

Overexpression and activation of EGFR and VEGFR2 in medullary thyroid carcinomas is related to metastasis

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Abstract

Therapeutic options for patients with metastatic medullary thyroid carcinoma (MTC) are limited due to lack of effective treatments. Thus, there is a need to thoroughly characterize the pathways of molecular pathogenesis and to identify potential targets for therapy in MTC. Since epidermal growth factor receptor (EGFR) seems to play a crucial role for RET activation, a key feature of MTCs, and several promising EGFR/vascular endothelial growth factor receptor 2 (VEGFR2)-targeted drugs have been developed, the present study was designed to investigate whether these proteins are altered in MTCs. We used a well-characterized series of 153 MTCs to evaluate EGFR activation by sequencing and FISH analysis, and to perform EGFR and VEGFR2 immunohistochemistry. EGFR tyrosine kinase domain mutations were not a feature of MTCs; however, EGFR polysomy and a strong EGFR expression were detected in 15 and 13% of the tumors respectively. Interestingly, EGFR was significantly overexpressed in metastases compared with primary tumors (35 vs 9%, $P=0.002$). We also studied whether specific RET mutations were associated with EGFR status, and found a decrease in EGFR polysomies ($P=0.006$) and a tendency towards lower EGFR expression for the most aggressive RET mutations (918, 883). Concerning VEGFR2, metastasis showed a higher expression than primary tumors ($P=2.8 \times 10^{-8}$). In this first study investigating the relationship between EGFR, RET, and VEGFR2 in a large MTC series, we found an activation of EGFR and VEGFR2 in metastasis, using both independent and matched primary/metastasis samples. This suggests that some MTC patients may benefit from existing anti-EGFR/VEGFR2 therapies, although additional preclinical and clinical evidence is needed.

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Introduction

Medullary thyroid carcinoma (MTC) arises from parafollicular C-cells and comprises 4–10% of all thyroid cancers (Leboulleux *et al.* 2004). About 75% of cases are sporadic, and 25% are inherited as

a component of the multiple endocrine neoplasia type 2 (MEN2A, MEN2B, and familial MTC (FMTC)) syndromes. The best treatment option for patients with MTC is total thyroidectomy and lymph node dissection, with an overall survival rate after

10 years of the primary surgery of 69%, and with age and disease extent at initial presentation as the main prognostic factors (Bergholm *et al.* 1997). For local or regional recurrence in the neck and mediastinum, reoperation is the main treatment. However, cure is achieved only in 5–35% of the reoperated patients, and the remainder of patients are at high risk of developing distant metastasis (Wells *et al.* 1982, Giraudet *et al.* 2007). Distant metastases that are observed at presentation in 7–23% of unselected patients with MTC are the main cause of MTC-related death with survival rates of 25% at 5 years after their detection (Bergholm *et al.* 1997, Modigliani *et al.* 1998). To date, management of metastatic disease is primarily oriented towards the relief of symptoms, since no effective treatment exists. Thus, there is a great need for novel treatments and comprehensive clinical trials for patients with recurrent and metastatic MTC to improve therapy.

The *RET* proto-oncogene encodes a tyrosine kinase receptor that plays a major role in MTCs. Sporadic MTC forms are related to somatic mutations in the *RET* gene in 30–50% of these tumors (Uchino *et al.* 1999), while the vast majority of FMTC forms carry inherited mutations of *RET* (Schuffenecker *et al.* 1998, Randolph & Maniar 2000, Nagy *et al.* 2004). These are gain-of-function mutations and activate the kinase activity of RET, which provides mitogenic and survival signals to the calcitonin-producing C-cells. The type of *RET* mutation correlates with the presentation and aggressiveness of the tumor, thus allowing for genetic screening and recommendations for preventive surgical management in the familial cases (Brandi *et al.* 2001, Cote & Gagel 2003).

In addition to RET, other kinase receptors may play a role in the development and progression of MTCs (Liu *et al.* 2008). The epidermal growth factor receptor (EGFR) has been shown to be frequently overexpressed in various solid tumors, and plays a key role in cancer development and progression. Dimerization of the receptor following ligand binding results in transphosphorylation and subsequent activation of several downstream signal transduction pathways. Overexpression of EGF and EGFR has been detected in thyroid carcinomas, mainly in the extremely aggressive but rare anaplastic tumors. The inhibition of EGFR kinase activity by anti-EGFR antibodies and small molecule inhibitors has shown anti-proliferative effects in thyroid carcinoma cell lines *in vitro* and *in vivo*, including cells with activated RET mutants characteristic of sporadic and FMTCs (Croyle *et al.* 2008). Furthermore, recent findings show that EGFR stimulates RET phosphorylation (Croyle *et al.* 2008).

