Duplication of the Mutant RET Allele in Trisomy 10 or Loss of the Wild-Type Allele in Multiple Endocrine Neoplasia Type 2-associated Pheochromocytomas

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Abstract

Inherited mutations of the RET proto-oncogene are tumorigenic in patients with multiple endocrine neoplasia type 2 (MEN 2). However, it is not understood why only few of the affected cells in the target organs develop into tumors. Genetic analysis of nine pheochromocytomas from five unrelated patients with MEN 2 showed either duplication of the mutant RET allele in trisomy 10 or loss of the wild-type RET allele. Our results suggest a "second hit" causing a dominant effect of the mutant RET allele, through either duplication of the mutant allele or loss of the wild-type allele, as a possible mechanism for pheochromocytoma tumorigenesis in patients with MEN 2.

Introduction

MEN 2 is an autosomal dominant inherited cancer syndrome, characterized by the development of pheochromocytoma, medullary thyroid carcinoma, and parathyroid hyperplasia/adenoma. The gene responsible for MEN 2, the RET proto-oncogene, has been localized to chromosome 10q11.2 (1). RET is expressed in human neural crest-derived and neuronal tissues such as Schwann cells, sympathetic ganglia, adrenal medulla, astrocytes, and cerebral cortical neurons (2). In patients with MEN 2, germ-line mutations of RET are commonly identified and are believed to be activating, i.e., causing ligand-independent dimerization of the receptor. Although the inherited RET mutation is related to tumorigenesis in patients with MEN 2, it is unknown by which mechanism(s) only few of the affected cells in the target organs develop into tumors (3).

MET represents another proto-oncogene with structural and functional homology to RET. Upon activation, both proto-oncogenes, MET and RET, act similarly through stimulation of a receptor tyrosine kinase. For MET, trisomy 7-harboring nonrandom duplication of the mutant MET allele has been implicated recently in tumorigenesis of patients with hereditary papillary renal carcinoma (4). The functional homology between MET and RET led us to investigate whether activation of the RET proto-oncogene in patients with the familial tumor syndrome MEN 2A occurs by a similar mechanism.

Materials and Methods

Patients and Tumors. All five patients are unrelated. Results of biochemical screening for catecholamines combined with abdominal imaging by computed tomography were indicative of an intra-adrenal pheochromocytoma. Subsequently, these patients underwent either unilateral or bilateral adrenalectomy. Blood was drawn for DNA extraction, and tumors from all five patients were removed at the time of surgery and frozen at -80°C. DNA was extracted from lymphoblasts and tumor tissue by standard methods.

Preparation of Tumor Imprints and Interphase Nuclei from Patients' Blood for FISH. FISH analysis of tumors was performed using a chromosome 10-specific centromeric α-satellite probe, as described by Zhuang et al. (4). Tumor touch preparations were performed by attaching slightly thawed tumor to a glass slide and air-dried. The air-dried tumor touch preps were fixed in an ethanol series (70, 80, 90, and 100% for 10 min each), followed by collagenase A (0.01%) treatment in Gurr-Ringer buffer for 20 min at room temperature. Metaphase and interphase slides from patients' leukocytes were made by routine protocol for metaphase harvesting.

FISH. Chromosome 10-specific α-satellite probe, biotin-labeled (Oncor), was used for the detection of the chromosome copy number. In situ hybridization and detection procedures were carried out as described (5). In brief, slides were denatured (70°C formamide, 2× SSC) at 72°C for 2 min, dehydrated in a cold (−20°C) ethanol series (70, 80, 90, 100%) for 2 min, and air-dried. α-satellite repetitive DNA, specific for chromosome 10, was denatured for 6 min at 76°C, and overnight hybridization was done in a humidified chamber at 37°C. Posthybridization washes were at 45°C in 50% formamide/2× SSC (3×5 min), 1× SSC (2×5 min), and 0.1× SSC (2×5 min). Detection was performed using avidin-FITC (30 min at 37°C), followed by washing in 4× SSC/0.1% Tween 20 solution at 45°C and counterstaining with propidium iodide.

Hybridization signals were scored using a Zeiss Axioskop epifluorescence microscope, and two-color images were captured on a Photometrics CCD camera (Sensys) using IPLab Image software (Scanalytics, Inc.).

