Influence of \textit{RET} mutations on the expression of tyrosine kinases in medullary thyroid carcinoma

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Abstract

The therapeutic options for patients with metastatic medullary thyroid carcinoma (MTC) have recently increased due to the development of tyrosine kinase inhibitors (TKIs), some of which have achieved remarkable clinical responses in MTC patients. However, the molecular basis for the large variability in TKI responses is unknown. In this exploratory study, we investigated the expression of eight key TKI target proteins (EGFR, KIT, MET, PDGFRB, VEGF (VEGFA), VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4)) by immunohistochemistry in 103 molecularly characterized MTC samples and identified the associated clinical and molecular features. A number of MTC samples exhibited a high expression of VEGFR2 and VEGFR3, which were overexpressed in 57 and 43\% of the MTC samples respectively. VEGFR1, PDGFRB, VEGF, KIT, and MET were present in 34–20\% of the cases, while EGFR was highly expressed in only 10\% of the MTC samples. Some proteins exhibited large differences in expression between sporadic and familial cases, suggesting that different \textit{RET} mutations may be associated with the immunohistochemical profiles. MTC samples with the C634 \textit{RET} mutation exhibited a higher expression of VEGFR3 and KIT than the M918T \textit{RET}-mutated and non-mutated \textit{RET} tumor samples ($P=0.005$ and $P=0.007$ respectively) and a lower expression of VEGFR1 ($P=0.04$). Non-mutated \textit{RET} MTC cases exhibited a lower expression of PDGFRB ($P=0.04$). Overall, this is the first study, to our knowledge, to show that multiple TKI targets are highly expressed in a subset of MTCs, suggesting that molecular stratification of patients may have the potential to improve TKI therapies for MTC.
Introduction

Medullary thyroid carcinoma (MTC) arises from the parafollicular cells of the thyroid gland. Sporadic MTC accounts for 75% of the cases, and the remaining 25% is inherited in nature as part of multiple endocrine neoplasia type 2 syndrome. The RET proto-oncogene plays a major role in MTC development, with 30–50% of the sporadic MTCs carrying somatic RET mutations (Uchino et al. 1999). RET mutations involved in MTC are gain-of-function alterations that increase RET kinase activity, resulting in a constant activation of downstream signaling pathways that ultimately lead to tumor growth (Schuffenecker et al. 1998, Randolph & Maniar 2000, Nagy et al. 2004). The presence of specific RET mutations determines the age of presentation and aggressiveness of the tumor, allowing for genetic screening and recommendations for preventive surgical management in familial cases (Brandi et al. 2001, Cote & Gagel 2003).

Patients with MTC undergo total thyroidectomy and lymph node dissection. However, because MTC is derived from neuroendocrine cells, it is unresponsive to radioiodine and TSH suppression, and it is unclear as to which is the most appropriate treatment for patients with residual or recurrent disease after primary surgery and for those with distant metastasis. Until recently, management of metastatic disease has primarily been oriented toward the relief of symptoms (Wells et al. 1982, Giraudet et al. 2007), but in the last few years, much effort has been devoted to developing clinical trials using targeted therapies.

In addition to RET mutations, vascular endothelial growth factor (VEGF)-mediated angiogenesis, leading to increased tumor growth and invasiveness, has been recognized as an important feature in MTC. Thus, targeted molecular therapies that inhibit oncogenic kinases such as RET and tyrosine kinase receptors involved in angiogenesis could be important for the treatment of metastatic or locally advanced MTC. In this regard, a number of small-molecule inhibitors that selectively inhibit tyrosine kinase receptors, such as vandetanib, sorafenib, sunitinib, axitinib, motesanib, and cabozantinib (XL184), have shown remarkable clinical responses in MTC patients, inducing partial responses and stabilization of the disease in a subset of MTCs in a RET mutation-dependent manner (Rodriguez-Antona et al. 2010). However, currently such information is lacking for most of the tyrosine kinase inhibitor (TKI) targets, and it is unknown whether they are expressed in MTC and whether this expression is associated with specific clinical and molecular features. Therefore, we set out to investigate the expression of eight key TKI target proteins in an outstanding series of 103 molecularly characterized MTC cases. This is the first study, to our knowledge, to demonstrate that multiple TKI targets are highly expressed in a subset of MTCs in a RET mutation-dependent manner. This information might be critical for the inclusion of MTC patients in future clinical trials and, ultimately, for improving treatment response.