All together, this suggests that EGFR might be important for the proliferation and metastasis of thyroid carcinoma and, in particular, of MTC. Recently, several EGFR-targeted molecules, such as the tyrosine kinase inhibitors, gefitinib and erlotinib, and the monoclonal antibody cetuximab, have been successfully used in the treatment of some EGFR-dependent cancers. The response rate to the novel EGFR-targeted drugs has been associated, among other factors, to the presence of somatic activating mutations in *EGFR*, to *EGFR* amplification/polysomy, and to EGFR protein overexpression (Ciardiello & Tortora 2008, Zhang & Chang 2008). However, the potential benefit that these drugs could represent for MTCs has not been assessed, since the role of EGFR in MTCs has not been investigated. The only phase II trial evaluating the efficacy of an EGFR inhibitor (gefitinib) in patients with advanced thyroid cancer, included only four MTC patients (Pennell *et al.* 2008), thus reflecting the enormous difficulty of studies on low incidence tumors, such as MTC, and the necessity of previous exploratory work as this one. Other tyrosine kinase receptors such as VEGFR2 have been associated with increased growth, progression, and invasiveness of papillary thyroid carcinoma (PTC; Klein *et al.* 1999). Thus, drugs with multiple targets, such as vandetanib that inhibits VEGFR2, RET and EGFR, could represent a promising therapeutic approach for MTC (Wells *et al.* 2007, Haddad *et al.* 2008).

The present study was designed to investigate EGFR and VEGFR2 expression and activation in MTCs, using an outstanding series of 153 samples. We found that *EGFR* mutations in the tyrosine kinase domain are rare in MTC. On the other hand, EGFR expression was high and gene copy number increased in a substantial number of cases; metastases showed the highest EGFR and VEGFR2 expression. Overall, this is the first study that proves that EGFR and VEGFR2 are activated in a subset of MTCs, suggesting that these patients might benefit by novel drugs targeting these receptors.

Patients and methods

Human medullary thyroid carcinoma samples

A total of 153 paraffin-embedded MTC samples were available from the Pathology Departments of the Hospital Universitari Arnau de Vilanova, Hospital de Sant Pau, and the Spanish National Cancer Center. Institutional review board approval was obtained for the study, and informed consents were obtained from all patients. The patients were aged from 15 to 82 years (mean age 48 years) and 64% were females.

Table 1 Characteristics and mutational status of *RET* of the 153 medullary thyroid carcinoma (MTC) samples included in the study

MTC samples	<i>n</i>
Primary tumor	133
Metastasis	20
Sporadic	111
<i>RET</i> exon 10 (C618, C620)	2
<i>RET</i> exon 11 (C634)	5
<i>RET</i> exon 15 (A883)	1
<i>RET</i> exon 16 (M918)	28
No mutation in <i>RET</i> ^a	49
Undetermined <i>RET</i> mutation ^b	26
Familial	39
<i>RET</i> exon 10 (C618, C620)	5
<i>RET</i> exon 11 (C634)	31
<i>RET</i> exon 14 (V804)	2
<i>RET</i> exon 15 (S891)	1
Unknown	3
<i>RET</i> exon 11 (C634)	1
Undetermined <i>RET</i> mutation ^b	2

^aNo mutations found in *RET* exons 10, 11, 13, 14, 15, and 16.

^bTumors in which *RET* exons 10, 11, 13, 14, 15, or 16 could not be assessed.

Of the 153 MTC samples, 133 corresponded to primary tumors and 20 corresponded to metastases (Table 1), and in seven cases, primary/metastatic samples were from the same patient (Supplementary Table 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). The mutational status of the *RET* proto-oncogene in exons 10, 11, 13, 14, 15, and 16 was assessed from genomic DNA using standard PCR conditions, primers, and automated sequencing as previously described (Ceccherini *et al.* 1993). The tumor samples corresponded to patients diagnosed as sporadic or familial based on the analysis of the *RET* proto-oncogene initially performed in peripheral blood samples. Familial cases carried germline mutations in exons 10, 11, 14, or 15, while sporadic cases carried somatic *RET* mutations in exons 10, 11, 15, or 16, or were classified as ‘no mutation in *RET*’ (*RET* negative) when no somatic mutations were found in the exons 10, 11, 13, 14, 15, or 16 (Table 1). Among the apparently sporadic cases (without *RET* mutations in blood samples), 26 tumors were classified as ‘undetermined *RET* mutation’ due to failure of the PCR analysis caused by low tumoral DNA quality. Finally, only in three cases, the blood sample was not available and could not be classified as sporadic or familial (referred to as ‘unknown’).

