0021-972X/01/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 86, No. 1
Printed in U.S.A.

Loss of Heterozygosity at the *RET* Protooncogene Locus in a Case of Multiple Endocrine Neoplasia Type 2A*

LOREDANA QUADRO, OLIMPIA FATTORUSO, MARIA PIA COSMA, UBERTA VERGA, ANTONIO PORCELLINI, ALFONSO LIBROIA, AND VITTORIO COLANTUONI

Dipartimento di Biochimica e Biotecnologie Mediche and CEINGE Centro di Ingegneria Genetica (L.Q., O.F., M.P.C., V.C.), and Dipartimento di Patologia e Biologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli Federico II (A.P.), 80131 Naples; Divisione di Endocrinologia Ospedale Niguarda (U.V., A.L.), 20100 Milan; and Facoltà di Scienze Matematiche, Fisiche e Naturali, Università del Sannio (L.Q., V.C.), 82100 Benevento, Italy

ABSTRACT

We describe a patient affected by multiple endocrine neoplasia type 2A (MEN 2A) bearing a heterozygous germline mutation ($Cys^{634}Arg$) in exon 11 and an additional somatic mutation of the *RET* protooncogene. A large intragenic deletion, spanning exon 4 to exon 16, affected the normal allele and was detected by quantitative PCR, Southern blot analysis, and screening of several polymorphic markers. This deletion causes *RET* loss of heterozygosity exclusively in the metastasis, thus suggesting a role for this second mutational event in tumor progression. No additional mutations were found in the other exons analyzed. We provide the first evidence that *RET*, a dominant oncogene, is affected by a germline mutation and by an additional somatic deletion of the wild-type allele. This unusual genetic profile may be related to the clinical course and very poor outcome. (*J Clin Endocrinol Metab* **86:** 239–244, 2001)

MEDULLARY THYROID carcinoma originates from the C cells of the thyroid gland and presents in a sporadic form [medullary thyroid carcinoma (MTC)] or as part of the inherited cancer syndromes, such as multiple endocrine neoplasia type 2A (MEN 2A). This syndrome, transmitted in an autosomic dominant fashion, is characterized by MTC, pheochromocytoma, and hyperplasia of the parathyroid glands (1).

The gene responsible for these tumors is the *RET* protooncogene (2–6), coding for a membrane tyrosine kinase receptor expressed in neural crest-derived cells (7–9). Ninety-five percent of MEN 2A cases bear point mutations of the *RET* gene (10) mainly in exons 10 and 11 (2, 3, 5, 6). Thirty to 50% of sporadic MTCs cases bear a *RET* mutation in exon 16 (4, 11–15); less frequent are mutations in other exons (11, 12, 15, 16).

Duplication or deletion of segments of various lengths of the *RET* gene take place only occasionally; 9- or 12 bp duplications in exon 11 have been reported in a few MEN 2A cases (17, 18). A 27-bp deletion in exon 10 (19) and a deletion-insertion in exon 11 have been reported in sporadic MTCs (20).

In other hereditary tumors, a germline mutation in a predisposing gene is usually followed by functional loss of the homologous allele in the tumor tissue (21). In these cases the affected gene acts as a tumor suppressor, and the disease is established only when both copies of the gene are altered (22). In MEN 2 tumor DNA, no gross abnormalities are found at the *RET* locus, nor are other markers in proximity of the gene affected. In a percentage of MEN 2-related tumors, loss of heterozygosity (LOH) on chromosomes 1, 3, 11, 17, and 22 has been described and related to loss of function of putative suppressor genes (23–29). However, the role of these LOH in the tumor progression process is not known yet.

Here we report a MEN 2A case affected by a typical heterozygous germline mutation ($Cys^{634}Arg$) in exon 11 and by an additional somatic deletion of the wild-type allele. The deletion spans exon 4 to exon 16, does not include extragenic sequences, and is not associated with base pair changes in any other mutation-bearing *RET* exons. This is the first report of a MEN 2A case with loss of heterozygosity of the *RET* gene. This genetic profile may be related to the early onset, the clinical course with local and distant metastases, and the very poor outcome.

Materials and Methods

DNA analysis

DNA was extracted from the primary tumor, a metastatic node, and peripheral blood lymphocytes and used as a template for PCR reactions performed as previously described (30). *RET* exons 10, 11, 13, 14, 15, and 16 were amplified using as primers the oligonucleotides reported previously (4, 5, 31–34). The purified PCR products were sequenced on both strands with the Sanger method, using the Sequenase PCR product sequencing kit (U.S. Biochemical Corp., Cleveland, OH) and the primers used for the amplification. Sequence reactions were then run on 6% denaturing polyacrylamide gels and autoradiographed overnight. In some cases, 100–200 ng of the PCR products of exons 2, 11, 13, 14, and 16 amplicons were digested with 2–5 U *Eag*I, *Ban*I and *Hha*I, *Alu*I, *Nla*III,

Received December 29, 1999. Revision received May 23, 2000. Rerevision received August 10, 2000. Accepted October 1, 2000.

