Heterogeneous Mutation of the RET Proto-oncogene in Subpopulations of Medullary Thyroid Carcinoma

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ABSTRACT

Mutations in the RET proto-oncogene are associated with the pathogenesis of medullary thyroid carcinoma (MTC). In an attempt to understand this process, we examined microdissected subpopulations from MTC and multiple metastases from these tumors. Approximately 80% of sporadic MTCs had at least one subpopulation with the RET codon 918 mutation, which is a mutation previously detected in sporadic MTC as a somatic mutation and in multiple endocrine neoplasia type 2B as a germ-line mutation. However, the distribution of this mutation was nonhomogeneous, occurring only in subpopulations in most tumors and among subsets of multiple metastases, thus implying that although the codon 918 mutation could be an early event, it is not necessarily an early or essential event in tumorigenesis. This heterogeneity suggests either that the codon 918 mutation can arise as an event in progression within a metastatic clone or within a single tumor, or that MTC can be of polyclonal origin. Of significance, one of two multiple endocrine neoplasia type 2A MTCs or within a single tumor, or that MTC can be of polyclonal origin. Of significance, one of two multiple endocrine neoplasia type 2A MTCs carried a somatic mutation at codon 918, in addition to the RET mutation present in the germ-line. We found no correlation between the presence of other somatic genetic events, such as loss of heterozygosity on chromosomes arms 1p and 22q, and RET mutation status in the various subpopulations of MTC.

INTRODUCTION

MTC,3 a neoplasm of the calcitonin-secreting thyroid C cells, may occur sporadically or as a component of the inherited cancer syndrome MEN 2 (1). The majority of all three clinical subtypes of the MEN 2 syndromes are caused by germline mutations in the RET proto-oncogene (2), which encodes a receptor tyrosine kinase expressed predominantly in tissues and tumors of neural crest origin (3, 4). MEN 2A, which comprises MTC, pheochromocytoma, and parathyroid hyperplasia, is a consequence of a germline missense mutation in one of five cysteine codons in the cysteine-rich extracellular domain of RET (5–9). FMTC, characterized by the presence of MTC as the only phenotype in the family, is associated with mutations similar to those in MEN 2A and, rarely, with a missense mutation in codon 768 or 804 in the tyrosine kinase domain (5–7, 10, 11). MEN 2B, which is similar to MEN 2A, except for the presence of developmental abnormalities and a typical habitus, is caused by a germline mutation in codon 918, which lies in the substrate specificity pocket of the RET tyrosine kinase catalytic core (9, 12–15).

Like that of other tumors, the pathogenesis of MTC probably involves the accumulation of a somatic loss of tumor suppressor function, as manifested most frequently by LOH on chromosome arms 1p and 22q (16), and/or activation of proto-oncogenes (reviewed by Eng and Ponder; Ref. 17). Widely varying frequencies (between 23 and 66% in series with 10 or more) of sporadically occurring MTCs have been reported to have a somatic RET mutation in the MEN 2B-specific codon 918 (12, 13, 18–20), an alteration that has been hypothesized to be the initiating or one of the early events in the tumorigenesis of sporadic MTC. In contrast, two other somatic mutations, at codons 768 (exon 13) and 883 (exon 15), are relatively rare, detected in ≤10% of sporadic MTCs (10, 19, 20).

We examined the RET mutation status in MTC metastases and subpopulations within individual tumors from sporadic and MEN 2 cases. The aims of the study were to determine whether MTCs are monoclonal with respect to RET codon 918 mutation status, and whether the patterns of other genetic events allow these to be assigned to a sequence in tumor progression.

MATERIALS AND METHODS

MTC Tumors. Eighty-seven MTC blocks from 33 patients, 82 from 28 sporadic cases, 2 from 2 MEN 2A cases, and 3 from 3 MEN 2B cases, were analyzed. All were obtained as paraffin-embedded tissue.

MTC was considered sporadic if the patient did not have multiple primary tumors, and there was no history of a first- or second-degree relative with MTC or pheochromocytoma. Information regarding the presence of C-cell hyperplasia (a relative indicator of hereditary disease) in the thyroidectomy specimen is incomplete and, therefore, has been omitted for clarity. The MEN 2 cases belong to families in which the diagnosis had been made based on pathology of thyroid and adrenal tumors and the presence of a germline mutation in either a MEN 2A- or MEN 2B-specific RET codon.

