Somatic Mutations of the RET Proto-oncogene Are Not Required for Tumor Development in Multiple Endocrine Neoplasia Type 2 (MEN 2) Gene Carriers


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Abstract

Germ line mutations in one allele of the RET proto-oncogene predispose to the multiple endocrine neoplasia type 2 (MEN 2) syndromes. To investigate whether these inherited mutations alone can cause the development of tumors in vivo (oncogene model) or whether somatic mutations in the homologous RET allele are required for tumorigenesis (tumor suppressor gene model), we analyzed the entire coding region of both alleles of the RET gene in two MEN 2A and two MEN 2B tumors by reverse transcription-PCR and direct sequencing. No tumor-specific mutations could be detected in either allele of the RET gene in these tumors. Unlike the molecular mechanism in other hereditary tumor syndromes, somatic mutations in the homologous allele are apparently not required in MEN 2 tumorigenesis. Thus, RET genes with MEN 2-specific germ line mutations act as dominantly transforming oncogenes in vivo.

Introduction

The dominantly inherited cancer syndromes MEN 2A and MEN 2B were found to be associated with specific germ line mutations in the RET gene (1–5). MEN 2A is characterized by MTC, pheochromocytoma (in about 50% of gene carriers), and sometimes parathyroid adenoma (6). MEN 2B is also characterized by MTC and pheochromocytoma, but with an earlier age of onset, and also by mucosal ganglioneuromas and a marfanoid habitus (7). MEN 2 resembles other hereditary tumor syndromes in the dominant pattern of inheritance. A germ line mutation of one allele of a tumor suppressor gene leads to a cancer predisposition in these other diseases. However, tumors will only develop after functional loss of the homologous allele (8). It was conceivable that the MEN 2 gene would also act as such a tumor suppressor gene. However, loss of heterozygosity for markers near the disease locus, characteristic of tumor suppressor genes, was found in only a small percentage of MEN 2-related tumors (9, 10). The RET locus itself was also investigated for loss of constitutional heterozygosity in MEN 2 tumor DNA, but again no gross abnormalities could be detected (11). Apparently, the etiology of MEN 2 at a chromosomal level was different from what had been found in other hereditary tumor syndromes.

The RET gene encodes a transmembrane receptor tyrosine kinase and had been identified as a proto-oncogene (12). Thus, the finding of congenital mutations in the RET gene in families with MEN 2A and MEN 2B suggested that the classical tumor suppressor gene model might not apply for these traits (1, 13). In almost all MEN 2A families investigated, a missense mutation in either exon 10 or exon 11 of the RET gene has been detected, invariably affecting one of five cysteine residues in the extracellular region of the RET protein (1, 2, 14). In almost all MEN 2B-families, as well as in de novo MEN 2B patients, a specific missense mutation in exon 16 affecting the tyrosine kinase domain 2 of the RET protein has been described (3–5). A schematic representation of the RET gene with the positions of the MEN 2-specific germ line mutations is given in Fig. 1. In vitro studies by Santoro et al. (15) and Asai et al. (16) have shown that in NIH 3T3 cells transfected with expression vectors harboring RET cDNA with MEN 2 mutations, Ret kinase activity is constitutively stimulated and associated with cellular transformation. In these transfected cell lines, the mutant RET genes thus act as dominantly transforming oncogenes.

Affected MEN 2 family members are heterozygous for the family-specific RET mutation also in their tumor DNA. However, it was conceivable that other mutations, perhaps in the initially unaffected (homologous) RET allele, are a prerequisite for tumors to develop, in which case the “two-hit” model for tumor suppressor genes would still apply. Therefore, we studied expression of the RET gene in tumors from MEN 2 patients by reverse transcription-PCR and direct sequencing to investigate whether the MEN 2-specific germ line mutations are the only RET gene mutations responsible for tumor development in MEN 2 gene carriers in vivo. We have analyzed the entire coding region of both RET gene alleles in four tumor specimens derived from MTC and pheochromocytoma from two different MEN 2A patients and from one MEN 2B patient, as well as in a normal thyroid gland.

PATIENTS AND METHODS

Patients. The four investigated tumors were specimens from MEN 2A and MEN 2B patients. The MEN 2A MTC was from a patient belonging to a family with a germ line mutation of codons 634 and 635 (TGC to TGG GGC) of the RET proto-oncogene. The MEN 2A pheochromocytoma came from a patient with a germ line mutation of codon 634 (TGC to TGG). The other two tumors were an MTC and a pheochromocytoma from one MEN 2B patient. This patient had a de novo germ line mutation of RET codon 918 (ATG to ACG). The normal thyroid gland, investigated as a control for analytical procedures and providing a reference RET cDNA sequence, was an autopsy specimen from a non-MEN 2 subject.

