a shift in the extent of RET analysis in clinical practice. The objective of the present study is to report the results of diagnostic RET analysis performed in a clinical setting at a single centre over a 21-year period.

2 | METHODS

2.1 | Patients

Between 1997 and 2018, 1,058 index patients with MTC and other MEN2-related clinical features were referred from across the United Kingdom to the Exeter Genomics Laboratory at the Royal Devon and Exeter NHS Foundation Trust for diagnostic germline RET testing. An online request form was used to collect clinical data including age at diagnosis, MTC and other MEN2-related clinical features and information on family history. In addition, 551 clinically unaffected family members of patients found to have germline RET pathogenic variants underwent predictive testing. Informed consent was obtained by the treating clinician from all patients prior to genetic testing.

2.2 | Genetic analysis

Genomic DNA was analysed for variants in selected exons of the RET gene using sequence-specific primers (sequences available on request). PCR products were sequenced using Big Dye Terminator chemistry on an ABI DNA sequencer (Applied Biosystems, Warrington, UK), and the sequences were compared with the published sequence (NM_020975).

During the period of this study, the extent of RET sequencing has evolved in line with reports of new causative pathogenic variants. When diagnostic RET sequencing was introduced at our institution in 1997, only exons 10 - 11 were included, with subsequent expansion to include exons 13, 14, 15 and 16 in the same year. Testing for exon 8 was introduced in 2007, exon 5 in 2010 and exon 7 in 2016 in response to reports of novel pathogenic variants in those regions.\(^11,\)\(^12,\)\(^13\) Patients with a possible diagnosis of MEN2B are tested for exons 15 and 16 only. Unaffected relatives of a proband with a germline RET pathogenic variant are routinely offered predictive testing.

3 | RESULTS

The clinical indications for germline RET analysis in the 1,058 index patients are shown in Table 1. Overall, pathogenic variants were identified in 108 index patients (10.2%). The detection rate was higher amongst the 514 patients referred after 1 December 2010 (n = 63, 11.9%) than amongst the 544 patients referred before this date (n = 45, 8.3%); however, the indication for testing also varied according to date, with 65.1% of those referred prior to December 2010 having MTC compared with 80.2% of those referred after December 2010. The prevalence of individual variants according to clinical features is shown in Table S1. Of the 551 family members who underwent predictive testing, 160 (29%) were found to carry the familial variant.

3.1 | Index patients with MTC

Of the 766 UK patients with MTC, 433 were female (56.5%). The median age at referral was 51 years (Range 1-94 years). In total, 92 patients (12%) were found to harbour a germline RET pathogenic variant. Variants in exons 10, 11 and 14 of the RET gene made up...
the majority, with the most commonly affected codons overall being 634 followed by 804 (Figure 1). As expected, pathogenic variants affecting the cysteine residues were the most common (58.2%, n = 53). The p.(Val804Met) variant was identified in 14 of the 91 patients (15.4%). Of the 766 index patients with MTC, 690 had isolated MTC with no other features of MEN2A or MEN2B.

### TABLE 1 Pathogenic variant rates in UK patients according to clinical features and family history (MTC = medullary thyroid carcinoma; PHPT = primary hyperparathyroidism; MEN2B = multiple endocrine neoplasia type 2B)