Isolation of Genomic DNA. DNA was extracted from archival tissue as described (21, 22). After examination of a H&E-stained section, serial sections of paraffin-embedded tissue were initially divided into normal tissue and MTC. The MTC was then microdissected into further sections based on division by connective tissue septae. When septae were not present, which was rare, the MTC was randomly divided.

PCR Amplification and Mutation Detection. An initial genomic amplification encompassing exon 16 of the RET proto-oncogene was carried out by the PCR and the primer pair CRT 5G and CRT 5H or CRT 6 and CRT 6 RET 16 (12, 13). Ten to 50 ng genomic DNA were amplified using the conditions described previously (12, 13) or according to the recommendation of the manufacturer (Red Hot Thermus aquaticus DNA polymerase; Advanced Biotechnologies, Surrey, United Kingdom). No more than 25–30 cycles of amplification were carried out. A secondary amplification was performed with the primers f16Rsa (22) and either CRT 5H or CRT 16 RET under similar conditions, except for an annealing temperature of 60°C. Similarly, amplicons encompassing RET exons 13 and 15 were created with the primer pair CRT 4E and CRT 4F and CRT 17S and CRT 17A, respectively (10, 23), under conditions identical to those described above, except for an annealing temperature of 55°C for the exon 13 primers. PCR products were purified through low-melting-point agarose and thereafter sequenced.

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3 The abbreviations used are: MTC, medullary thyroid carcinoma, MEN 2, multiple endocrine neoplasia type 2, FMTC, familial MTC; LOH, loss of heterozygosity.

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eluted using the Wizard PCR preparation kit (Promega, Southampton, United Kingdom). The final exon 16 PCR products were subject to digestion with Rsal and fractionated on 3% agarose gels. Digestion with Rsal denotes the presence of the codon 918 ATG→ACG missense mutation (22). Lack of digestion denotes the presence of the wild-type sequence. This technique has been validated previously against samples with known sequence data (22). The molecular sensitivity of this technique is such that the codon 918 mutation can be detected at a minimal dilution of 1:200 (data not shown). As a control, these products were also subject to Sau3AI digestion, and all were digested with this enzyme. This site is ubiquitously present within exon 16 and is not dependent on the presence or absence of the codon 918 mutation.

The exon 13 and 15 amplicons were digested with Alul. The absence of the site denotes the presence of the codon 768 and codon 883 mutations, respectively. No subpopulation had a codon 768 mutation.

LOH Analyses. LOH was assessed using the markers D1S211 and D1S233, the markers D2S272 and D2S284, which are in proximity to the NF2 locus, as described previously (16), and D10S141 and ZNF22, markers flanking RET (24–26).

RESULTS

RET Mutations in Subpopulations of MTC. To determine whether somatic RET mutations occurred in subpopulations of MTC, we carried out analyses in separate metastatic clones and in dissected subpopulations from individual tumors. Eighty-two MTC blocks from 28 patients with sporadic MTC, 80% had at least 1 subpopulation that had a somatic codon 918 mutation. In addition, of the 248 subpopulations from these 28 patients, 38% had the codon 918 mutation, whereas 62% did not. Interestingly, one MTC from 1 of the 2 MEN 2A patients with the germline mutation in codon 634 TGC→CGC also had a somatic codon 918 mutation (Fig. 2, Lanes 9–13).

As a positive control, the MEN 2B MTCs were shown to have the codon 918 mutation in every subpopulation of MTC and in areas of normal thyroid tissue, as expected (Fig. 2, Lanes 18–24). As negative controls, available nonneoplastic thyroid tissues within some of the sections from non-MEN 2B patients were shown not to have codon 918 mutations in all subpopulations.

To determine whether the nonuniform presence of the codon 918 mutation within subpopulations of a single tumor or within some metastases from a single individual was due to subsequent loss of one or the other RET allele, LOH analysis was performed with markers closely flanking the RET locus. No LOH was observed (data not shown).