Subjects and methods

Human MTC samples

A total of 103 paraffin-embedded MTC samples from 101 patients were obtained from the Spanish National Cancer Centre in collaboration with the CNIO tumor Bank. Institutional Review Board approval was obtained for the study, and informed consent was obtained from all the patients. The patients were aged from 11 to 80 years (median age 50 years), and 56% were females. Of the 103 MTC samples, 92 corresponded to primary tumors and 8 to metastases (see Table 1). 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 described previously (Ceccherini et al. 1993). The tumor samples corresponded in most cases to patients diagnosed as sporadic or familial, based on the analysis of the RET proto-oncogene in peripheral blood samples. Familial cases carried germline mutations in exon 10, 11, 14, or 15, while sporadic cases carried somatic RET mutations in exon 10, 11, 15, or 16, or were classified as ‘no mutation in RET’ when no somatic mutations were found in exon 10, 11, 13, 14, 15, or 16 (Table 1). Among the apparently sporadic cases (without RET mutations in blood samples), four tumors were classified as ‘undetermined RET mutation’ due to the failure of the PCR analysis caused by low tumor DNA quality. Among the familial cases, one
tumor derived from a patient with a family history of multiple neoplasia type 2 (second-degree relative diagnosed with a pheochromocytoma and mother with a MTC) was classified as ‘undetermined RET mutation’ because no blood sample was available and the tumor DNA was of low quality. Five cases could not be classified as sporadic or familial (referred to as ‘unknown’) because blood samples were not available.

Immunohistochemical study

Hematoxylin and eosin-stained sections of each tumor sample were examined by two pathologists to confirm the diagnosis and to select MTC areas representative of each tumor to construct tissue microarrays (TMAs). Three TMAs containing all the tumor samples (103) were constructed as described previously with two cores of each tumor placed at different positions in the TMAs (Cascon et al. 2005).

The three paraffin-embedded TMAs were used for the detection of EGFR, KIT, MET, PDGFRB, VEGF (VEGFA), VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4) proteins by immunohistochemistry (IHC) using specific antibodies. The suppliers, dilutions, visualization systems, and immunostainers used for the antibodies are given in Supplementary Table 1, see section on supplementary data at the end of this article. Two independent experienced pathologists (I Munoz-Repeto and M Cañamero) evaluated the intensity and extension of staining for all the antibodies by visual examination under a microscope. Not only the tumoral cells but also the stroma (fibroblasts, inflammatory cells, and blood vessels) were evaluated, taking into account the fact that these tumors are highly cellular with low stromal component, mostly consisting of blood vessels. Since each TMA included two different tumor cylinders from each case, immunohistochemical scoring was done after examining both samples.