| Clinical features                                    | N   | Germline pathogenic variants (%) |
|------------------------------------------------------|-----|----------------------------------|
| All index patients with MTC                          | 766 | 92 (12.0)                        |
| Index patients with isolated MTC                     | 690 | 68 (9.9)                         |
| Isolated MTC with no known family history of endocrine tumours | 657 | 56 (8.5)                        |
| Isolated MTC with family history of MEN2 components  | 33  | 12 (36.4)                        |
| Index patients with multiple MEN2-related tumours    | 91  | 21 (23.1)                        |
| MTC and pheochromocytoma or PHPT                      | 69  | 20 (29.0)                        |
| Phaeochromocytoma and PHPT                           | 22  | 1 (4.5)                          |
| Index patients with phaeochromocytoma only           | 165 | 6 (3.6)                          |
| Index patients with PHPT only                        | 56  | 0 (0)                            |
| Index patients with MEN2B phenotype                   | 44  | 13 (29.5)                        |
| With MTC only                                        | 6   | 3 (50)                           |
| With phaeochromocytoma only                          | 4   | 2 (50)                           |
| With MTC and phaeochromocytoma                       | 1   | 1 (100)                          |
| With no endocrine tumour                             | 33  | 7 (21.2)                         |
| Index patients with C-cell hyperplasia                | 5   | 0 (0)                            |
| Index patients with unspecified paraganglioma         | 4   | 0 (0)                            |
| Index patients with carcinoid                        | 1   | 0 (0)                            |
| Index patients with pituitary adenoma                 | 1   | 0 (0)                            |
| Index patients with lichen amyloidosis                | 1   | 0 (0)                            |
| Unaffected family members undergoing predictive testing| 551 | 160 (29)                        |

*All index patients with MTC include index patients with isolated MTC (n = 690), MTC and pheochromocytoma or PHPT (n = 69), MTC with MEN2B phenotype (n = 6), and MTC with phaeochromocytoma and MEN2B phenotype (n = 1)."

3.2 | Patients with isolated MTC and no known family history of endocrine tumours

Of the 657 patients with confirmed isolated MTC and no family history (therefore presumed to have sporadic disease), 56 were found to harbour germline RET pathogenic variants (8.5%, Table 1). The median age of these patients was 58 years (age range: 2-94 years), and there was a 1.3:1 female to male ratio. One patient in this group who was diagnosed with isolated MTC at the age of 30 was found to harbour a pathogenic variant at codon 883 - p.(Ala883Phe) which is associated with MEN2B. She had no known family or personal history of endocrine tumours and no phenotypic features of MEN2B. In addition, three patients in this group aged seven, 13 and 19 were found to have the MEN2B-associated p.(Met918Thr) variant. According to their referrals, their family history was unknown and they had no personal history of phenotypic features of MEN2B or pheochromocytoma. A variant of uncertain significance (VUS), p.(Val292Met), was identified in a female with isolated MTC. This variant has previously been described in 44-year-old man with pheochromocytoma and MTC. In vitro assays indicated a low-grade transforming potential, and the authors proposed that other genetic determinants may also have contributed to tumorigenesis.

3.3 | Patients with isolated MTC and a family history of MTC or MEN2 components

This subgroup was formed of patients with a diagnosis of MTC and a positive family history of MTC, C-cell hyperplasia, pheochromocytoma or primary hyperparathyroidism, and included 33 patients. There were 12 pathogenic variants identified in this group (36.4%, Table S1).

3.4 | Patients with multiple MEN2-associated conditions

A clinical diagnosis of a MEN2 syndrome was suspected based on the presence of 2 or more of MTC, pheochromocytoma and primary hyperparathyroidism. This group included 91 patients, of whom 6 were reported to have all three diagnoses, 25 had MTC and primary hyperparathyroidism, 38 had MTC and pheochromocytoma and 22 had pheochromocytoma and primary hyperparathyroidism.

Amongst patients with multiple MEN2-associated conditions, 21 (23.1%) were found to harbour a germline RET pathogenic variant, and one was found to have a variant of uncertain significance (Table S1).

3.5 | Patients with MTC plus pheochromocytoma or primary hyperparathyroidism

In those with MTC and Pheochromocytoma, the pathogenic variant detection rate was 44.7% (17 out of 38), compared with 8% (2 out of 25) in those with MTC and primary hyperparathyroidism. We identified a previously reported variant, p.(Glu818Lys), in a patient in this group. This variant has been described in a patient with MTC.
however, further functional analysis is required to determine the significance of this variant on the transforming potential of RET and it was therefore classified as being of uncertain significance. A pathogenic RET variant was identified in only one of the six patients reported by the referring clinician to have all three diagnoses. However, of the five patients with no variant identified, one had undergone testing of exons 10, 11, 13, 14 and 15 elsewhere and only underwent testing of exons 1-7, 9, 12 and 16-20 at our laboratory so the possibility of a missed variant cannot be excluded. The remaining four patients were referred as having MTC, phaeochromocytoma and primary hyperparathyroidism but on further inquiry none had confirmed MTC, but rather raised calcitonin along with confirmed phaeochromocytoma and primary hyperparathyroidism.