Quantitative PCR Amplification of Microsatellites on Chromosome 10. Three polymorphic markers, D10S677, D10S1239, and D10S141 (Research Genetics), were used in quantitative PCR analysis with genomic DNA extracted from peripheral blood and microdissected tumor tissue. PCR amplifications in the presence of [α-32P]dCTP (0.1 μCi/μl; DuPont) were performed in a Hybaid Omnigene thermal cycler using Ampli-Taq Gold DNA polymerase (Perkin-Elmer Roche). PCR conditions were as follows: initial denaturation at 95°C for 10 min, then 30 cycles, each with 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C; PCR was completed with a final extension at 72°C for 10 min. The amplicons were resolved on a 6% polyacrylamide gel. Gels were dried and exposed to Kodak XAR film. Quantitative analysis of allelic imbalance was performed using PhosphoImager analysis (Molecular Dynamics). All PCR reactions were performed in triplicate and were repeated twice. Each densitometry measurement was performed four times.

Sequence Analysis. The primers for sequencing analysis of RET were as follows: exon 10 (IIF, 5'-GGG GGA TTA AAG CTC ATG GCT AT and IR, 5'-CTC AGA TGT GCT GTC CAG AC), and exon 11 (IF, 5'-TCA CAC CAC...
Table 1 Genetic alterations in MEN 2-associated pheochromocytoma

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor</th>
<th>RET mutation (WT/mutant)</th>
<th>Allelic analysis marker*</th>
<th>Phosphorimage ratio (±0.2)</th>
<th>Copy number of chromosome 10 by FISH</th>
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<tr>
<td>1</td>
<td>1A</td>
<td>Codon 634 TGC/AGC</td>
<td>D10S677</td>
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<td>Trisomy 10</td>
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<td>Disomy 10</td>
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<td>Codon 620 TGC/CCG</td>
<td>D10S141</td>
<td>1.3</td>
<td>Disomy 10</td>
</tr>
</tbody>
</table>

*Representative markers that were informative.

CCAC CCA CAG and IIR, 5′-TGG TAG CAG TGG ATG CAG AA). The AmpliCycle sequencing kit (Perkin-Elmer Roche) was used according to the manufacturer’s protocol.

Restriction Enzyme Digestion Analysis. The composition of the wild-type and the mutated RET in tumor 1B, bearing a germ-line mutation at codon 634 (TGC/AGC), was resolved by subjecting a 124-bp amplicon (primers: IF, 5′-ACA CTG GCA TCA AAT TGA GAC TAC-3′; IIR, 5′-TGG TAG CAG TGG ATG CAG AA) of exon 11 to overnight 9°C DdeI digestion at 37°C. Only the amplicon from the mutant allele has a unique DdeI site 40-bp from the 5′ end.

SSCP Analysis. To confirm the absence of the wild-type RET allele in tumor 5A, we used SSCP analysis. The amplicon (primers: F, 5′-ACA CTG CCC CCA TAA TAT GG and R, 5′-CTG AGA TGT GCT GTG GAG AC) of exon 10 from tumor DNA and a DNA sample extracted from the peripheral blood of a healthy individual were analyzed on a MDE gel (FMC Bioproducts) according to the manufacturer’s protocol.

Results

We studied nine pheochromocytomas from five unrelated patients with MEN 2A. In all patients, we identified RET germ-line mutations in blood DNA (Table 1). Five of the tumors from four patients (1A, 2A, 2B, 3A, 4; Table 1) were trisomic for chromosome 10 by FISH analysis with chromosome 10-specific centromeric α-satellite probes (Fig. 1A). As a control, lymphoblasts from these patients showed disomy for chromosome 10. To analyze the copy number of the mutant and the wild-type RET allele in trisomy 10, we performed quantitative PCR on tumor DNA, using chromosome 10 microsatellite markers D10S677 and D10S1239. In two tumors (2A and 2B), the mutant RET allele on chromosome 10 was identified from the affected patient’s family pedigree study (Fig. 1B). Upon direct visualization, the band representing the mutant allele displayed greater intensity than the one representing the wild-type allele. Quantitative analysis using phosphorimage densitometry revealed an intensity ratio of 2:1 between the mutant and the wild-type alleles in all five trisomic tumors (1A, 2A, 2B, 3A, 4; Table 1). In contrast, blood DNA from the same patients (nos. 1, 2, 3, and 4) revealed equal allelic intensities for both chromosome 10 markers (Fig. 1C).

To further characterize the nature of allelic imbalance in the three trisomic tumors (1A, 3A, 4) without available linkage data, we...
compared sequencing data from the patients’ tumor and blood DNA. Both the wild-type and the mutant sequence of each affected codon (codon 634: TGC/AGC in patient 1, TGC/GGC in patient 4; codon 631: GAC/TAC in patient 3) were present in both tumor and blood DNA (Table 1). However, the mutant nucleotide (adenine in patient 1) guanine in patient 4, and thymidine in patient 3) displayed a more intense signal as compared with the wild-type nucleotide (Fig. 1D).