The IHC scoring used was as follows: for EGFR, VEGFR2, and PDGFRB, tumor samples with moderate/strong staining were considered positive (Maderna et al. 2007, Rodriguez-Antona et al. 2010); for KIT, both the intensity and extent of staining were evaluated (Miliaras et al. 2004), and the intensity of staining was graded as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong), and the extent of staining was evaluated semiquantitatively and categorized as 0 (0% of cells), 1 (<10%), 2 (between 10 and 50%), 3 (between 50 and 80%), and 4 (more than 80%), and aggregate scores were obtained for each case (range 0–7) and cases with scores >3 were regarded as KIT-positive; MET protein was considered positive when its expression was positive for 30% of the tumor cells with moderate/strong staining (Lee et al. 2010); for VEGF, the intensity of staining was estimated on a four-tiered scale encoded as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong), and the extent of staining was evaluated semiquantitatively and categorized as 0 (0% of cells), 1 (<10%), 2 (between 10 and 50%), 3 (between 50 and 80%), and 4 (more than 80%), and aggregate scores were obtained for each case (range 0–7) and cases with scores >3 were regarded as VEGF-positive; for VEGFR3, the percentage of cells with positive staining was evaluated for each case and the median was calculated, and tumor samples with a percentage of positive cells higher than the median (50% of the positive tumor cells) were considered to have a high expression of VEGF3. The staining intensity for each protein marker analyzed and the number of tumor samples included in each category are given in Supplementary Table 2, see section on supplementary data at the end of this article.
Clustering
Hierarchical unsupervised cluster analysis of the 92 primary tumor cases was carried out by the average linkage clustering method using GeneCluster 3.0 (mean centered; de Hoon et al. 2004) and viewed in a visualizer (TreeView) that displays cluster profiles and relevant cluster member information. Immunohistochemical results were represented by a range of colors from green to red, with the brightest green representing the lowest staining intensity for each marker and the brightest red the highest one (Fig. 1). Three samples in which more than three antibodies failed to yield results were excluded from the analysis.

Statistical analyses
All statistical analyses were carried out using SPSS version 17.0 statistical software. The $\chi^2$ test or Fisher’s exact test was used to compare variables representing patient characteristics (male/female) and tumor types (primary tumors/metastases and sporadic/familial) with IHC protein expression (Tables 2 and 3). In addition, logistic regression analyses were carried out to obtain odds ratios (ORs) and 95% CI, which are given in Supplementary Table 4, see section on supplementary data given at the end of this article. For RET mutation analysis, we considered independently each of the major RET-mutated groups: C634, M918T, and no RET mutation. The C634 RET-mutated group included both familial and sporadic forms with germline and somatic mutations respectively; the M918T RET-mutated group corresponded exclusively to sporadic forms with M918T somatic mutation; and the ‘no mutation in RET’ group corresponded exclusively to sporadic forms in which germline mutations were discarded and no somatic RET mutation was detected (see Table 1). Other less frequent RET mutations were not considered for further analysis as they represented a heterogeneous group. To calculate the correlation between the expression of the different proteins evaluated, we used the Spearman’s test. Since this is an exploratory study, no correction of $P$ values due to multiple testing was performed, and bilateral $P$ values <0.05 were considered significant.

Figure 1
Hierarchical clustering of 89 primary MTC samples. The staining intensity for each immunohistochemical marker is represented as a range of colors between the brightest green (lowest expression) and the brightest red (highest expression). Gray squares indicate that no data was available.
Results

Immunohistochemical study in MTC cases

The different TKI targets tested in the 103 MTC samples available for the study presented a variable expression (Supplementary Figure 1, see section on supplementary data given at the end of this article). Many MTCs showed positive staining with 57, 43, 34, 33, 32, 23, 20 and 10% positive cases for VEGFR2, VEGFR3, VEGFR1, PDGFRB, VEGF, KIT, MET and EGFR, respectively. The IHC intensity observed for each marker is summarized in detail in Supplementary Table 2.

The expression of several TKI targets was correlated. The strongest correlations corresponded to PDGFRB, which exhibited a positive correlation with VEGFR3 (correlation coefficient of 0.51 and $P=0.046$) and a negative correlation with VEGFR1 (correlation coefficient of -0.34, $P=9 \times 10^{-8}$ and 0.002 respectively). A positive correlation was also detected between VEGF and VEGFR2 (correlation coefficient of 0.37, $P=0.0006$). The expression of EGFR correlated with that of MET and VEGFR3, and the expression of MET correlated with that of VEGFR2 and VEGF (see Supplementary Table 3, see section on supplementary data given at the end of this article). Similar results were observed when only primary tumor samples were analyzed.