Hematoxylin and eosin-stained sections of each sample were examined by a pathologist to confirm the diagnosis and to select MTC areas representative of each tumor to construct tissue microarrays (TMA).

TMA construction was carried out as previously described with 2–3 cores of each tumor placed at different positions in the TMA and including, whenever possible, surrounding normal tissue (Cascon *et al.* 2005). Three TMAs contained most of the tumor samples (109), and the remaining samples (44) were analyzed using whole tumor sections.

Immunohistochemical study of EGFR and VEGFR2 protein expression

The membranous protein expression of EGFR was determined by means of immunohistochemistry (IHC) using an EGFR monoclonal antibody (dil 1:10, EGFR.113, Novocastra) and the NovoLink Polymer Detection System (Novocastra, Newcastle upon Tyne, UK). Sections of the paraffin-embedded tissue were immersed in boiling 10 mM sodium citrate at pH 6.5 for 2 min in a pressure cooker for antigen retrieval. The results were analyzed by two experienced pathologists (X M-G and J P). The intensity of the staining was evaluated by visual examination under a microscope. Each case was scored by estimating the fraction of positively membrane-stained tumor cells (0, no staining was observed, or membrane staining was observed in <10% of the tumor cells; 1) a faint/barely perceptible membrane staining was detected in >10% of tumor cells. The cells exhibited incomplete membrane staining; 2) a weak to moderate complete membrane staining was observed in >10% of tumor cells; 3) a strong complete membrane staining was observed in >10% of tumor cells). The tumor was considered positive for EGFR expression when the score was 2+ or 3+. The IHC results obtained using the TMAs were validated independently in whole tumor sections from 20 samples (5 positive and 15 negative), and a complete agreement in the results was observed.

For VEGFR2 evaluation, TMA blocks were sectioned at a thickness of 3 μm and dried for 16 h at 56 °C. They were dewaxed in xylene, rehydrated through a graded ethanol series, and washed with PBS. Antigen retrieval was achieved by heat treatment in a pressure cooker for 3 min in 10 mM citrate buffer (pH 6.5). Endogenous peroxidase was blocked. A monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (clon A3, dilution 1/100) was used. This primary antibody was incubated overnight at 4 °C in a humidified chamber. The reaction was visualized with the EnVision Detection Kit (DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as substrate. Sections were counterstained with Harris hematoxylin. Immunohistochemical results were evaluated by three different members of the team

(X M-G, E C, and J P). The staining was cytoplasmic. Uniform pre-established criteria were used to assign an intensity score (Pallares *et al.* 2006). Immunoreactivity was graded semi-quantitatively by considering the percentage and intensity of the staining of the tumor cells. A histological score was obtained from each sample, which ranged from 0 (no immunoreaction) to 300 (maximum immunoreactivity). The score was obtained by applying the following formula: $H \text{ score} = 1X (\% \text{ light staining}) + 2X (\% \text{ moderate staining}) + 3X (\% \text{ strong staining})$. Since each TMA included two different tumor cylinders from each case, immunohistochemical evaluation was done after examining both samples.

EGFR gene copy number analysis by FISH

Fluorescence *in situ* hybridization (FISH) studies were performed with a commercial probe for EGFR (Kreatch Cat # KBI-10702 EGFR, Her1 (7p11)/SE 7, Amsterdam, The Netherlands). This probe labeled the EGFR locus with a red fluorochrome and the centromere of chromosome 7 that served as a ploidy control, with a green fluorochrome. The laboratory process was performed following the recommendations of the manufacturer. The slides were observed under an Olympus BX fluorescence microscope, and cell images were captured using a CCD camera (Photometrics SenSys, Tucson, AZ, USA) connected to a computer running the Chromofluor image analysis system (Cytovision, v3.1. software package, Applied Imaging Corporation, San Jose, CA, USA). Non-overlapping tumor cell nuclei were analyzed to estimate the number of red (*EGFR*) and green (*CEP7*) signals. FISH scoring of *EGFR* fluorescence signals was carried out in each sample by counting the number of red and green signals in an average of 100 nuclei. Since no *EGFR* gene amplifications were found, we assigned the level of ploidy for each sample as follows: tumors with four or more copies of *EGFR* and their respective centromeres in $\geq 40\%$ of the cells were considered to be FISH positive for polysomy, whereas tumors with the majority of cells carrying two or three copies of *EGFR* were considered to be FISH negative for polysomy. The FISH results obtained in the TMAs were validated independently in sections for eight samples (four positive and four negative), and a complete agreement in the results was observed.

