MULTILOCATIONS OF CODON 918 IN THE RET PROTO-ONCOGENE CORRELATE TO POOR PROGNOSIS IN SPORADIC MEDULLARY THYROID CARCINOMAS


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ABSTRACT

The hereditary multiple endocrine neoplasia syndromes types 2A and B (MEN 2A and B) were recently linked to germline mutations in the RET proto-oncogene, altering one of five cysteine residues in exon 10 or 11 (MEN 2A), or substituting a methionine for a threonine at codon 918 in exon 16 (MEN 2B). The latter mutation also occurs somatically in some sporadic medullary thyroid carcinomas (MTC), and has in a previous study been correlated with a less favorable clinical outcome. In the present study, 46 MTCs were selected for investigation of the codon 918 mutation. The mutation was found in 29 tumors (63%), and was significantly correlated with a poor outcome, with regard to distant metastasis or tumor recurrence (p<10⁻⁴). Two tumors showed multifocal growth and C-cell hyperplasia, and these patients were therefore also investigated for germline mutations in exons 10, 11 and 16. The codon 918 mutation was found only in the tumors, thus of somatic origin. The RET codon 918 mutation may have prognostic impact, and therefore preoperative assessment may influence decision-making in the treatment of patients suffering from MTC.

Mutations of the RET proto-oncogene are responsible for the hereditary multiple endocrine neoplasia syndromes (MEN), which are characterized by the development of tumors in distinct endocrine organs. MEN 2A and MEN 2B are caused by germline mutations in the RET proto-oncogene, with the latter mutation being particularly associated with a poor prognosis.

In the present study, 46 medullary thyroid carcinomas (MTCs) were selected for investigation of the codon 918 mutation. The mutation was found in 29 tumors (63%), and was significantly correlated with a poor outcome, with regard to distant metastasis or tumor recurrence (p<10⁻⁴). Two tumors showed multifocal growth and C-cell hyperplasia, and these patients were therefore also investigated for germline mutations in exons 10, 11, and 16. The codon 918 mutation was found only in the tumors, thus of somatic origin. The RET codon 918 mutation may have prognostic impact, and therefore preoperative assessment may influence decision-making in the treatment of patients suffering from MTC.
other clinical symptoms of recurrence. This patient's tumor was one of the two exhibiting multifocal growth and C-cell hyperplasia. The other patient with a tumor showing these phenomena was a 23 year old male with extensive metastases at initial operation.

DNA extraction

Three patients from group I and six patients from group II have been reported previously (11). Tumor DNA was, in these cases, extracted from fresh frozen tissue. In all other cases, tumor DNA was extracted from archival paraffin embedded tissue. In short, four 5 μm sections were de-paraffinized with xylene, and tumor cell areas dissected into tubes containing lysis buffer (0.05 M Tris-HCl pH 7.9, 0.15 M NaCl, 5 mM EDTA, 1% SDS, Proteinase K 500 μg/ml). The samples were then incubated at +45°C overnight, followed by phenol-chloroform extraction and ethanol precipitation.

Polymerase chain reactions (PCR) and mutation analysis

Amplification of exon 16 of RET was done by PCR using the primers 16F (5'-AGG GAT AGG GCC TGG GCT TC-3') and 16R (5'-TAA CCT CCA CCC CAA GAG AG-3') as previously described (11). If the DNA used in this PCR was obtained from archival tumor tissue, another PCR was performed using nested primers, one of them biotinylated: N16F (5'-CTT CAA TGC TTT ATT CCA TCT TCT C-3') and N16R (5'-CAA CAC CCA CAC TTA CAC ATC AC-3'), resulting in a 117 basepair product. Nested PCR was done with 8 μl of first PCR product as template and 1.5 mM MgCl2 in a final volume of 100 μl. Thermal cycling conditions were incubation at 95°C for 4 min, 35 step cycles at 95°C for 30 s, at 54°C for 30 s, and at 72°C for 30 s, with a final extension for 5 min at 72°C. Purified PCR products (Wizard PCR Prep, Promega) were subjected to (A) sequencing from both DNA strands using Dynabeads (Dynal) and the Sequenase kit (USB), and (B) restriction enzyme digest by FokI as described (11). Sequence reactions were run on 6% denaturing polyacrylamide gels, and FokI digests on 1% LMP/1% normal agarose gels.

Blood, normal thyroid tissue and tumor tissue from two patients were subjected to sequence analysis of RET exons 10,11 and 10 for reasons described above. These analyses were performed as described (11).

Statistical analysis

Fisher's exact test was used for statistical analysis.

Results

In this study, genomic DNA from 46 sporadic MTCs was analyzed by direct sequencing of RET exon 16. To increase the reliability of the analysis both DNA strands were sequenced. Informative results were obtained from 41 of the tumors. In the remaining five cases, mutation analysis was based on FokI digest results.