Unsupervised clustering analysis of the primary MTCs based on the eight immunohistochemical markers evaluated clustered tumor samples into two main groups (Fig. 1). The left branch, which grouped 35% of the MTC cases, was characterized by positive PDGFRB and VEGFR3 staining and included most cases with EGFR overexpression. The right branch of the cluster included some of the VEGFR2-, VEGF-, MET-, KIT-, and VEGFR1-positive cases and cases displaying a negative expression for all the tested proteins. The left branch tended to be enriched in cases carrying the C634 RET mutation, either germline or somatic (35 vs 21%, in the left and right branches respectively), while the right branch was apparently enriched in tumors with the M918T RET somatic mutation (14 vs 6%).

Protein expression of TKI targets in different MTC types

Gender was associated with the expression of VEGFR1 (45% of female MTC cases exhibited positive staining vs 16% of the male cases, $P=0.007$) and VEGF (23 and 46% positive cases for females and males respectively, $P=0.017$), but age at diagnosis was not associated with IHC staining. We next determined whether the expression of the markers could be influenced by the type of tumor: primary tumors vs metastases and sporadic vs familial. MET displayed a higher expression in the metastatic cases than in the primary tumor cases ($P=0.049$; Table 2), similar to EGFR, PDGFRB, and VEGF, although differences for the latter proteins did not reach statistical significance (see Table 2). On the other hand, VEGFR1 displayed a higher expression in the primary tumor samples ($P=0.046$), similar to KIT, although this protein did not show any statistically significant difference. Some of the

Table 2  Protein expression of EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 according to the type of MTC

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary n (%)</th>
<th>Metastatic n (%)</th>
<th>$P$</th>
<th>Sporadic n (%)</th>
<th>Familial n (%)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR - $^b$</td>
<td>83 (91)</td>
<td>5 (71)</td>
<td>NS</td>
<td>58 (92)</td>
<td>24 (89)</td>
<td>NS</td>
</tr>
<tr>
<td>EGFR + $^c$</td>
<td>8 (9)</td>
<td>2 (29)</td>
<td></td>
<td>5 (8)</td>
<td>3 (11)</td>
<td></td>
</tr>
<tr>
<td>KIT -</td>
<td>65 (75)</td>
<td>8 (100)</td>
<td>NS</td>
<td>50 (83)</td>
<td>14 (54)</td>
<td>0.0040</td>
</tr>
<tr>
<td>KIT +</td>
<td>22 (25)</td>
<td>0 (0)</td>
<td>0.049</td>
<td>10 (17)</td>
<td>12 (46)</td>
<td></td>
</tr>
<tr>
<td>MET -</td>
<td>63 (83)</td>
<td>4 (50)</td>
<td></td>
<td>48 (84)</td>
<td>14 (78)</td>
<td>NS</td>
</tr>
<tr>
<td>MET +</td>
<td>13 (17)</td>
<td>4 (50)</td>
<td>0.046</td>
<td>9 (16)</td>
<td>4 (22)</td>
<td></td>
</tr>
<tr>
<td>PDGFRB -</td>
<td>60 (70)</td>
<td>3 (38)</td>
<td></td>
<td>46 (78)</td>
<td>13 (50)</td>
<td>0.010</td>
</tr>
<tr>
<td>PDGFRB +</td>
<td>26 (30)</td>
<td>5 (63)</td>
<td></td>
<td>13 (22)</td>
<td>13 (50)</td>
<td></td>
</tr>
<tr>
<td>VEGF -</td>
<td>62 (69)</td>
<td>4 (50)</td>
<td></td>
<td>41 (65)</td>
<td>20 (77)</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF +</td>
<td>28 (31)</td>
<td>4 (50)</td>
<td></td>
<td>22 (35)</td>
<td>6 (23)</td>
<td></td>
</tr>
<tr>
<td>VEGFR1 -</td>
<td>45 (62)</td>
<td>8 (100)</td>
<td>0.046</td>
<td>31 (58)</td>
<td>14 (78)</td>
<td>NS</td>
</tr>
<tr>
<td>VEGFR1 +</td>
<td>27 (38)</td>
<td>0 (0)</td>
<td></td>
<td>22 (41)</td>
<td>4 (22)</td>
<td>NS</td>
</tr>
<tr>
<td>VEGFR2 -</td>
<td>32 (43)</td>
<td>2 (29)</td>
<td></td>
<td>23 (42)</td>
<td>8 (44)</td>
<td></td>
</tr>
<tr>
<td>VEGFR2 +</td>
<td>42 (57)</td>
<td>7 (11)</td>
<td></td>
<td>32 (58)</td>
<td>10 (56)</td>
<td></td>
</tr>
<tr>
<td>VEGFR3 -</td>
<td>50 (57)</td>
<td>4 (57)</td>
<td></td>
<td>39 (65)</td>
<td>10 (38)</td>
<td>0.022</td>
</tr>
<tr>
<td>VEGFR3 +</td>
<td>37 (43)</td>
<td>3 (43)</td>
<td></td>
<td>21 (35)</td>
<td>16 (61)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Only primary tumor cases included in the analysis.