Address all correspondence and requests for reprints to: Dr. Loredana Quadro, Facoltà di Scienze Matematiche, Fisiche e Naturali, Università del Sannio, 82100 Benevento, Italy. E-mail: quadro@dbbm.unina.it; or Dr. Vittorio Colantuoni, Dipartimento di Biochimica e Biotecnologie Mediche and CEINGE Centro di Ingegneria Genetica, 80131 Naples, Italy. E-mail: colantuoni@dbbm.unina.it.

* This work was supported by a grant from MURST PRIN '97.

or *Fok*I restriction enzymes, respectively (31, 32, 35, 36). Samples were then run on 2% or 3% agarose gels, and DNA bands were visualized after ethidium bromide staining. The D10S94 locus was amplified using the primers described previously (37), and 100–200 ng of the purified PCR product were digested with 2–5 U *Pvu*II restriction enzyme. Genomic DNA was also analyzed by Southern blot.

Quantitative PCR procedure

The quantitative PCR analysis was performed as previously described (38). The duplex PCR reaction for all *RET* exons was performed by amplifying as internal control exon 10 of the piruvate kinase (PK) gene (39). The primers used for the PK gene were: 10F, 5′-CTCGTTCACCACTTTCTTGC-3′; and 10R, 5′-GGGAAGCTGGGTTGGGGGC-3′, both of which were derived from the intronic sequence. The primers used to amplify the *RET* exons 10, 11, 13, 14, 15, and 16 were reported previously (4, 5, 31–34); the others were designed according to the gene sequence (1). The duplex PCR reactions were performed in a 50-μL final volume, containing 100–200 ng genomic DNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 5 mmol/L NH_4Cl, 200 μmol/L of each deoxy-NTP, 30 pmol of each primer, 4 μCi (3000 Ci mM) of ^{32}P-labeled deoxy-CTP, and 2.5 U *Taq* polymerase (AmpliTaq, Perkin-Elmer Corp., Norwalk, CT). The PCR cycles were as follows: denaturation step for 7 min at 94 C; 22 cycles of denaturation, annealing, and extension performed at 94 C for 1 min, 52 C for 2 min, and 72 C for 5 min; and a final elongation step at 72 C for 7 min. The samples were stored at 4 C until required. Four microliters of each PCR product were pipetted from each tube and mixed with 1 μL loading dye. The samples were electrophoresed on a 5% polyacrylamide gel (39:1 acrylamide/bisacrylamide ratio) in 1 × Tris Borate EDTA buffer for 1 h at 1800 V. The gel was dried and exposed on the GS-250 Molecular Imager (Bio-Rad Laboratories, Inc., Hercules, CA) for about 15–18 h.

Results

Case report

In 1986, a 18-yr-old woman was referred for evaluation to the Niguarda Hospital and underwent fine needle aspiration biopsy of a lateral cervical lymph node that had rapidly enlarged. Histological examination revealed MTC, as confirmed by elevated serum calcitonin (314 μg/ml) and carcinoembryonic antigen (44.5 ng/mL) levels. Total thyroidectomy with central compartment resection was immediately performed. The thyroid was bilaterally affected by the tumor that had invaded the neighboring vessels. Local nodes were metastasized. No clinical signs or symptoms of a pheochromocytoma were present. After treatment and a period of well-being, the patient complained of general discomfort due in part to enlargement of the cervical nodes that compressed the adjacent veins. They were removed, but later the patient was again hospitalized for respiratory problems due to several metastatic infiltrates in both lungs, as revealed by computed tomography. The patient's general condition progressively worsened, and in February 1995 she died at the age of 27 yr. Her family history was negative for relevant thyroid pathology.

Identification of RET proto-oncogene mutations

DNA from peripheral blood lymphocytes, primary (1986 surgery) and metastatic (1991 surgery) tumors were analyzed for *RET* protooncogene mutations. Exon 11 from peripheral blood DNA was PCR amplified and sequenced. A heterozygous TGC to CGC mutation was detected at codon 634 causing a Cys to Arg substitution (Fig. 1, A and B, patient blood). The heterozygous mutation of germline DNA was also found in the DNA from the primary tumor (data not shown). In contrast, DNA from the metastatic node revealed only the sequence corresponding to the mutated allele (Fig. 1, A and B, patient metastatic tumor). The specific mutation