LOH lp and 22q. LOH at lp and 22q has been shown to occur with some frequency in both familial and sporadic MTCs (16). Thus, we determined whether the RET mutation status in each MTC subpopulation was related to LOH at these loci. Of the 274 MTC subpopulations that were analyzed for mutation status and LOH, 105 did not have LOH at either lp or 22q; 26 were uninformative at one or the other locus (i.e., homozygous or PCR failure); and 143 had LOH at either lp or 22q (Table 2). Within any single tumor, LOH was not clonal, but certain subpopulations within that tumor exhibited loss, whereas others did not (Fig. 3). Two hundred forty-eight tumor subpopulations were informative for codon 918 and codon 883 mutation status and the presence or absence of LOH at lp or 22q markers. Overall, there was no significant correlation between the presence or absence of the RET codon 918 mutation and LOH at lp or 22q markers.

The RET codon 918 mutation status and LOH at lp and 22q were further examined in each of the three individuals with 9 or more metastases. For this part of the analysis, each of the metastases was divided into subpopulations. The first patient (Table 1, patient F), with 9 metastases, could not be analyzed, because seven of these metastases were uninformative (i.e., homozygous or had PCR failure) at either all lp or 22q markers or both markers. The second (Table 1, patient D) with 11 others did not (e.g., Fig. 2, Lanes 14–17). In sum, of 28 patients with sporadic MTC, 80% had at least 1 subpopulation that had a somatic codon 918 mutation. In addition, of the 248 subpopulations from these 28 patients, 38% had the codon 918 mutation, whereas 62% did not. Interestingly, one MTC from 1 of the 2 MEN 2A patients with the germline mutation in codon 634 TGC→CGC also had a somatic codon 918 mutation (Fig. 2, Lanes 9–13).

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**Table 1** Number of MTC metastases with RET mutation in six individuals

<table>
<thead>
<tr>
<th>Patient</th>
<th>Primary MTC</th>
<th>With mutation</th>
<th>Total</th>
<th>918 Mutation+</th>
<th>883 Mutation+</th>
<th>918− and 883−</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>1 (918 mutation+)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>NA†</td>
<td>NA</td>
<td>11</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>NA†</td>
<td>NA</td>
<td>32</td>
<td>11</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

†+, positive; −, negative.

‡NA, primary tumor not available.
metastases, had 33 subpopulations that were scored for RET codon 918 mutation status and LOH. Of the 33 subpopulations, 11 had the codon 918 mutation, and 22 did not. Of the 11 with a codon 918 mutation, 9 also had LOH at a 22q marker. Of the 22 without the mutation, 11 exhibited LOH at a 22q marker. In contrast, only 4 subpopulations had LOH at a 1p marker, and all 4 did not have a codon 918 mutation. The third individual (Table 1, Patient E) had 64 subpopulations that were scorable for mutation status and LOH. Of the 23 subpopulations that were codon 918 mutation positive, 2 and 6 had LOH at 1p and 22q, respectively. Of the 41 subpopulations without a codon 918 mutation, 8 and 6 had LOH at 1p and 22q, respectively.

DISCUSSION

By analyzing subpopulations of MTC, either several metastases from a single individual or different regions within a primary MTC, we have shown that 80% of individuals with sporadic MTC have at least one tumor cell subpopulation with a mutation in RET codon 918. The nonuniform distribution of codon 918 mutations within MTC may account for the wide range of reported frequencies of codon 918 mutations in MTCs, ranging from a low of 23% to a high of 66% in series that comprise 10 or more tumors (12, 13, 18-20). Indeed, 38% of our subpopulations had the codon 918 mutation. In light of our data, it seems that the detection of codon 918 mutations may depend on which region of a primary tumor or which metastasis is analyzed. Conceivably, these observations could be common to other tumors and other genes as well.

Table 2. Somatic RET mutation status and LOH in MTC subpopulations

<table>
<thead>
<tr>
<th>No. of informative subpopulations sampled</th>
<th>No. RET 918 or 833 Mutation+ (%)a</th>
<th>No. RET Mutation− (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>No LOH 1p or 22q</td>
<td>105</td>
<td>38 (36)</td>
</tr>
<tr>
<td>LOH 1p</td>
<td>71</td>
<td>25 (35)</td>
</tr>
<tr>
<td>LOH 22q</td>
<td>37</td>
<td>16 (46)</td>
</tr>
<tr>
<td>LOH 1p and 22q</td>
<td>37</td>
<td>15 (41)</td>
</tr>
<tr>
<td>Total</td>
<td>248</td>
<td>94 (38)</td>
</tr>
</tbody>
</table>

*+*, positive; −, negative.