Amongst patients with MTC and phaeochromocytoma, variants at codon 634 were the commonest, being present in 76.5% of those with a pathogenic variant. This is in contrast to patients with only MTC, amongst whom, p.(Val804Met) was the most common variant. Furthermore, when the whole series was grouped according to pathogenic variants by codon, 20 of the 31 patients (64.5%) with pathogenic variants at codon 634 had phaeochromocytoma compared with only 7 of 75 patients (9.3%) with pathogenic variants at all other codons. One patient in this group who was diagnosed with MTC and phaeochromocytoma at the age of 13 years with uncertain family history was heterozygous for the MEN2B-associated germ-line p.(Met918Thr) pathogenic variant.

3.6 | Index patients with phaeochromocytoma and primary hyperparathyroidism

Of the 22 index patients with a diagnosis of both phaeochromocytoma and primary hyperparathyroidism, only 1 (4.5%) was found to have a germline RET pathogenic variant (p.Cys634Arg). None of these patients had a family history of MEN2-related conditions.

3.7 | Index patients with isolated phaeochromocytoma

165 patients were referred for germline RET testing with a diagnosis of isolated phaeochromocytoma, with a median age of 39 years (range: 1-88 years), and only 6 were found to harbour pathogenic variants (3.6%). Of these, 4 were found to have pathogenic variants at codon 634, and one had a family history of MEN2-associated conditions.

3.8 | Index patients with isolated primary hyperparathyroidism

There were 56 patients with isolated primary hyperparathyroidism. The median age at referral was 36 years (range: 12 - 73 years), and 11
patients (19.6%) had a family history of MEN2-associated conditions. No patients with isolated primary hyperparathyroidism were found to harbour a pathogenic RET variant. One patient had a previously reported RET variant, p.(Asn783Ser), which is currently of uncertain clinical significance. This variant has previously been reported in a patient with MTC.\textsuperscript{15}

### 3.9 Index patients with the MEN2B phenotype

The final group comprised 44 patients with a clinical suspicion of MEN2B based on phenotypic features such as mucosal neuromata, intestinal ganglioneuromata, corneal nerve thickening and marfanoid body habitus, with or without MTC or phaeochromocytoma. Six of these patients had MTC only, four had phaeochromocytoma only, and one had both MTC and phaeochromocytoma; however, 33 patients did not have MTC or phaeochromocytoma at the time of referral (Table 1). The median age of this group without MTC or phaeochromocytoma was 12 years, and they were referred purely on the basis that their phenotype was suspicious for MEN2B. Germline RET pathogenic variants were identified in six of the 11 patients with phenotypic features of MEN2B plus MTC and/or phaeochromocytoma (54.5%), and seven of the 33 patients with phenotypic features of MEN2B but no diagnosis of MTC of phaeochromocytoma at the time of referral (21.2%). The p.(Met918Thr) variant accounted for every pathogenic variant in this latter group, and the median age at testing was 9 years. There was one patient with an isolated phaeochromocytoma and marfanoid features included in this group who was heterozygous for the p.(Cys634Tyr) variant, which is not associated with MEN2B.

### 4 DISCUSSION

This study represents the largest series of germline RET testing in a clinical diagnostic setting in the United Kingdom. 1,058 index patients were referred over a 21-year period, and a further 551 unaffected family members underwent predictive testing. Amongst the 690 index patients with a diagnosis of isolated MTC, the principle finding was of a lower than expected overall pathogenic variant rate (9.9%). This may be due to less stringent testing criteria in a clinical setting than in the research setting; however, the rates of pathogenic variant detection in those with other MEN2-associated conditions and with a positive family history were also lower than expected (29% and 36.4%, respectively). We did find an increase in detection rate amongst those referred since 2010 compared with those referred prior to 2010, and this may reflect evolution in testing over recent decades, although the indications for tests before and after 2010 were not comparable so this information should be interpreted with caution. Our finding that 29% of unaffected family members were found to have pathogenic variants on predictive testing suggests that this is a valuable use of resources.