Detection of mutations in the tyrosine kinase-coding domain of the EGFR gene

Genomic DNA was extracted from paraffin-embedded tumor tissue using the DNA easy tissue kit (Qiagen).

EGFR exons 18, 19, and 21 were selected for mutational analysis because most functional mutations cluster in this region (encoding the tyrosine kinase domain; Sharma *et al.* 2007), and were amplified by nested PCR using specific primers: exon 18 (f-PCR1 CAAATG-AGCTGGCAAGTGCCGTGTC, r-PCR1 GAGTTTC-CCAAACACTCAGTGAAAC; f-PCR2 CAAGTGCC-GTGTCTGGCACCCAAGC, r-PCR2 CC-AAA-CACTCAGTGAAACAAAGAG); exon 19 (f-PCR1 GCAATATCAGCCTTAGGTGCGGCTC, r-PCR1 CATAGAAAGTGAACATTTAGGATGTG, f-PCR2 GTGCATCGCTGGTAACATCC, r-PCR2 TGTGGA-GATGAGCAGGGTCT); and exon 21 (f-PCR1 CTAA-CGTTCCGCCAGCCATAAGTCC, r-PCR1 GCTGCG-ACTCACCCAGAATGTCTGG, f-PCR2 GCTCA-GAGCCT-GGCATGAA, r-PCR2 CATCCTCCCCTG-CATGTGT). After purification of the PCR products, direct DNA sequencing was performed (sequencer 3730, Applied Biosystems, Alcobendas, Madrid, Spain).

Statistical analysis

All statistical analyses were performed using SPSS (Chicago, IL, USA) version 17.0 statistical software. The χ^2 test was used to compare variables representing tumor types (primary tumors/metastasis and sporadic/familial), EGFR IHC and FISH data (positive/negative samples). For *RET* mutations analysis, we considered independently each mutation type according to activation (very high: 918 and 883; high: 634 and 618; moderated: 620, 804, and 891; and no *RET* mutation). Unconditional lineal regression was applied for the comparison of the continuous variable VEGFR2 IHC with all other variables. *P* values < 0.05 were considered significant.

Results

Immunohistochemical, gene copy number, and mutational study of EGFR in MTCs

From the 153 MTCs available for the study, 17 cases were not evaluable for EGFR IHC analysis. MTCs exhibited a variable immunopositivity for EGFR (Fig. 1A and B): 18 cases (13%) presented a high membranous EGFR expression (eight cases scored 3+ and ten cases scored 2+), while in 118 cases the expression of EGFR was absent or very limited (92 cases scored 0 (68%) and 26 scored 1+(19%) respectively). EGFR overexpression (2–3+) was compared with the characteristics of the tumor, finding a statistically significant difference between primary tumors and metastases ($P=0.002$). Strong EGFR positivity occurred in 9% of the primary tumors versus