Four tumors (1B, 3B, 5A, and B) from three patients did not reveal increased copy numbers of chromosome 10 by FISH analysis (Fig. 2A) and failed to display any imbalance between the two heterozygous alleles by microsatellite analysis with markers D10S677 and D10S141 (Fig. 2B). However, loss of the wild-type RET sequence was detected in two tumors. In tumor 1B (codon 634 TGC/AGC), only the mutant sequence was present, as demonstrated by mutation site-specific restriction enzyme digestion analysis (Fig. 2C) and sequencing analysis (Fig. 2D). In tumor 5A (codon 620 TGC/GGC), SSCP and sequencing analyses showed loss of the wild-type RET sequence (data not shown).

In the two remaining tumors (3B and B), normal wild-type and mutant RET sequences were present and were equally intense by phosphorimage densitometry.

Discussion

In patients with MEN 2, it is believed that inherited mutations of the RET proto-oncogene are responsible for the development of MEN 2-associated tumors, but the mechanism(s) explaining why only few of the affected cells (although all cells have the RET germ-line mutation) undergo tumorigenesis are unknown (3). Furthermore, it remains puzzling why patients with a germ-line mutation in RET do not have MEN 2-associated tumors such as pheochromocytoma from birth on, but rather develop these tumors over time, sometimes as late as the 7th decade (1, 6). In this study, we provide evidence that activation of RET and subsequent tumor formation may occur by a “second hit” that causes a dominant effect of the mutant RET allele, either through duplication of the mutant allele in trisomy 10 or loss of the normal wild-type RET.

In experimental mouse tumors, imbalance between the wild-type and the mutant oncogene has been shown to play a mechanistic role during early tumorigenesis (7). Only recently, such imbalance has also been demonstrated in a human hereditary papillary renal cell carcinoma syndrome (4). Zhuang et al. (4) proposed that inherited mutations of MET may render the cells more susceptible to errors in chromosomal replication during cell division, resulting in nonrandom chromosomal duplication of the mutant allele in those cells. Our finding of allelic copy changes of RET in MEN 2-associated pheochromocytoma is consistent with previous studies of chromosomal instability in other tumors (8). Cells that contain two mutant RET alleles may gain growth advantage and eventually develop into tumors. Similarly, loss of the wild-type RET allele may provide a dominant effect of the mutant RET allele. In support of the loss of the wild-type RET allele causing tumorigenesis, other investigators have shown allelic loss on chromosome 10 in MEN 2-associated tumors, in one case with the entire copy of chromosome 10 absent (9). Interestingly, various MEN 2-associated tumors from the same patient can have different genetic alterations; in patient 1, one pheochromocytoma (1A) harbored trisomy 10, and the second tumor (1B) showed loss of the wild-type RET allele (Table 1). This result suggests that the selection between duplication of the mutant RET and loss of the wild-type RET allele is random.

The reason why the majority of cells carrying a germ-line RET mutation in patients with MEN 2A fail to undergo tumorigenesis could be that the wild-type RET gene product dimerizes with the mutant counterpart, exerting a neutralizing effect and thereby partially compensating for the activating effects of the mutant RET allele. Once allelic imbalance occurs with an increased “dosage” of the mutant allele, either by duplication of the mutant allele or by loss of the wild-type allele, the protective effect of wild-type protein may be
overridden, leading to an increased chance of dimerization between the mutant monomers. In patients with MEN 2A, either mechanism, duplication of the mutant allele in trisomy 10 or loss of the wild-type RET allele, may represent an early and fundamental event in the development of MEN 2-associated pheochromocytoma. Tumorigenesis may have been initiated by a RET germ-line mutation, followed by duplication of the mutant RET allele or loss of the wild-type allele as a “second activating hit,” a mechanism similar to the “two hit” hypothesis of tumor-suppressor inactivation (10).

In our study, two tumors failed to show either duplication of the mutant or loss of the wild-type RET allele. At present, it is not clear by which mechanism(s) these two tumors developed. Other oncogenic mechanisms that cause differences in expression of mutant and wild-type RET, such as RET rearrangement through translocation or inversion (11) or gross genomic changes, might be involved in tumorigenesis of MEN 2-associated tumors and should be further investigated.

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References

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