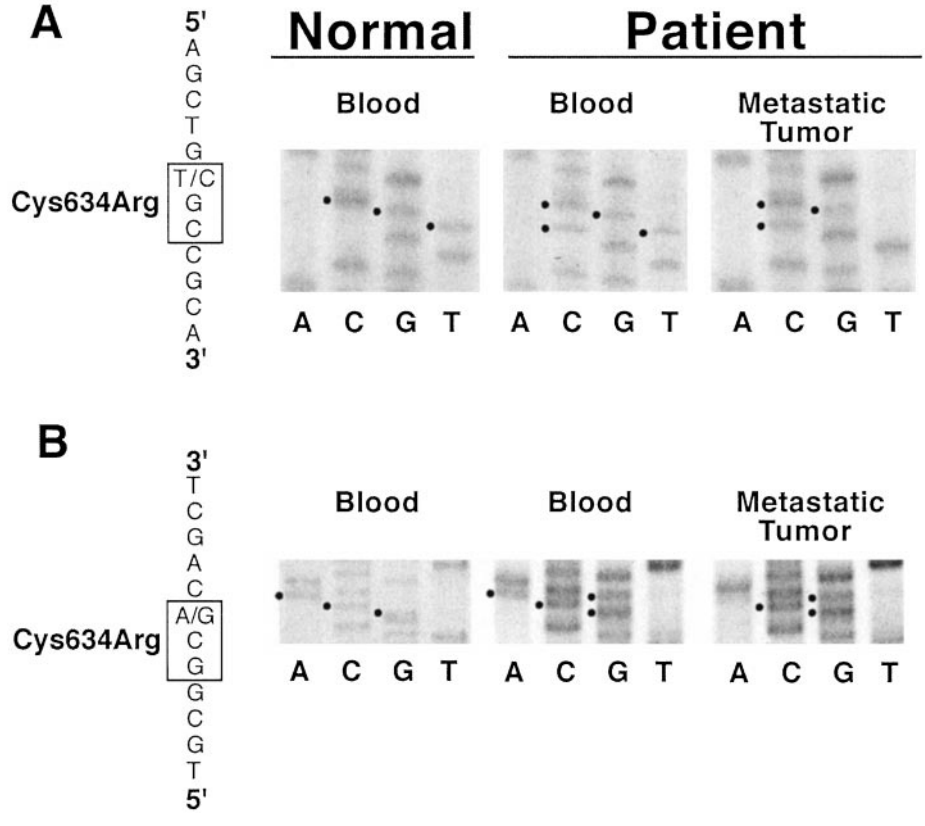


FIG. 1. Detection of the Cys^{634}Arg mutation in *RET* protooncogene. *RET* exon 11 was amplified from constitutive and metastatic DNA, and the bands were sequenced on both strands. In A and B, the products of the direct sequencing of the coding and noncoding strand, respectively, are shown. The mutated codon and the corresponding amino acid change are *boxed* on the *left*. The sequencing from a normal individual is also reported as a control.

was confirmed by *Hha*I restriction enzyme digestion (data not shown). A new site for this enzyme is, in fact, generated by the $Cys^{634}Arg$ mutation (36).

Quantitative PCR analysis identifies RET LOH

We performed phosphorimager-based quantitative PCR analysis to establish whether the genomic profile detected in the metastatic DNA was due to *RET* LOH (38). This assay entails the quantitative analysis of two ^{32}P-labeled PCR products, a possibly deleted exon *vs.* a nondeleted exon. Each *RET* exon from germline, primary tumor, and metastatic DNA was coamplified with exon 10 of the PK gene (39) as an internal control. This gene was chosen because it maps on the long arm of chromosome 1, and it should not be involved in any mutation affecting the *RET* gene located in the centromeric region of chromosome 10 (1). Each experiment was carried out on DNA from the patient and from a normal individual. The radioactive amplified products were quantified by phosphorimager scanning, and the ratio of the intensity of the *RET* exon *vs.* the internal control was referred to as *R*. The ratio of the *R* value of the patient *vs.* that of a normal individual was the diagnostic index (DI). If the DI ranges between 0.8–1, the tested exon is present in two copies; if the DI ranges between 0.4–0.6, the exon is present in a single copy. All *RET* exons amplified from germline and primary tumor DNA gave a DI between 0.8–1, clearly indicating that they were present in two copies, as in normal DNA. In contrast, the DI for exons 4–16 amplified from the metastatic DNA ranged between 0.4–0.6, indicating that they were in a single copy. *RET* exons 1–3 and 17–21 had normal values, indicating that they were preserved on both alleles. Figure 2 illustrates the results obtained with some representative exons, *i.e.* 3, 11, 16, and 17. The histogram shows the DI obtained with normal DNA and with constitutive, primary, and metastatic tumor DNA from the proband.