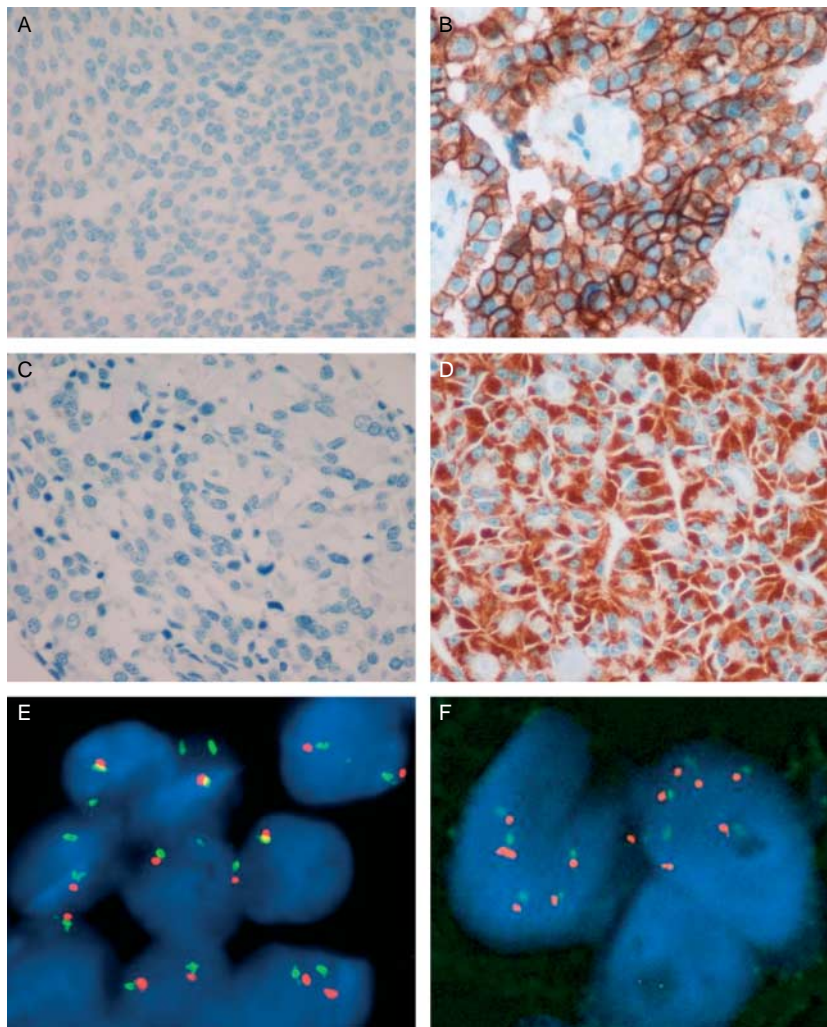


Figure 1 MTCs show variability in EGFR and VEGFR2 protein expression and *EGFR* gene copy number. EGFR and VEGFR2 protein expression was analyzed by IHC. Lack of membrane staining corresponded to EGFR-negative cases (A), while strong EGFR staining corresponded to positive cases (B). Representative cases with low (C) and high (D) VEGFR2 protein expression. The number of *EGFR* gene copies was analyzed by FISH analysis. Diploid tumor cells showed two red signals (EGFR), and two green (CEP7) signals in most cells (E), whereas cells with high polysomy showed four or more red and green signals in more than 40% of the tumor cells (F).

35% of the metastases (Table 2). On the other hand, the sporadic/familial character of the tumors did not have an influence on EGFR IHC.

We then determined the number of copies of the *EGFR* gene in the MTCs by FISH analysis (Fig. 1E and F). No gene amplifications were found, but some tumors showed an elevated number of copies (four or more) detected by both the *EGFR* and centromeric probes. This was interpreted as polysomy for chromosome 7 and was observed in 16 samples (14%), whereas 94 samples (86%) were negative (the remaining 43 cases were not evaluable). *EGFR* polysomy was similar in metastasis and primary tumors, and in sporadic versus

familial disease (Table 2). However, as was expected, there was a significant correlation between *EGFR* gene polysomy and strong (2/3+) EGFR protein expression ($P=0.011$), with 37% of the FISH-positive cases having a strong IHC signal, whereas 12% of the FISH-negative cases had a strong EGFR IHC signal (Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>).

We also determined whether the *EGFR* gene might be carrying activating mutations in the tyrosine kinase domain in MTCs, using tumoral DNA and sequencing those *EGFR* exons where the mutations cluster in non-small cell lung cancer. The most frequently

Table 2 Epidermal growth factor receptor (EGFR) protein expression (immunohistochemistry, IHC) and polysomy (fluorescence *in situ* hybridization, FISH) according to the type of medullary thyroid carcinoma

EGFR	Primary n (%)	Metastasis n (%)	P value	Sporadic n (%)	Familial n (%)	P value
IHC negative (0/1) ^a	105 (91)	13 (65)	0.002	90 (89)	25 (78)	NS
IHC positive (2/3)	11 (9)	7 (35)		11 (11)	7 (22)	
FISH negative ^b	83 (87)	11 (79)	NS	66 (84)	27 (90)	NS
FISH positive	13 (13)	3 (21)		13 (16)	3 (10)	

^aEGFR immunohistochemistry was considered positive for strong membrane staining (scores 2+ and above), and negative for absent or very limited expression (scores 0 and 1+).

^bFISH positive referred to *EGFR* polysomy, and FISH negative referred to lack of polysomy.

mutated exon 19 was analyzed in 58 cases, and exons 21 and 18 in 17 and 16 cases respectively. We found only one case carrying two exon 19 nucleotide changes not described before and of unknown significance (P741H and I759V).

RET mutational status and EGFR expression and copy number

Since it has been shown that EGFR contributes to RET kinase activation, which is a key event in all familial and most sporadic MTCs, we investigated whether EGFR expression or gene copy number could be related to specific *RET* mutations. MTCs were grouped according to the aggressiveness of the *RET* mutation (i.e. very high: 918 and 883; high: 634 and 618; moderate: 620, 804, and 891; and no *RET* mutation, when no mutations were found in *RET* exons 10, 11, 13, 14, 15, and 16), and this was compared with the EGFR IHC and FISH data.

