CASE REPORT

RET proto-oncogene mutation in a mixed medullary-follicular thyroid carcinoma

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ABSTRACT. A case of a patient with an uncommon thyroid carcinoma, showing histological and immunohistochemical features of both follicular and parafollicular cells is described. Somatic point mutation (ATG to ACG heterozygotic mutation at codon 918) of the *RET* proto-oncogene was detected in tumor tissue, as confirmed by immunohistochemical expression of *RET* oncoprotein. Our findings suggest that constitutive *RET* proto-oncogene activation may be involved in the development of mixed medullary-follicular thyroid carcinoma.

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INTRODUCTION

The thyroid gland displays two distinct endocrine cell types: the thyroglobulin-producing follicular cells and the calcitonin-producing parafollicular cells. Neoplastic transformation may involve both cell types. Follicular and papillary carcinomas develop from follicular cells, whereas medullary carcinomas (MTC) develop from parafollicular cells (1). MTC may occur sporadically or as familial syndrome, with or without extrathyroid involvement (2). Somatic or germline mutations of *RET* proto-oncogene have been identified in sporadic and familial MTC, respectively (3-5).

An uncommon thyroid carcinoma, sharing histological and/or immunohistochemical features of both follicular and parafollicular cells, has been identified and classified as mixed medullary-follicular thyroid carcinoma (MMFTC) (6-9). A co-expression of thyroglobulin (TG) and neuroendocrine peptides (calcitonin, calcitonin gene related protein, chromogranin, neurotensin and others) is the hallmark of this tumor. The histogenesis and the pathogenesis of mixed tumors are matter of debate: they may arise from uncommitted stem cells of the ultimo-branchial body (10) or may represent a special type of thyroid tumor undergoing divergent differentiation. Alternatively, they may originate from neoplastic transformation of different cell populations under common oncogenic stimuli (11); finally, the so called "collision hypothesis" suggests that the coexistence of different tumors may realize a confluence in a single nodule (12, 13). Some cases of MMFTC have been reported in the setting of MEN 2A syndrome (6). Since somatic *RET* mutations have been recently described in MMFTC (16), we found of interest to confirm this novel observation in a patient with a thyroid tumor sharing follicular-parafollicular phenotypic appearance.

CASE REPORT

A 76-year-old woman, with no familial history of thyroid tumors, was admitted to our hospital because of a nodule in the right lobe of the thyroid. Fine needle aspiration cytology was consistent with a follicular lesion. Routine laboratory tests, FT3 and FT4 were in normal range, while high serum levels of both calcitonin (CT) (6230 pg/ml, range: 0-10 pg/ml; ELSA-hCT kit, CIS Bio International, CEDEX, France) and carcinoembryonic antigen (130 ng/ml, range: 0-5 ng/ml) were found.

The patient underwent total thyroidectomy and central neck lymph node dissection. Histopathological e-

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xamination revealed a medullary carcinoma, consisting of solid areas with polygonal cells, organized in compact lobules and trabeculae; the stroma was devoid of amyloid deposits. In the same nodule an histopathological pattern of follicular carcinoma, with vascular invasion and concomitant immunohistochemical expression of thyroglobulin and calcitonin was observed (Fig. 1, Fig. 2 panel A). The surrounding thyroid tissue did not show any sign of C-cell hyperplasia. The final diagnosis was mixed medullary-follicular thyroid carcinoma. After thyroidectomy, radioiodine ablation therapy was given (^{131}I, 30 mCi; 1,11 GBq) and the following total body scintiscan was negative. Ultrasound examination and computed tomography scan excluded residual disease and/or metastatic lesions. L-thyroxine suppressive treatment was begun. During the 5-year follow-up, specific serum markers for differentiated thyroid cancer (TG<1.5 ng/ml with negative anti-thyroglobulin antibody) and for MTC (CT<10 pg/ml and CEA<5 ng/ml) were undetectable, excluding disease progression.

METHODS

DNA extraction and PCR amplification

Genomic DNA was extracted from peripheral leukocytes and from paraffin embedded tissue using DNA extraction kit, under conditions suggested by the supplier (Amersham, Milano, Italy).
Genomic amplicons encompassing exons 10, 11, 13, 14, 15 and 16 of the *RET* proto-oncogene were obtained by PCR using primers designed on the basis of the intronic sequence (14). The primers were the following:
10S: 5′-GAATTCGCTGAGTGGGCTACGTCT-3′
10A: 5′-CTGCAGACCCACTCACCCTGGATG-3′
11S: 5′-GTGCCAAGCCTCACACCA-3′
11A: 5′-GGAGTAGCTGACCGGGAA-3′
13S: 5′-CTCTCTGTCTGAACTTGGGC-3′
13A: 5′-TCACCCTGCAGCAGGCCTTA-3′
14S: 5′-TGGCTCCTGGAAGACCCA-3′
14A: 5′-AGAGCCATATGCACGCAC-3′
15S: 5-CTCGTTCATCGGGACTTGGCA-3′
15A: 5-CTGGCTCCTCTTCACGTAGGA-3′