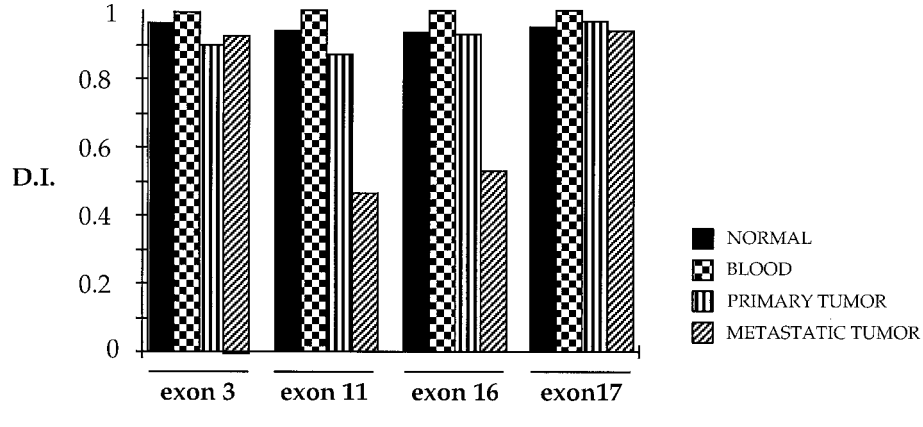
Analysis of polymorphic sites at the RET locus confirms LOH

To further confirm the results of the quantitative PCR, two *RET* intragenic heterozygous polymorphisms were analyzed. One maps at the Ala^{45} codon in exon 2, and the second maps at the Gly^{691} codon in exon 11 (35). The former polymorphism eliminates the restriction site for the enzyme *Eag*I, the latter eliminates that for *Ban*I. Patient DNA from peripheral blood and metastatic tumor were heterozygous for exon 2 polymorphism (Fig. 3A). Exon 2 PCR-amplified products digested with *Eag*I produced, in both cases, a band of 265 bp, corresponding to the undigested allele and two bands of 203 and 62 bp derived from the digested allele. In contrast, a different profile was detected on DNA from peripheral blood and metastatic tissue analyzed for the polymorphism at exon 11 (Fig. 3B). *Ban*I digestion of the exon 11 PCR product from peripheral blood yielded a band of 338 bp, corresponding to the undigested allele and two bands of 269 and 69 bp generated from the digested allele. In metastatic tumor DNA, the *Ban*I digestion profile gave only a single high molecular weight band, corresponding to the undigested allele, indicating that the polymorphism was not retained. In both digestions, DNA from a normal individual showing the same polymorphism in heterozygosity was used as a positive control.

A heterozygous polymorphism at the D10S94 locus, located at the 3′-region of the *RET* gene (37), was also analyzed and shown to be retained in metastatic tumor DNA, indicating that deletion of the wild-type *RET* allele of the metastatic node does not involve extragenic sequences (data not shown).

Southern blot analysis confirms RET LOH

The proband's genomic DNA from peripheral blood and metastatic tumor tissue was analyzed by Southern blot. Figure 3C shows the results of *Eco*RI and *Eco*RI/*Sal*I digestions of patient's DNAs hybridized with the *Xho*I/*Bgl*II *RET* complementary DNA (cDNA) fragment, spanning from exon 14



$$R = \frac{\text{Tested RET exon}}{\text{Reference exon}} \qquad D.I. = \frac{\text{R patient}}{\text{R normal}}$$

FIG. 2. Quantitative PCR analysis. The DI reported in the histogram was calculated on the germline, primary, and metastatic tumor DNA from the proband. The DNA from a normal individual was used as a control. The DI was calculated on each *RET* exon and on exon 10 of the PK gene (39), which was coamplified as internal control, as described in *Materials and Methods*. A DI between 0.8–1 indicates that the tested exon is present in two copies; if it is between 0.4–0.6, the exon is present in a single copy. The results refer to four representative *RET* exons (no. 3, 11, 16, and 17) and to one of three independent experiments.