When the sporadic and familial character of the MTCs was not taken into account in the analysis, EGFR expression and *EGFR* polysomy in MTCs were not significantly associated to specific *RET* mutations. However, there seemed to be a tendency toward a lower proportion of EGFR IHC positive cases among the tumors with the most aggressive mutations (4.2% *RET* 918/883 mutated tumors were 2+/3 IHC versus 18% of the rest of *RET*-mutated tumors, and 14% of the negative tumors; see Supplementary Table 3, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). When we performed this analysis subdividing the MTCs into sporadic and familial cases, we found that in the sporadic forms, *EGFR* polysomy was negatively correlated with the most aggressive *RET* 918/883 mutations. In the primary sporadic tumors, only 9.1% (2/22) of the MTCs carrying *RET* 918/883 mutations had *EGFR* polysomy, in contrast to 57% (4/7) of the other cases carrying less aggressive *RET* mutations (804, 891, 620,

618, and 634), $P=0.006$ (Supplementary Table 3). When metastatic cases were included in the study, the differences remained significant ($P=0.016$). With respect to the *RET*-negative tumors, 13% (3/23) had *EGFR* polysomy (Supplementary Table 3). In familial tumors, no *RET* 918/883 cases were available for the study, and in less aggressive *RET* mutants, only 7% (2/29) showed *EGFR* polysomy, suggesting that the familiar or sporadic context in which the mutation appears is important for *EGFR* copy number.

Immunohistochemical study of VEGFR2

VEGFR2 protein expression in the MTC was evaluated by IHC (Fig. 1C and D). The expression of VEGFR2 was higher in the metastases compared with the primary tumors with a very significant P value ($P=2.8 \times 10^{-8}$, see Fig. 2A). In addition, we found greater VEGFR2 IHC staining in: sporadic cases ($P=0.030$), *RET* mutation-negative tumors ($P=0.008$), and tumors without *RET* 634/618 mutations ($P=0.012$). When performing a multivariate analysis including all significant variables, only the metastatic nature remained as an independent feature associated to VEGFR2 IHC. To further analyze these variables, we performed the analysis only in the primary cases. We found that the presence of *RET* 634/618 mutations was negatively associated to VEGFR2 IHC ($P=0.036$; Fig. 2B). These results suggest that in addition to metastatic/primary characteristics, mutations affecting *RET* residues 634/618 could also be predictors of VEGFR2 protein expression.

No statistically significant association between VEGFR2 IHC and EGFR IHC, or EGFR copy number was found. However, 25% of the metastases simultaneously showed a strong EGFR (2/3+) and VEGFR2 (within the 25% top intensity score) expression. Among the primary tumors, none of the cases showed simultaneously a strong expression of these proteins (Supplementary Table 1).

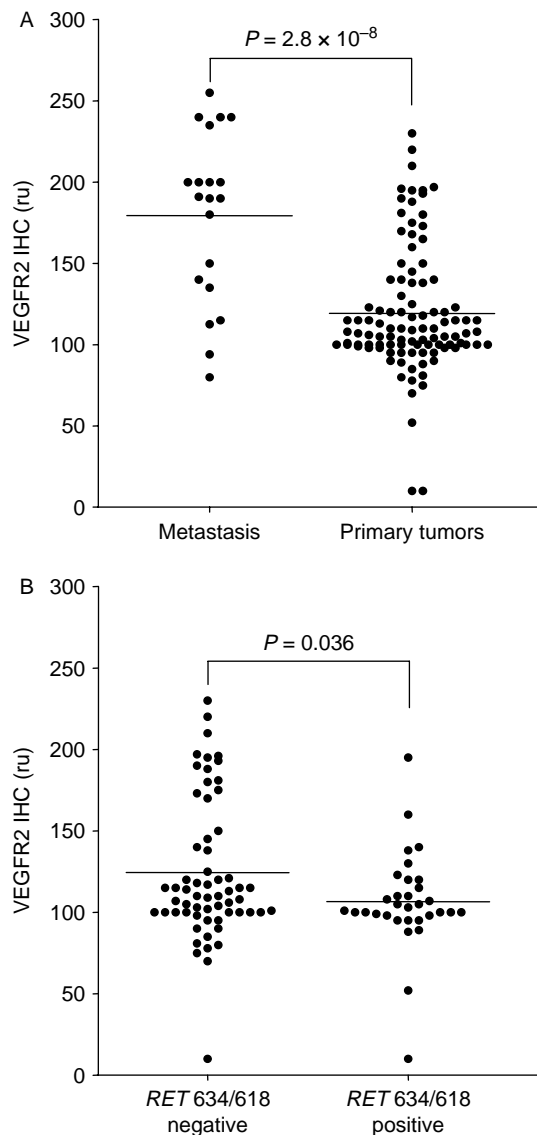


Figure 2 VEGFR2 protein expression in MTC. VEGFR2 protein expression was analyzed by IHC. Both hereditary and sporadic tumors were included in the analysis. Metastasis showed a significantly higher VEGFR2 protein expression than primary tumors, $P=2.8 \times 10^{-8}$ (A). Analysis of *RET* mutations in MTC primary tumors showed that the presence of *RET* 634/618 mutations was negatively associated with VEGFR2 IHC ($P=0.036$) (B).