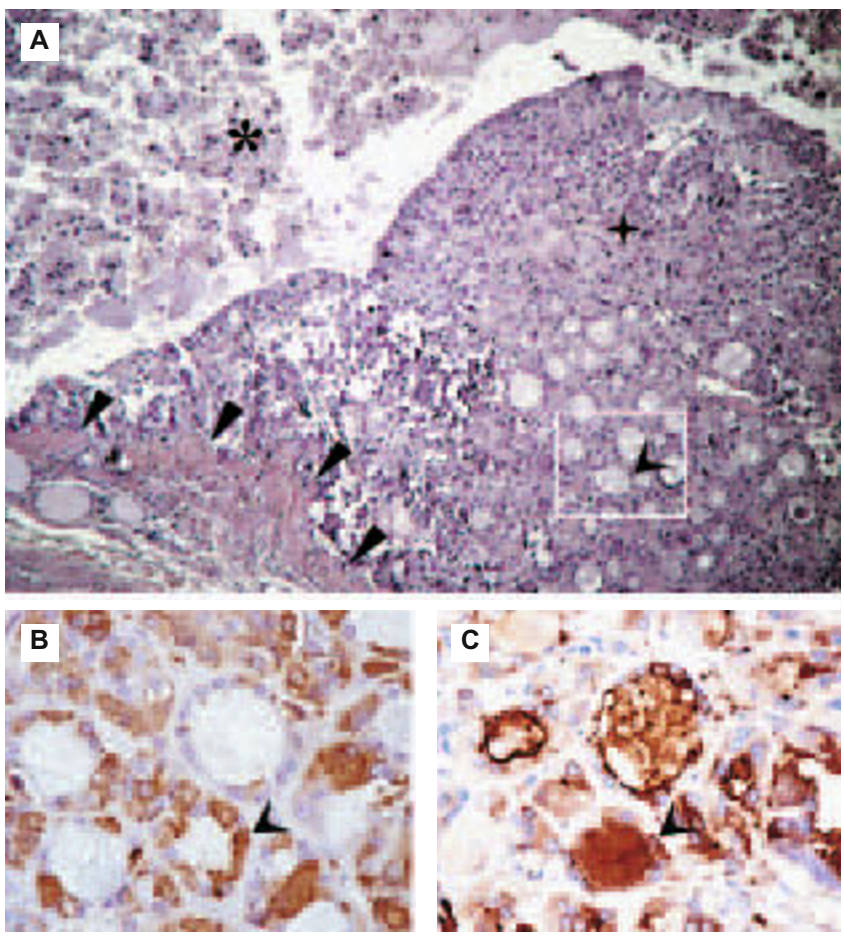

Fig. 1 - *Panel A: Mixed medullary-follicular carcinoma with wide vascular invasion (⇑). Vessel lumen (*); Vessel wall (▼) (H&E 100x). Panel B, C: Higher magnification (400x) of the framed area marked in Panel A. Panel B: Immunohistochemical expression of calcitonin immunoreactivity (3,3′-diaminobenzidine tetrahydrochloride). Panel C: Immunohistochemical expression of thyroglobulin immunoreactivity (3-amino-9-ethylcarbazole). The arrows (▼) in Panels A, B, C indicate the same follicle in paraffin embedded sections consecutively obtained.*