RNA Isolation. MTC, pheochromocytoma, and normal thyroid gland tissue were snap-frozen in liquid nitrogen after surgery or autopsy. Tissue specimens (300 mg) were homogenized in 3 ml of RNA-SR (Biogenesis, Bournemouth, United Kingdom) using an Ultra-Turrax (IKA Labortechnik, Staufen, Germany). Chloroform (0.1 volume) was added and mixed, and the
Human RET gene

| exons | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|-------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|

Human RET mRNA

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<th>3</th>
<th>4</th>
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<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
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Amino acid substitutions in MEN 2A/FMTC and MEN 2B

<table>
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<tr>
<th>Cys 609</th>
<th>Cys 611</th>
<th>Cys 618</th>
<th>Cys 620</th>
<th>Cys 634</th>
<th>Glu 768</th>
<th>Val 804</th>
<th>Met 918</th>
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Fig. 1. Schematic representation of the RET proto-oncogene, mRNA, and protein. The intron-exon structure of the RET gene is shown in the upper panel. Exons, ■. Noncoding regions within exons are white. The Ret protein structure is shown in the lower panel, where the positions of the amino acids mutated in MEN 2A and MEN 2B are indicated. S, signal peptide; Cd, cadherin-like domain; Cys, cysteine-rich domain; TM, transmembrane domain; TK1 and TK2, tyrosine kinase subdomains 1 and 2, respectively.

Results and Discussion

Five DNA sequence polymorphisms in the coding region of the RET gene were revealed by sequencing this small panel of five tissues from four individuals (Table 1). One of these polymorphisms involves an amino acid substitution at codon 691 but is most probably not related to tumor development because it was also demonstrated in DNA from control subjects. However, because it is not known which amino acid residues are essential for proper functioning of the Ret protein, it is conceivable that one or more of such amino acid substitutions can modulate disease phenotype in MEN 2 gene carriers. Apart from the five polymorphisms revealed in this study, several more have been reported (1, 17).

Table 1 DNA sequence polymorphisms revealed by sequencing the coding region of RET cDNA from four MEN 2 tumors and a normal thyroid gland

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Restriction siteb</th>
<th>Amino acid change</th>
<th>Allele frequenciesv</th>
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<tr>
<td>45</td>
<td>GCG → GCA</td>
<td>− HaeIII</td>
<td>None</td>
<td>ND</td>
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<tr>
<td>432</td>
<td>GCG → GCA</td>
<td>+ BamHI</td>
<td>None</td>
<td>0.72/0.28</td>
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<tr>
<td>691</td>
<td>GGT → AGT</td>
<td>+ BsrI</td>
<td>Gly → Ser</td>
<td>0.78/0.22</td>
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<tr>
<td>769</td>
<td>CTT → CTG</td>
<td>− TaqI</td>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>769</td>
<td>TCC → TCG</td>
<td>+ Koi</td>
<td>None</td>
<td>ND</td>
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</table>

a Codon number 1 is the Met translational start codon at positions 196-198 of the RET cDNA, according to Ref. 12.

b The first number is the generation of a new cleavage site for the restriction endonuclease; −, the disappearance of an existing cleavage site.

c The first number is the frequency of the allele without the respective restriction site; the second number is the frequency of the allele with the respective restriction site. Allele frequencies were determined by restriction enzyme digestion of PCR products generated on DNA from 35 (for codon 432) and 75 (for codon 691) random subjects, respectively. Mendelian inheritance of the polymorphisms was confirmed in three families. ND, not determined.
coding region of the RET cDNA sequence in the four tumors investigated. The RET gene promoter and intronic sequences were not screened for mutations. However, it is unlikely that such mutations were present because both alleles of the RET gene were expressed at similar levels, and no indications for tumor-specific RNA splice-variants were obtained. In vitro studies by Santoro et al. (15) and Asai et al. (16) have shown that Ret proteins harboring MEN 2A- or MEN 2B-specific missense mutations are constitutively activated in NIH 3T3 cells transfected with the respective mutant RET cDNAs. In addition, the mutant cDNAs in these cell lines have transforming activity. Our present results also demonstrate that in vivo, in MTC and pheochromocytoma from both MEN 2A and MEN 2B patients, the mutated RET genes act as dominantly transforming oncogenes.

The differences in the clinical manifestation of disease among MEN 2A patients, however, are not readily explained by a single RET mutation. Different phenotypes of MEN 2A in different families, and even within one family, can be observed for one specific germ line RET mutation. Variability is observed with respect to the aggressiveness of tumor growth and the prevalence of pheochromocytoma and hyperparathyroidism, but also to concurrent manifestation of cutaneous lichen amyloidosis and Hirschsprung’s disease (19). Although the inherited mutations may also result in constitutively activated Ret kinase activity in vivo and thus predispose to tumor development, these inherited mutations may not be sufficient for aggressive tumor growth. Additional mutations of the RET proto-oncogene, in casu somatic mutations of Met codon 918, were recently described for 2 of 14 investigated MEN 2-related MTCs as well as 1 case of MEN 2A-related C-cell hyperplasia (20). It was not clear whether the allele harboring the germ line RET mutation or the homologous allele acquired this additional mutation. Although somatic RET gene mutations may contribute to MEN 2 tumor progression by further impeding normal Ret function, our data show that such additional mutations of the RET gene are not required for tumorigenesis. Phenotypic variability observed both between and within MEN 2A families may be due to inherited conditions other than a mutated RET gene, somatic mutations at other loci in the genome, or nurture. For example, in MTC and pheochromocytoma, frequent loss of heterozygosity has been observed for loci on chromosomes 1p and 22 (21). The finding that the inherited mutations are the only RET gene mutations required for tumor development in MEN 2 has clinical consequences. All the MEN 2 target cells in which the constitutively activated Ret kinase is already expressed during prenatal development (22) are prone to neoplastic changes. Therefore, presymptomatic removal of the thyroid gland at a young age is indicated in all MEN 2 gene carriers.

References

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