EGFR and VEGFR2 protein expression changes during metastasis

We were able to collect matched primary tumor/metastasis from the same individual in seven cases, allowing us to determine whether EGFR and VEGFR2 status changed during this progression. With respect to EGFR, we found that in four cases, the expression was the same in primary tumor and metastasis, but three cases exhibited an increased EGFR expression in the

corresponding metastasis. In two cases, the expression greatly increased from very weak expression (1+) to strong (2+), and in one case, the expression changed from absence of protein (0) to weak expression (1+) in the metastasis (Table 3). With respect to the *EGFR* FISH analysis, we could only obtain the results of five pairs: three of them were negative in the primary tumor and in the metastasis, while two cases, coincident with those showing increased EGFR expression, acquired polysomy in the metastatic stage (Table 3). VEGFR2 protein expression increased more than 1.5-fold in three cases, two of them also showing an increase in EGFR expression and copy number. Two cases showed a moderate increase and two showed a moderate decrease in VEGFR2 expression in the corresponding metastasis (Table 3).

Discussion

Surgery is the only curative treatment of MTC, and unfortunately, 10–15% of sporadic and index familial cases present at diagnosis a palpable thyroid nodule, which is related to cervical lymph node metastases, and in some cases, distant metastases affecting most frequently lungs, bones and liver. In retrospective series, survival rates when distant metastases have already occurred at diagnosis are around 25% at 5 years and 10% at 10 years, since there is no effective treatment. The *RET* oncogene plays a crucial role in thyroid tumorigenesis, with somatic *RET* rearrangements being a prevalent feature of PTCs, whereas activating germline point mutations are responsible for MEN2-associated MTCs and somatic mutations for sporadic MTCs. Similarly, the tyrosine kinase receptor EGFR is associated with the regulation of cell growth, proliferation, and apoptosis. Croyle *et al.* (2008) recently showed that EGFR and RET coimmunoprecipitate and that ligand-induced activation of EGFR results in phosphorylation of RET. In addition, several EGFR inhibitors have been shown to markedly inhibit the growth of the MTC cell line TT, carrying the C634W *RET* mutation, and of *RET*-transfected cell lines expressing C634W and M918T mutated *RET* (Croyle *et al.* 2008). These data suggest that EGFR status determination in MTCs might be crucial for a possible EGFR-targeted therapy for these tumors. In addition, VEGFR2 has been associated with increased growth, progression, and invasiveness of PTC (Klein *et al.* 1999), and drugs simultaneously inhibiting VEGFR2, RET and EGFR have been developed and have promising therapeutic results in MTC (Wells *et al.* 2007, Haddad *et al.* 2008). However, the expression and activation of EGFR and VEGFR2 have not been

Table 3 Epidermal growth factor receptor (EGFR) expression and copy number and VEGFR2 expression in primary tumors and metastasis from the same patient

Matched MTC tumors	EGFR IHC		EGFR FISH		VEGFR2 IHC		Fold change
	Primary	Metastasis	Primary	Metastasis	Primary	Metastasis	
87-10505/88-1607	1+	2+	Neg	Polysomy	100	200	2.0
07B8365/07B8386	1+	2+	Neg	Polysomy	120	180	1.5
83-8179/83-8179-6C	0	1+	NE	NE	190	235	1.2
91-8321-2RA/91-91-10971	2+	2+	Neg	Neg	95	190	2.0
01B6049-3/01B6049-2	1+	1+	Neg	Neg	220	255	1.2
05B7152-4/05B7152	1+	1+	Neg	Neg	180	135	0.8
06T197.1/06T197.2	0	0	Neg	NE ^a	230	200	0.9

^aNot evaluable. Bold indicates significant increases.

investigated in MTCs. This is the first study providing this data in MTC, by using a large well-characterized panel of primary and metastatic samples.