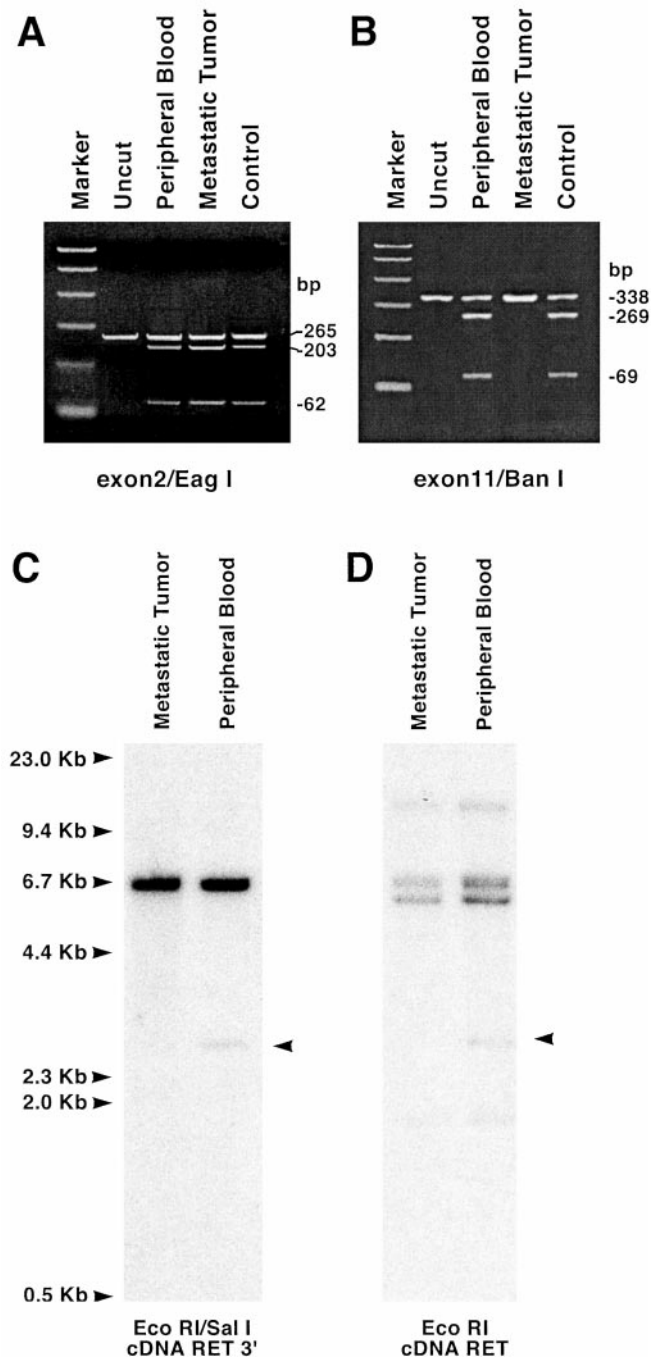


FIG. 3. Analysis of polymorphic sites at the *RET* gene and Southern blot. Two polymorphisms were analyzed on the germline and metastatic DNA of the proband and on the DNA from a normal individual bearing the same heterozygous polymorphisms as a control (35). A, The polymorphism at the Ala^{45} codon in *RET* exon 2. B, The polymorphism at the Gly^{691} codon in exon 11. Uncut, The PCR product before digestions in both cases. The migration of a molecular size marker (Promega Corp. PCR marker G3161) is also indicated. C, Fifteen micrograms of the proband's genomic DNA extracted from peripheral blood and metastatic tumor were digested with *Eco*RI and *Sal*I and hybridized with a *Bgl*II/*Xho*I fragment derived from the 3′-portion of *RET* cDNA as a probe. D, Fifteen micrograms of the same DNA samples were digested with *Eco*RI and hybridized with the full-length *Eco*RI *RET* cDNA as a probe. The migration of a size marker (λ *Hin*dIII) is reported on the *right*. An *arrow* on the *left* indicates the 2.8-kb band absent in the metastatic tumor DNA.

to the 3′-end of the cDNA (1). Figure 3D shows the results of the hybridization with the full-length *RET* cDNA of the same DNA sample digested with *Eco*RI (1). The pattern observed corresponds to that expected, except for a 2.8-kb band present in the germline DNA and absent in the metastatic tumor DNA. The 2.8-kb band was also observed on the DNA from two independent normal subjects digested with the same enzymes. The disappearance of this band in the tumor DNA could be due to a polymorphic *Eco*RI site present in the wild-type allele, downstream from exon 14. This confirms the *RET* LOH, because the site is no longer retained in the metastatic DNA as a consequence of the interstitial deletion on the wild-type allele. However, from the data obtained, we cannot establish the type of rearrangement that occurred, *i.e.* translocation of part of the gene to another chromosome or rejoining of the two portions of the gene.

Discussion

Heterozygous point mutations of the *RET* protooncogene are responsible for type 2 MENs and some sporadic MTCs. In this respect, *RET* functions as a dominant oncogene after activation by these specific mutations. The pathogenetic mechanisms underlying these tumors have been clarified. In the case of MEN 2A, the mutations lead to constitutive activation of the RET receptor through a ligand-independent dimerization mechanism. Constitutive phosphorylation and altered substrate specificity are invoked in most sporadic MTCs (40).

The *RET* mutations underlying all inherited tumor syndromes cause thyroid C cell hyperplasia, which does not fully explain either tumor progression or the phenotypic variations observed not only among families with the same mutations, but also among individuals in the same family bearing the same mutation (41). Accumulation of genetic alterations, functional loss of tumor suppressor genes, and activation of other oncogenes could contribute to tumor development. In fact, LOH on chromosomes 1, 3, 11, 17, and 22 has been reported in MEN 2 cases (23–29).