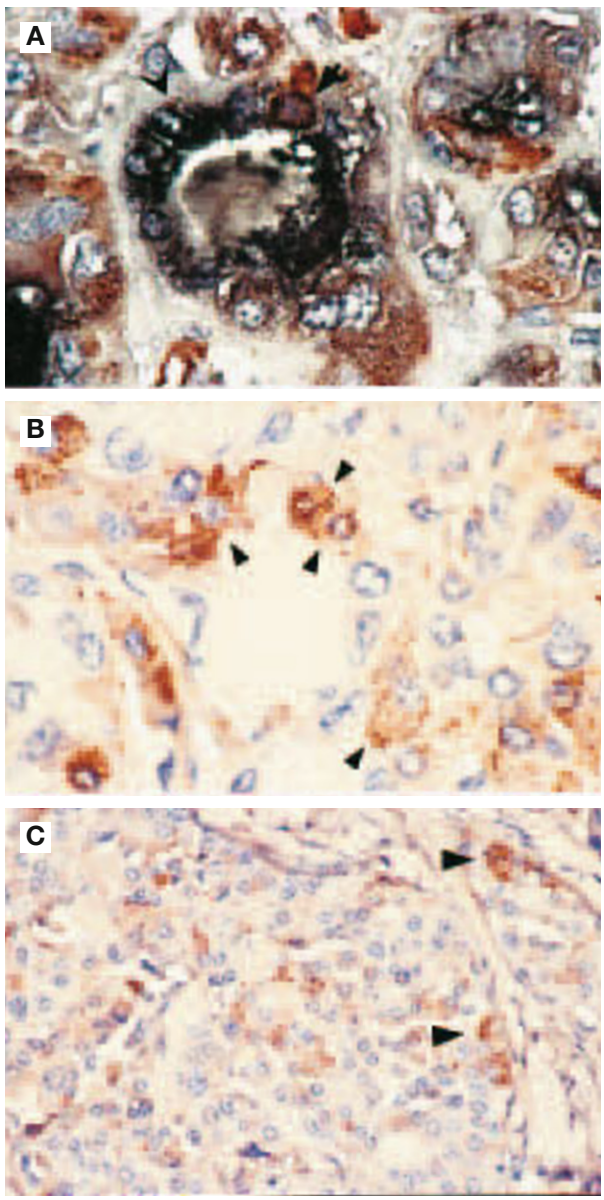

Fig. 2 - *Panel A: Thyroglobulin (black, ⮟) and calcitonin (red, ⮝) double staining immunoreactivity of the neoplastic cells showing their dual expression in the same follicle. Thyroglobulin is revealed by 5-Bromo-4-Chloro-3-Indolyl-Phosphate/Nitro Blue Tetrazolium in alkaline phosphatase procedure, whereas calcitonin by 3-amino-9-ethylcarbazole in peroxidase procedure. Panels B-C: Immunohistochemical immunoexpression of* RET *oncoprotein. Some neoplastic cells (red, ▼) are stained both in follicular (panel B) and in solid areas (panel C).*

16S: 5′-AGGGATAGGGCCTGGGCTTC-3′
16A: 5′-TAACCTCCACCCCAAGAGAG-3′
PCR amplification was performed as previously described (15).

Single strand conformation polymorphism (SSCP) analysis

Amplicons of exons 10, 11, 13, 14 and 15 were screened for mutations by SSCP analysis. An aliquot of each sample was incubated with specific buffer (10 ml formamide, 0.5M etilendiamminetetraacetate (EDTA), 0.3% bromophenol blue, 0.3% xylene cyanol) for 8 min at 100 C and run on 15% Tris-Borate-EDTA gel using a Minigel apparatus (Bio-Rad Laboratories, Segrate, Milano, Italy), at 100 V at 4°C. After electrophoresis, the gel was examined by DNA Silver Staining Kit (Pharmacia Biotech, Cologno Monzese, Milano, Italy) according to the manufacturer's instructions.

Restriction analysis

Amplicon of exon 16 was directly screened for mutations by restriction enzyme analysis with Fok 1. The samples were incubated for 3 h at 37°C and run on 3% agarose-Tris-Borate-EDTA ethidium bromide gel containing.

RET *immunohistochemical analysis*

Tissue sections (5 µm) were first dewaxed in xylene, rehydrated in decreasing ethanol concentrations and microwave processed for three times in citrate buffer (pH 6.0) for 5 min. Endogenous peroxidase activity was inhibited by immersing the sections in 0.5% hydrogen peroxide for 30 min. Samples were briefly rinsed in phosphatase buffer saline (PBS), incubated with blocking serum and subsequently with primary antibody anti-*RET* oncoprotein (Clone 3F8, Novocastra Laboratories Ltd., Newcastle, UK) diluted 1:100 in PBS at 4°C overnight. After a thorough washing in PBS the sections were incubated with biotinylated secondary antibody (DAKO, Copenhagen, Denmark) for 30 min and processed for peroxidase-conjugated streptavidin (LSAB2, DAKO, Copenhagen, Denmark). The sections were washed three times and incubated in AEC solution, washed in tap water for 10 min, counterstained with hematoxylin and mounted in glycerin.