It does not seem that the codon 918 mutation represents an early event in tumorigenesis in the majority of MTCs examined here, despite the majority of these tumors having at least one tumor cell population with the mutation. If the codon 918 mutation were an early event, then we would expect the majority of subpopulations within individual primary MTCs to have this mutation (27), unless the allele bearing the mutation is lost once a selective advantage has been gained, and there was no evidence of this. The observations suggest that codon 918 mutations have arisen during clonal evolution of the tumor; i.e., they arise within an established primary tumor or within a metastatic clone. An alternative, which is less likely in the case of sporadic disease, is that MTC and MTC metastases could have a polyclonal origin. Our finding that somatic codon 918 mutations seem to occur frequently and in subsets of cells within sporadic MTC suggests that they may not necessarily be associated with a strong selective advantage. Otherwise, one might expect that cells with the mutation would form a larger proportion of the tumor. Indeed, one individual had a primary tumor without the codon 918 mutation and subsequently developed eight metastases, two of which contained the codon 918 mutation. At least in this case, the codon 918 mutation seemed to develop during clonal evolution during the metastatic process. However, in just over one-half of the metastatic subpopulations sampled, there was no codon 918 or codon 883 mutation detected; therefore, there is no strong evidence that these events are frequent accompaniments of metastatic tumor progression.

MTCs are commonly slow growing, and it may be that clonal evolution of the tumor occurs over a protracted time scale. The finding of the codon 918 mutation or codon 883 mutation in different metastatic deposits in a single individual suggests that either mutation can play a role in tumor progression and is an unequivocal demonstration of clonal heterogeneity.

The presence of a somatic codon 918 mutation in an MTC from a MEN 2A patient with a germline mutation at codon 634 is noteworthy. In earlier studies, nonmicrodissected MTCs from MEN 2A and FMTC patients were examined for the presence of somatic codon 918 mutations, and none were found (18–20, 28). These observations had
suggested that the germline RET mutation in one of the cysteine codons would be sufficient for transformation, as evidenced by in vitro transfection studies (9, 29). However, the presence of both germline and somatic mutations of RET in the single MEN 2A MTC is unlikely to be redundant; more likely, the somatic mutation adds to the effect of the germline mutation (30). Additional studies of the somatic RET mutation in MEN 2A and FMTC tumors are in progress to determine whether this is observed more frequently, especially at the subpopulation level, whether both mutations are allelic, and whether these reflect more aggressive tumors.

Just as the RET codon 918 mutation is heterogeneous within MTCs, LOH of 1p and 22q markers is not homogeneous within a single tumor or among metastases in the majority of the tumors studied. The nonhomogeneous presence of LOH is usually attributed to events related to tumor progression, either as a cause or an effect. For example, in the well-worked-out model of colon carcinoma progression, the paucity of 17p losses in colonic adenomas and the high frequency in frank carcinoma and metastases are explained by the loss of a gene or genes, including the tumor suppressor gene p53, leading to tumor advancement (reviewed by Fearon and Vogelstein; Ref. 27). This model may be extended to explain our observations. In MTC, we showed that the nonhomogeneous presence of an RET mutation and/or LOH occurred even within a single tumor or a single metastasis, suggesting a less rapid overgrowth of the clones bearing these changes. The distribution of 1p and 22q losses across the subpopulations in primary and metastatic tumors shows no evidence of which one could construct a sequence of these events and a codon 918 mutation in tumor progression.

This opportunity to look for specific mutations in microdissected tumors has revealed that somatic mutations in RET may be more common than thought and has revealed an unsuspected degree of heterogeneity, most clearly seen in the tumor metastases with codon 918 mutation and codon 883 mutation-positive clones. This underscores the need to be aware of the heterogeneity, whether in molecular diagnosis using tumor templates or in potential therapy directed at molecular targets.

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