Of the 46 patients with MTCs selected for the study, 31 (67%) showed signs of incurable or recurrent disease. Of these, 26 (84%) showed the mutation in codon 918 of RET, which substitutes a methionine (ATG) with a threonine (ACG). Of the tumors from the 15 patients without recurrent MTC, 12 (80%) did not have any codon 918 mutation. A statistically significant correlation was made between the presence of the RET codon 918 mutation and a poor outcome (p<10^-4) (Table 1). The mean age of the patients with mutations in the tumor tissue was similar to that in patient group II. The mutations were apparently heterozygous, as the wildtype sequence in all cases was visible to at least the same extent as the mutant (Figure 1).

The three patients in Group I whose tumors showed the RET codon 918 mutation, did not significantly differ from the patients in Group II with regard to age at diagnosis, tumor size, or the surgical procedures used. Similarly, the five patients in Group II whose tumors did not show the mutation, were not different from the patients in Group I with regard to clinicopathological data, or surgical treatment.

In the two cases exhibiting multifocal tumor growth and C-cell hyperplasia, no mutation was found in exons 10,11 and 16 when analyzing DNA from blood and normal thyroid tissue. However, the codon 918 mutation was in both cases found in the tumor tissue, thus proven to be of somatic origin. These patients were 23 and 61 years of age at diagnosis, respectively.

![Fig. 1. Autoradiograms of the partial sequencing of RET exon 16 in two tumors. In the tumor to the left, a methionine (ATG) is substituted with a threonine (ACG) at codon 918, as indicated by the arrow.](image-url)

| Table 1. Summary of the RET codon 918 mutations in the 46 sporadic MTCs with regard to the patients clinical outcome. The correlation is statistically significant (p<10^-4). For definition of patient groups, see Material and Methods. |
|-----------------|-----------------|-----------------|
|                  | Group I. Disease-free | Group II. Recurrent disease | Total |
| No mutation     | 12               | 5               | 17    |
| Mutation        | 3                | 26              | 29    |
| Total           | 15               | 31              | 46    |
Discussion

Somatic RET mutations have previously been reported in sporadic MTCs (6, 10, 11). These mutations affect codon 918, substituting a methionine (ATG) with a threonine (ACG), the same mutation found constitutionally in virtually all MEN 2B patients. About one third of sporadic MTCs so far reported exhibit this mutation. Recently, a missense mutation of codon 768 was reported in some sporadic MTCs without the codon 918 mutation (12). With further studies, it may turn out that the majority of MTCs carry a mutation somewhere in RET.

In this study, we have only investigated for the codon 918 mutation. The RET codon 918 mutation was strongly correlated to poor prognosis (p<10^{-4}). The comparably high proportion of tumors with this mutation in the material (63%) probably reflects our selection of patients with mainly recurrent MTC, and is thus not in disagreement with earlier reports where a lower general mutation rate was given (6, 10).

The exact mechanism by which this mutation acts on the cellular level is not clearly understood. However, recently presented data show that this specific mutation, which alters an amino acid within a highly conserved substrate-recognition site in the catalytic core of the tyrosine kinase, probably results in a shift of substrate specificity (9). A methionine at this site is found in cytoplasmatic tyrosine kinases rather than in receptor tyrosine kinases, e.g. RET. It has been shown that the MEN 2B mutation in vitro results in a shift in peptide substrate specificity, thus involving downstream signalling proteins not normally activated by RET (13). This phenomenon could explain the reason for MEN 2B tumors being more aggressive than their counterparts (2, 3). Subsequently, it may explain why the mutation is correlated to poor prognosis in sporadic MTC.

Our study shows that the detection of a RET codon 918 mutation in sporadic MTC indicates a high risk of recurrence. Pre-operative detection of the mutation may implicate the need for extended lymph node dissection at initial surgery, or a more careful search for yet undetected metastases. (A rapid method for pre-operative analysis of RET codon 918 mutation on fine needle aspirates will be presented elsewhere, and is available from the authors.) RET codon 918 mutation analysis together with other clinical data will help individualize the treatment for patients with MTC. In addition, the detection of this somatic mutation makes it highly unlikely that the patient carries a germline mutation elsewhere in RET which predisposes to pheochromocytoma (10). The risk of an undetected pheochromocytoma by the time of surgery would therefore be markedly reduced. As an example, the only two tumors in this material resembling a hereditary MTC variant (multifocal growth pattern and C-cell hyperplasia) were not due to a germline mutation in RET, but showed the somatic codon 918 mutation. However, since a stem cell mutation due to an early mitotic event cannot be excluded, it may be appropriate to screen for the corresponding germline mutation in the offspring of such patients.

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References