Amongst patients with presumed sporadic MTC, there was an 8.5% rate of germline RET pathogenic variants, which is in keeping with figures from other studies, which range from 6.2% to 14.9%,\textsuperscript{7,16-18} and justifies international guidelines to offer germline RET testing to all patients diagnosed with MTC.\textsuperscript{1,8} The use of testing somatic RET mutations in presumed sporadic MTC in order to inform prognosis has been discussed extensively elsewhere, and there is evidence that certain somatic RET mutations predict a poorer outcome.\textsuperscript{19-23} Despite this increasing body of evidence, guidelines on the management of sporadic MTC disagree on the use of somatic RET analysis in the routine clinical management of sporadic MTC.\textsuperscript{1,24} Another use for somatic RET analysis is to confirm the true sporadic nature of the disease in cases where a somatic variant is identified that was not present on germline analysis, thus providing reassurance to family members regarding their risk of hereditary disease. In addition, it can guide the use of targeted therapy in advanced disease.

The rate of detection of germline pathogenic variants in patients with confirmed hereditary MTC has been reported as high as 98.3%.\textsuperscript{16} In our series, 39.4% of index patients with a family history of either MTC or another MEN2-related condition were found to harbour pathogenic variants in RET. The rate was 29% in those with MTC plus phaeochromocytoma and/or primary hyperparathyroidism, with 80% of these being in codon 634. These rates are lower than expected given the high likelihood of a MEN2 syndrome in patients with both MTC and phaeochromocytoma, and raise the possibility that either the clinical information provided by referring clinicians was not accurate, or that a RET pathogenic variant was indeed present and not detected in a proportion of cases. Patients with no variant identified were referred from across the UK with no evidence of geographical clustering, and it is notable that despite the introduction of screening for exons 5 and 7, no additional pathogenic variants were identified. In the case of familial MTC, it is possible that either hitherto unidentified variants in RET, or variants in genes other than RET have a role in tumorigenesis. For example, Smith et al identified a novel germline frameshift deletion in the ESR2 gene using exome sequencing in a patient with MTC and 3 family members with C-cell hyperplasia but no identifiable germline RET pathogenic variant.\textsuperscript{25} They showed that the effect was loss of ERβ and indirect upregulation of RET, which raises the possibility of alternative pathways in the development of MTC.

Overall, the most commonly affected codons amongst the UK patients with MTC were 634 in exon 11, followed by 804 in exon 14. The largest studies on RET analysis in MTC are from Italy, and they consistently find codon 804 to be most commonly mutated in MTC,\textsuperscript{16,26} however, codon 634 has been reported as the most commonly affected in both Germany and Brazil.\textsuperscript{27,28} Interestingly, a large Danish study found that variants at codon 611 were most prevalent.\textsuperscript{29} In keeping with reports from Italy, we also found that cysteine pathogenic variants at codon 634 were more common in those with MTC with a phaeochromocytoma.\textsuperscript{16} The lowest pathogenic variant detection rates in the current series were in the subgroups of index patients with isolated primary hyperparathyroidism (0%) and isolated phaeochromocytoma (3.6%).
A previous series of 271 patients with apparently sporadic phaeochromocytoma reported a germline RET pathogenic variant rate of 4.8%.\textsuperscript{30} Furthermore, our finding that 21 out of 32 patients (65.6%) with pathogenic variants at codon 634 had phaeochromocytoma compared with only 7 of 76 patients (9.2%) with pathogenic variants at all other codons supports findings by other authors that pathogenic variants at codon 634 are associated with phaeochromocytoma in patients with MEN2.\textsuperscript{31,32} Primary hyperparathyroidism is a relatively common condition, with an incidence of 6.72 per 1000 in the UK population.\textsuperscript{23} This may account for the low rates of pathogenic variant detection in those with primary hyperparathyroidism, even in addition to MTC or phaeochromocytoma. These patients may in fact have sporadic MTC or sporadic phaeochromocytoma with incidental co-existing primary hyperparathyroidism, rather than MEN2A. Our finding that none of the 56 patients with isolated primary hyperparathyroidism had a pathogenic variant in the RET gene (which is included in the gene panel testing for primary hyperparathyroidism in the UK) is consistent with a recent study showing that primary hyperparathyroidism is a rare presenting manifestation of MEN2.\textsuperscript{34} These low rates in patients with isolated phaeochromocytoma and primary hyperparathyroidism should be borne in mind when counselling these patients for genetic testing.