$^b$A negative expression is indicated by ‘−’.

$^c$A positive expression is indicated by ‘+’.

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markers exhibited large differences in expression in relation to the inherited character of the disease. We found that positive KIT, PDGFRB, and VEGFR3 staining was more frequently associated with familial forms than with sporadic forms ($P$ values of 0.0040, 0.010, and 0.022 respectively; Table 2; see also Supplementary Table 4). On the other hand, EGFR, MET, VEGF, VEGFR1, and VEGFR2 exhibited similar positive staining in familial and sporadic forms. Because familial cases are associated with specific mutations, these data suggest that different RET mutations can predict differences in the expression of some of these proteins.

**Expression of TKI targets according to RET mutations**

Because the metastatic samples exhibited altered expression of some of the proteins studied (see Table 2), we examined only primary tumor cases to determine the effect of specific RET mutations on protein levels. Cases with the C634 RET mutation included both germline and sporadic tumors, while cases with the M918T RET mutation and cases with no mutation in RET were all sporadic forms. We found that 67% of the MTC cases with the C634 RET mutation expressed VEGFR3, compared with 36 and 29% respectively of the MTC cases with the M918T RET mutation and the MTC cases without the RET mutation ($P=0.0047$; see Table 3; OR = 4.1, 95% CI = 1.5–11; Supplementary Table 4). The expression of KIT was also more frequently found in the C634 RET mutation cases than in the M918T RET mutation cases and non-mutated RET cases ($P=0.0069$; Table 3; OR = 4.1, 95% CI = 1.4–11; Supplementary Table 4). By contrast, VEGFR1 exhibited a lower expression in the C634 RET mutation cases than in the M918T or non-mutated RET cases (18, 56, and 39% respectively, $P=0.044$; Table 3). The expression of PDGFRB was higher in the C634 and M918T RET mutation cases than in the non-mutated RET cases ($P=0.040$; Table 3). Other proteins such as EGFR, VEGF, and VEGFR2 did not display differences in expression among the different types of RET-mutated tumors, while MET had a tendency toward a lower expression in MTC cases without RET mutations (Table 3).

The expression of KIT, PDGFRB, VEGFR1, and VEGFR3 was also examined in stroma: blood vessels, inflammatory cells, and fibroblasts (data not shown). KIT was only present in the inflammatory population in scattered cells; PDGFRB was expressed in blood vessels and fibroblasts, with an expression level ranging from moderate to high; and VEGFR1 and VEGFR3 were expressed in most blood vessels and occasionally in inflammatory cells. In addition, the expression of KIT was significantly lower in MTC cases with the C634 mutation ($P=0.0018$); no statistically significant differences for the expression of PDGFRB, VEGFR1, and VEGFR3 were found.