Despite the consolidated idea that *RET* acts as a dominant oncogene due to single mutations, other alterations of this gene have recently been reported in MTC. Simultaneous germline mutations at exon 10 or 11 and somatic mutations at codon 918 in exon 16 have been described (34, 42). Miyauchi *et al.* (43) also found a germline mutation at codon 768 in exon 13 and a concomitant somatic mutation at codon 919 in exon 16 in a familial MTC. These reports, however, did not show on which allele the additional somatic mutations occurred. More interestingly, Uchino *et al.* (44) reported two sporadic MTCs with point mutations at codons 918 and 919 of exon 16 and at codon 778 of exon 13, respectively. In both cases, the wild-type allele was absent.

The case analyzed here bears a typical *RET* MEN/2A germline mutation in exon 11 ($Cys^{634}Arg$) and loss of the wild-type allele in the tumor. In particular, LOH was detected only in the metastasis and not in the primary tumor, suggesting that this second mutational event had occurred somatically and that it probably could have played a role in tumor progression. Our hypothesis is that this LOH may be related to the clinical course observed. No additional muta-

tions were found in other *RET* exons analyzed (data not shown). This represents the first report of a MEN 2A case characterized by a germline mutation and a somatic loss of the wild-type allele.

The alteration found was a large intragenic deletion of about 20 kb encompassing exons 4–16 of the *RET* locus. These results ruled out a gene conversion event that should transform the wild-type to the mutated allele. In fact, gene conversion usually involves larger chromosomal regions and is detected for genes present in clusters and among nonallelic copies (45). This was not the case, as the deletion did not extend to extragenic sequences at the 3′-end of the *RET* gene and was present only in the metastatic DNA. In addition, the loss of genetic material at the *RET* locus was a random event and was not due to patient's genome instability. LOH at other loci distributed either on chromosome 10 or on other chromosomes was tested, but no instability was detected (data not shown).

We analyzed the rearrangement that occurred at the wild-type locus by quantitative PCR, Southern blot, and RFLPs of chromosome 10. Furthermore, the expression study of the metastatic tissue ruled out that the rearranged wild-type allele was still transcribed, indicating that the deletion had irreversibly compromised its architecture and function. In fact, in addition to the transcripts derived from the mutated allele, no bands corresponding to shorter or longer wild-type *RET* messenger ribonucleic acid, derived from the rearranged copy, were detected by Northern blot or ribonuclease protection (data not shown).

LOH of tumor suppressor genes has been described in inherited cancers, classically represented by retinoblastoma. The genes responsible are inactivated by a germline mutation in one allele and by somatic loss of the second allele. The tumorigenic phenotype is manifested only when both copies are altered, according to Knudson's two-hit model (22). The case we report is unusual and novel, because two mutations were detected in a dominant oncogene. This event does not appear to be redundant. We hypothesize that the somatic LOH could add to the effects of the germline mutation, resulting in an aggressive phenotype, and could represent the second or one of the subsequent steps in tumor progression. It was, in fact, found only in two distinct consecutive metastases at different locations.

We propose a model in which the wild-type RET form influences the activity of mutated RET species, functioning as a mitigating factor in the transformation process. In classical heterozygous MEN 2A, the mutant RET may form either homodimers, whose activation is ligand independent or heterodimeric complexes with wild-type RET, whose activation may still be ligand dependent. In contrast, in our case the absence of the wild-type species could allow the mutant RET to form only constitutively activated homodimers, thus enhancing the transduction of mitogenic signals. In fact, wild-type RET can form complexes with mutant RET, influencing the amount of active heterodimers exposed on the cell surface (46). This mechanism could explain the rapid tumor growth, its wide dissemination, the distant and very invasive metastases, and the poor outcome of the disease. Experiments to test this hypothesis *in vitro* are underway. Moreover, a larger series of patients needs to be analyzed to make a correlation between severe symptomatic MTC and the pathogenetic mechanism we propose.

Acknowledgments

We are extremely grateful to the family who participated in our studies, without whom this investigation would have not been possible. We are indebted to Dr. M. Santoro for his support and suggestions during the course of these experiments and for critical reading of the manuscript. We thank J. Gilder for text editing.