In our series, patients with phenotypic features of MEN2B plus either MTC or phaeochromocytoma were most likely to harbour a RET pathogenic variant, with a detection rate of 54.5%. A single feature of MEN2B was sufficient for inclusion in this group, and some features such as a marfanoid habitus are somewhat subjective, meaning that it is inevitable that some patients in this group in fact had sporadic MTC or phaeochromocytoma, or MEN2A in the case of the individual with a pathogenic variant at codon 634. Furthermore, it is well recognized that some patients can present with the characteristic physical phenotypes of MEN2B but do not carry RET pathogenic variants or develop endocrinopathies (such as MTC or phaeochromocytoma), the condition termed ‘pure mucosal neuroma syndrome’.\textsuperscript{35} We have recently shown that several of these patients carry pathogenic variants in the SOS1 gene, providing evidence that this condition is a distinct clinical condition unrelated to MEN2B despite similar physical phenotype.\textsuperscript{36} On the other hand, seven index patients with phenotypic features of MEN2B but not known to have MTC or phaeochromocytoma at the time of referral were found to carry the MEN2B-associated pathogenic variant p.(Met918Thr) in this study, suggesting that the physical phenotype (such as, mucosal neuromas, corneal nerve thickening and Marfanoid body habitus) can be the first presenting feature of MEN2B.

4.1 Strengths and limitations

This study represents the largest UK series of germline RET analysis to date and gives a perspective on the realities of molecular genetic diagnostic testing in a clinical setting. The main limitations are firstly the limited clinical information regarding index patients and their family members on referral forms in some cases. This makes it difficult to confirm the clinical diagnosis and therefore correlate this with identified pathogenic variants. To try and capture as much clinical information as possible, including family history, ethnicity and age at diagnosis, the laboratory has a request form that can be downloaded from our website by the clinician for completion. Secondly, although patients referred from outside the UK were excluded in the analysis of specific pathogenic variants, we were not able to ascertain the ethnicity of the included patients. It is known that RET variants in MTC and MEN2 differ according to geographical population,\textsuperscript{37} and therefore, the ethnicity of patients in our series may have had an effect on the pathogenic variants identified. Finally, although samples from across the UK were tested, the applicability of our findings to other populations is limited, and we were unable to obtain follow-up data to identify metachronous MEN2-associated conditions diagnosed since referral for RET analysis.

5 CONCLUSIONS

In a UK clinical setting as in other settings, the rate of germline RET pathogenic variants in presumed sporadic MTC is significant, and all patients with a diagnosis of MTC should be screened in order to identify familial cases. In patients with clinical manifestations of MEN2A, the majority did not receive a confirmatory genetic diagnosis. The rate of detection in patients with isolated phaeochromocytoma is not insignificant, and therefore, it seems this is an appropriate indication for germline RET analysis; however, the absence of a single positive result amongst patients with isolated primary hyperparathyroidism suggests that this may not be an effective use of resources. Finally, the use of somatic RET analysis to confirm the diagnosis of sporadic MTC in patients with no identified germline RET variants may be a useful adjunct both in terms of reassuring family members about the lack of a heritable pathogenic germline variant, and risk-stratifying sporadic tumours based on somatic variants.

CONFLICT OF INTEREST

SE is a Wellcome senior investigator. No other author has any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

JMF contributed to data collection, analysis and manuscript preparation; JAS and RC contributed to manuscript preparation; CB contributed to analysis and manuscript preparation; SE and BV contributed to conceptualization and manuscript preparation; MO contributed to conceptualization, data collection, analysis and manuscript preparation.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.