With respect to EGFR and VEGFR2 protein expression, strong EGFR expression was found in 13% of all MTCs studied, with metastases having higher positivity than primary tumors ($P=0.002$, Table 2). Similarly, VEGFR2 was overexpressed in metastasis compared with primary tumors ($P=2.8 \times 10^{-8}$, Fig. 2). This was supported by primary/metastasis-matched cases from the same individual, where some tumors showed that EGFR and VEGFR2 overexpression developed during this progression (Table 3). In agreement with this, EGFR and VEGFR2 overexpression has been shown to be closely correlated to advanced tumor stage, metastasis, and poor clinical outcome in several human cancers. With respect to thyroid carcinomas, EGFR overexpression has been detected in most anaplastic tumors (Schiff et al. 2004, Lee et al. 2007, Wiseman et al. 2007, Elliott et al. 2008) and to a much lower extent in PTCs (Schiff et al. 2004, Murakawa et al. 2005, Mitsiades et al. 2006, Lim et al. 2007, Elliott et al. 2008). In PTC, EGFR expression has been associated to poor prognosis (Akslen & Varhaug 1995, Chen et al. 1999), although not always (Lim et al. 2007), and recent studies have suggested that EGFR inhibitors might be active against anaplastic thyroid carcinomas (Lopez et al. 2008). In thyroid neoplasias, overexpression of VEGF and platelet-derived growth factor receptor have been described, and disruption of VEGF signaling has been shown to inhibit thyroid cancer cell growth *in vitro* and *in vivo* (Tuttle et al. 2002, Turner et al. 2003, Lin & Chao 2005, Vieira et al. 2005).

With respect to the number of EGFR gene copies in the MTCs, we did not detect amplifications but found polysomies in 15% of the tumors examined. FISH positivity was not significantly different among primary

and metastatic tumors, although there was a tendency towards higher positivity in metastases (21 and 13% positivity for metastasis and primary tumors respectively, Table 2). EGFR copy numbers were independent of the familial/sporadic origin of the tumors. When comparing IHC and FISH results, there was not a complete agreement between EGFR protein expression and gene copy number, but as expected, there was a significant correlation ($P=0.01$). This is similar to the findings in anaplastic thyroid carcinoma (ATC) and follicular thyroid carcinoma (FTC), where EGFR gene amplification did not occur, but polysomy was detected with high frequency (46–61 and 32% respectively; Lee et al. 2007, Liu et al. 2008), and there was also a significant, although not complete correlation between IHC and FISH positivity in ATCs (Lee et al. 2007). Concerning EGFR mutations, none of the already described EGFR tyrosine kinase activating mutations were detected in the subset of MTCs studied, only two not previously described amino acid changes with unknown biological significance were found in one of the samples. These results are similar to those described for ATC and FTC in which no mutations were found (Lee et al. 2007, Liu et al. 2008).

Since RET activation seems to be influenced by EGFR (Croyle et al. 2008), we investigated whether EGFR activation could be related to specific RET mutations by comparing EGFR IHC and FISH results with the RET mutation of the MTCs. Depending on the location and the amino acids affected, the point mutations correlated with the presentation and aggressiveness of the tumor. We found that the tumors with the most aggressive RET mutations (in codons 883/918) had a significantly lower number of EGFR polysomies and a tendency toward less EGFR immunopositivity when compared with the other RET mutations. It could be speculated that the most aggressive RET mutations are less dependent on EGFR activation. In fact, in the

work by Fagin *et al.* (Croyle *et al.* 2008) in which *RET* codon 634 and codon 918 mutated cell lines are compared, the effect of EGFR inhibitors on the 918 mutated cell line seems smaller, in agreement with our data. Since EGFR activation status seems related to *RET* activation, we examined EGFR status in the *RET*-negative tumors. However, we found no differences in EGFR activation between *RET*-positive and -negative tumors, probably indicating that other molecular mechanisms leading to *RET* activation, such as *RET* gene copy gains, altered promoter activity, or increased transcription, might be acting in the *RET* mutation-negative tumors. Additionally, and although it is unclear whether VEGFR2 and *RET* are related, when we analyzed VEGFR2 expression in our series, a modest significant association with *RET* mutation status in primary tumors was found (Fig. 2B).

In conclusion, in our MTC series, we found a significant overexpression of VEGFR2 and EGFR in metastasis, and we provide evidence showing *EGFR* polysomy in a subset of MTC. This data suggests that therapies targeting these tyrosine kinase receptors could have a clinical impact on these patients. In fact, promising results with MTC have been reported with vandetanib, an antagonist of VEGFR, EGFR, and *RET* (Wells *et al.* 2007, Haddad *et al.* 2008). In conclusion, this is the first study including a large series of well-characterized MTCs that shows that EGFR and VEGFR2 participate in the progression of an important proportion of cases.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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