References

1. **Hoff AO, Cote GJ, Gagel RF.** 2000 Multiple endocrine neoplasias. Annu Rev Physiol. 62:377–411.
2. **Donis-Keller H, Dou S, Chi D, et al.** 1993 Mutations in the *RET* proto-oncogene are associated with MEN2A and FMTC. Hum Mol Genet. 2:851–856.
3. **Mulligan LM, Kwok JB, Healey CS, et al.** 1993 Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. Nature. 363:458–460.
4. **Hofstra RMW, Landsvater RM, Ceccherini I, et al.** 1994 A mutation in *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature. 367:375–376.
5. **Quadro L, Panariello L, Salvatore D, et al.** 1994 Frequent *RET* proto-oncogene mutations in multiple endocrine neoplasia type 2A. J Clin Endocrinol Metab. 79:590–594.
6. **Mulligan LM, Marsh DJ, Robinson BG, et al.** 1995 Genotype-phenotype correlation in MEN 2: report of the International Ret Mutation Consortium. J Intern Med. 238:343–346.
7. **Avantaggiato V, Dathan NA, Grieco M, et al.** 1994 Developmental expression of the *RET* proto-oncogene. Cell Growth Diff. 5:305–311.
8. **Pachnis V, Mankoo B, Costantini F.** 1993 Expression of the c-*ret* proto-oncogene during mouse embryogenesis. Development. 119:1005–1017.
9. **Takahashi M, Buma Y, Iwamoto T, Inaguma Y, Ikeda H, Hiai H.** 1988 Cloning and expression of the *ret* protooncogene encoding a tyrosine kinase with two potential transmembrane domain. Oncogene. 3:571–578.
10. **Eng C, Clayton D, Schuffenecker I, et al.** 1996 The relationship between specific *ret* proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2: international ret mutation consortium analysis. JAMA. 276:1575–1579.
11. **Eng C, Mulligan LM, Smith DP, et al.** 1995 Mutation of the *RET* protoncogene in sporadic medullary thyroid carcinoma. Genes Chrom Cancer. 12:209–212.
12. **Marsh D, Learuyd D, Andrew S, et al.** 1996 Somatic mutations in the *RET* proto-oncogene in sporadic medullary thyroid carcinoma. Clin Endocrinol (Oxf). 44:249–257.
13. **Wohllk N, Cote G, Bugalho M, et al.** 1996 Relevance of the RET proto-oncogene mutations in sporadic medullary thyroid carcinoma. J Clin Endocrinol Metab. 81:3740–3745.
14. **Zedenius G, Larsson C, Bergholm U, et al.** 1995 Mutations of codon 918 in the *RET* proto-oncogene correlate to poor prognosis in sporadic medullary thyroid carcinomas. J Clin Endocrinol Metab. 80:3088–3090.
15. **Romei C, Elisei R, Pinchera A, Ceccherini I, et al.** 1996 Somatic mutations of the *RET* proto-oncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. J Clin Endocrinol Metab. 81:1619–1622.
16. **Eng C, Mulligan LM, Smith DP, et al.** 1995 Low frequency of germline mutations in the *RET* proto-oncogene in patients with apparently sporadic medullary thyroid carcinoma. Clin Endocrinol (Oxf). 43:123–127.
17. **Hoppner W, Dralle H, Brabant G.** 1998 Duplication of 9 base pair in the critical cysteine-rich domain of the *RET* proto-oncogene causes multiple endocrine neoplasia type 2A. Hum Mut. 1(Suppl):S128–S130.
18. **Hoppner W, Ritter MM.** 1997 A duplication of 12 bp in the critical cysteine rich domain of the *RET* proto-oncogene results in a distinct phenotype of multiple endocrine neoplasia type 2A. Hum Mol Genet. 6:587–590.
19. **Kalinin V, Frilling A.** 1998 27-bp deletion in the ret proto-oncogene as a somatic mutation associated with medullary thyroid carcinoma. J Mol Med. 76:365–367.
20. **Marsh DJ, Andrew SD, Learoyd DL, Pojer R, Eng C, Robinson BG.** 1998 Deletion-insertion mutation encompassing RET codon 634 is associated with medullary thyroid carcinoma. Hum Mut. 1(Suppl):S3–S4.
21. **Friend SH, Horowitz JM, Gerber MR, Wang XF, Bogenmann E, Li FP, Weinberg RA.** 1987 Deletions of a DNA sequence in retinoblastomas and mesenchymal tumors: organization of the sequence and its encoded protein. Proc Natl Acad Sci USA. 84:9059–9063.
22. **Knudson AG.** 1993 Antioncogenes and human cancer. Proc Natl Acad Sci USA. 90:10914–10921.
23. **Mulligan LM, Gardner E, Smith BA, Mathew CGP. Ponder AJP.** 1993 Genetic events in tumor initiation and progression in multiple endocrine neoplasis type 2. Genes Chrom Cancer. 6:166–177.
24. **Mathew CGP, Smith BA, Thorpe K, et al.** 1987 Deletion of genes on chromosome 1 in endocrine neoplasia. Nature. 328:524–526.