RESULTS AND DISCUSSION

Thyroid cancer with simultaneous histological and immunohistochemical features of medullary and follicular thyroid carcinomas is a rare condition (16). In the management of MTC, the identification of somatic or germinal *RET* mutations is required to discriminate sporadic cases from unrecognized familial disease (17). In sporadic MTC, somatic point mutations of the *RET* proto-oncogene have been detected (3,18). Thus, the presence of somatic *RET* mutations was examined in the thyroid neoplastic tissue of our patient, carrying an histological diagnosis of MMFTC.
Genomic DNA was extracted from paraffin-embed-

ded cancer tissue and peripheral blood. A purified 198 bp DNA fragment was obtained by PCR amplification of a region containing codon 918 of exon 16 of the *RET* gene. Since point mutations at this codon determine the loss of a Fok1 restriction site, the fragment was digested with Fok1. Figure 3 shows the presence of a stronger undigested band and weak bands, a pattern in accordance with the loss of the Fok1 site in one allele of the gene. The nucleotide sequence of the same fragment confirmed the presence of ATG to ACG heterozygotic mutation at codon 918. Analysis of genomic DNA extracted from peripheral blood showed persistence of the Fok1 site, demonstrating, therefore, the somatic nature of the mutation. No evidence of mutations in other domains of the *RET* gene was found (data not shown).

A variable degree of cytoplasmic *RET* protein expression was observed by using a specific monoclonal antibody. The immunostaining was detected in about 25% of the neoplastic cells both in follicular (Fig. 2 panel B) and in solid areas (Fig. 2 panel C).

A lot of evidence supports the existence of MMFTC as a distinct entity. First, the co-expression of TG and CT in the same neoplastic cells (19) may indicate mixed cellular differentiation. Second, the presence of mRNAs for both TG and CT in the tumoral cells as detected by in situ hybridization or Northern Blotting (20) may support the concomitant expression of CT and TG by the same neoplastic cell. Third, the concurrent expression of these different proteins can be demonstrated in the metastatic tissues (19, 21). As in our case, the observation of concomitant CT and TG immunostaining in the same follicle on the site of vascular invasion may be considered as an adjunctive criterion for the diagnosis of MMFTC. The simultaneous immunoexpression staining in the area of vascular invasion does not fit with the hypothesis of normal thyroid tissue entrapped in medullary carcinoma and supports the diagnosis of true MMFTC.

However, the morphological criteria together with a molecular analysis of the microdissected cancer tissue, by examining the gene expression of both follicular and neuroendocrine cells, may allow a more certain and unambiguous diagnosis of mixed tumors. In this regard, Volante *et al*. (16), by using laser-based microdissection technique was recently able to isolate the two-cell type components of 12 mixed tumors. By analyzing them for mutations of the *RET* proto-oncogene, allelic losses and for clonal expression, an hyperplastic rather than a neoplastic histological feature and a policlonal pattern of the follicular structures were frequently detected. This suggests that a true MMFTC tumor is a very rare thyroid cancer. Furthermore, since medullary and follicular components exhibited a different pattern of mutations and allelic losses, the "stem cell theory" (derivation from a single progenitor cell) as responsible of MMFTC appears unlike. Our data show, in a mixed tumor, a somatic mutation of *RET* proto-oncogene at codon 918, which represents a common abnormality in sporadic MTC (17). Therefore, we confirmed the recent observation of Volante *et al*. (16), which reported the same *RET* mutation in two mixed sporadic tumors and a different mutation (at codon 883) in a third patient. These findings confirm the central role of malignant transformation of parafollicular cells in the oncogenesis of mixed tumors. However, MMFTC is a rare but polymorphic entity with different pathogenetic mechanisms that require further studies on larger number of tissues to better clarify the controversial pathogenesis.

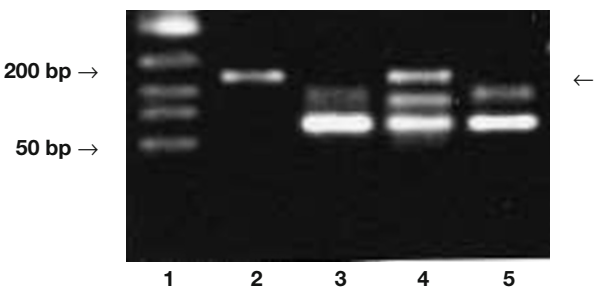

Fig. 3 - *Restriction analysis of PCR products of* RET *exon 16 after Fok 1 digestion (3 h at 37°C). Samples were run on 3% agarose-tris-borate-etilendiamminetetraacetate gel containing ethidium bromide. Lane 1: DNA size marker. Lane 2: undigested amplicon from unaffected subject. Lane 3: digested amplicon from unaffected subject. Lane 4: digested amplicon from tumoral tissue DNA of the patient (presence of heterozigotic point mutation at codon 918 determines loss of Fok 1 restriction site leading to pathological pattern). Lane 5: digested amplicon from blood DNA of the patient (presence of a normal restriction pattern indicates absence of germinal mutation).*

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