25. **Takai S, Tateishi H, Nishisho I, et al.** 1987 Loss of genes on chromosome 22 in medullary thyroid carcinoma and pheocromocytoma. Jpn J Cancer Res. 78:894–898.
26. **Yang K, Nguyen CV, Castillo SG, Samaan NA.** 1990 Deletion mapping on the distal third region of chromosome 1p in multiple endocrine neoplasia type 2A. Anticancer Res. 10:527–533.
27. **Yokogoshi Y, Yoshimoto K, Saito S.** 1990 Loss of heterozygosity on chromosome 1 and 11 in sporadic pheocromocytomas. Jpn J Cancer Res. 81:632–638.
28. **Khosla S, Patel VM, Hay ID, et al.** 1991 Loss of heterozygosity suggests miltiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. J Clin Invest. 87:1691–1699.
29. **Moley JF, Brother MB, Fong C-T, et al.** 1992 Consistent association of 1p loss of heterozygosity with pheochromocitomas from patients with multiple endocrine neoplasia type 2 syndromes. Cancer Res. 52:770–774.
30. **Scurini C, Quadro L, Fattoruso O, et al.** 1998 Germline and somatic mutations of the RET proto-oncogene in apparently sporadic medullary thyroid carcinomas. Mol Cell Endocrinol. 137:51–57.
31. **Bolino A, Schuffenecker I, Luo Y, et al.** 1995 RET mutation in exons 13 and 14 of FMTC patients. Oncogene. 10:2415–2419.
32. **Fattoruso O, Quadro L, Libroia A, et al.** 1998 A GTG to ATG novel point mutation at codon 804 in exon 14 of the *RET* proto-oncogene in two families affected by familial medullary thyroid carcinoma. Hum Mutat. 1(Suppl):S167–S171.
33. **Eng C, Smith DP, Mulligan LM, et al.** 1995 A novel point mutation in the tyrosine kinase domain of the *RET* proto-oncogene in sporadic medullary thyroid carcinoma and in a family with FMTC. Oncogene. 10:509–513.
34. **Marsh DJ, Andrew SD, Eng C, et al.** 1996 Germline and somatic mutations in an oncogene: RET mutations in inherited medullary thyroid carcinoma. Cancer Res. 56:1241–1243.
35. **Ceccherini I, Hofstra RMW, Luo Y, et al.** 1994 DNA polymorphism and conditions for SSCP analysis of the 20 exons of the RET proto-oncogene. Oncogene. 9:3025–3029.
36. **Zedenius J, Wallin G, Hamberger B, Nordenskjord M, Weber G, Larsson C.** 1994 Somatic and MEN 2A *de novo* mutations identified in the RET proto-oncogene by screening of sporadic MTCs. Hum Mol Genet. 3:1259–1262.
37. **Brooks-Wilson AR, Smailus D, Gilchrist D, Goodfellow PJ.** 1992 Additional RFLPs at D10S94 and the development of PCR. Based variant detection system: implications for disease genotype prediction in MEN 2A, MEN 2B and MTC families. Genomics. 13:233–234.
38. **Pastore L, Caporaso MG, Frisso G, et al.** 1996 A quantitative polymaresa chain reaction (PCR) assay completely discriminates between Duchenne and Becker muscular dystrophy deletion carriers and normal females. Mol Cell Probes. 10:129–137.
39. **Baronciani L, Buetler E.** 1993 Analysis of pyruvate kinase-deficiency mutations that produces nonspherocytic hemolytic anemia. Proc Natl Acad Sci USA. 90:4324–4237.
40. **Santoro M, Carlomagno F, Romano A, et al.** 1995 Germ-line mutations of MEN2A and MEN2B activate RET as a dominant trasforming gene by different molecular mechanisms. Science. 267:381–383.
41. **Eng C, Mulligan LM.** 1997 Mutations of the RET proto-oncogene in the multiple endocrine neoplasia type 2 syndromes, related sporadic tumors, and hirschsprung disease. Hum Mut. 9:97–109.
42. **Eng C, Mulligan LM, Healey CS, et al.** 1996 Heterogeneous mutation of the *RET* proto-oncogene in subpopulations of medullary thyroid carcinoma. Cancer Res. 56:2167–2170.
43. **Miyauchi A, Egawa S, Futami H, Kuma K, Obara T, Yamaguchi K.** 1997 Anovel somatic mutation in the RET proto-oncogene in familial medullary thyroid carcinoma with a germline codon 768 mutation. Jpn J Cancer Res. 88:527–531.
44. **Uchino S, Noguchi S, Adachi M, et al.** 1998 Novel ponit mutation and allele loss at the RET locus in sporadic medullary thyroid carcinomas. Jpn J Cancer Res. 89:411–418.
45. **Papadakis MN, Patrinos GP.** 1999 Contribution of gene conversion in the evolution of the human γ-like globin gene family. Hum Genet. 104:117–125.
46. **Cosma MP, Cardone M, Carlomagno F, Colantuoni V.** 1998 Mutations in the extracellular domain cause RET loss of function by a domain negative mechanism. Mol Cell Biol. 18:3321–3329.