**Table 3  Protein expression of EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 according to the RET mutation in primary MTCs**

<table>
<thead>
<tr>
<th>Protein</th>
<th>C634 mut$^*$</th>
<th>Rest$^b$</th>
<th>M918T mut$^c$</th>
<th>Rest$^b$</th>
<th>Wt RET$^d$</th>
<th>Rest$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR $^-e$</td>
<td>22 (92)</td>
<td>56 (90)</td>
<td>NS</td>
<td>14 (93)</td>
<td>64 (90)</td>
<td>NS</td>
</tr>
<tr>
<td>EGFR $^+f$</td>
<td>2 (8)</td>
<td>6 (10)</td>
<td>1 (7)</td>
<td>7 (10)</td>
<td>49 (71)</td>
<td>NS</td>
</tr>
<tr>
<td>KIT $-g$</td>
<td>13 (54)</td>
<td>48 (83)</td>
<td>0.0069</td>
<td>12 (92)</td>
<td>49 (71)</td>
<td>NS</td>
</tr>
<tr>
<td>KIT $+h$</td>
<td>11 (46)</td>
<td>10 (17)</td>
<td>1 (8)</td>
<td>20 (29)</td>
<td>6 (17)</td>
<td>15 (32)</td>
</tr>
<tr>
<td>MET $-$</td>
<td>13 (77)</td>
<td>46 (84)</td>
<td>NS</td>
<td>9 (75)</td>
<td>50 (83)</td>
<td>NS</td>
</tr>
<tr>
<td>MET $+$</td>
<td>4 (22)</td>
<td>9 (16)</td>
<td>3 (25)</td>
<td>10 (17)</td>
<td>3 (9)</td>
<td>10 (26)</td>
</tr>
<tr>
<td>PDGFRB $-$</td>
<td>13 (54)</td>
<td>43 (75)</td>
<td>0.058</td>
<td>10 (71)</td>
<td>46 (69)</td>
<td>NS</td>
</tr>
<tr>
<td>PDGFRB $+$</td>
<td>11 (46)</td>
<td>14 (25)</td>
<td>4 (29)</td>
<td>21 (31)</td>
<td>6 (18)</td>
<td>19 (40)</td>
</tr>
<tr>
<td>VEGF $-$</td>
<td>18 (75)</td>
<td>41 (67)</td>
<td>NS</td>
<td>10 (70)</td>
<td>49 (67)</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF $+$</td>
<td>6 (25)</td>
<td>20 (33)</td>
<td>5 (30)</td>
<td>21 (33)</td>
<td>13 (36)</td>
<td>13 (27)</td>
</tr>
<tr>
<td>VEGFR1 $-$</td>
<td>14 (82)</td>
<td>28 (55)</td>
<td>0.044</td>
<td>4 (44)</td>
<td>38 (64)</td>
<td>NS</td>
</tr>
<tr>
<td>VEGFR1 $+$</td>
<td>3 (18)</td>
<td>23 (45)</td>
<td>5 (56)</td>
<td>26 (44)</td>
<td>15 (45)</td>
<td>17 (46)</td>
</tr>
<tr>
<td>VEGFR2 $-$</td>
<td>7 (41)</td>
<td>25 (47)</td>
<td>NS</td>
<td>6 (54)</td>
<td>33 (56)</td>
<td>18 (55)</td>
</tr>
<tr>
<td>VEGFR2 $+$</td>
<td>10 (59)</td>
<td>28 (53)</td>
<td>5 (44)</td>
<td>33 (56)</td>
<td>18 (55)</td>
<td>20 (54)</td>
</tr>
<tr>
<td>VEGFR3 $-$</td>
<td>8 (33)</td>
<td>39 (67)</td>
<td>0.0047</td>
<td>9 (64)</td>
<td>38 (56)</td>
<td>NS</td>
</tr>
<tr>
<td>VEGFR3 $+$</td>
<td>16 (67)</td>
<td>19 (33)</td>
<td>5 (36)</td>
<td>30 (44)</td>
<td>10 (29)</td>
<td>25 (52)</td>
</tr>
</tbody>
</table>

$^*$Tumors with an activating mutation in RET residue C634, either germline or somatic.
$^b$The rest of the tumors that do not have the analyzed genetic characteristic.
$^c$Tumors with the activating somatic RET mutation M918T.
$^d$Sporadic tumors with no mutations in RET exons 10, 11, 13, 14, and 16.
$^e$A negative expression is indicated by ‘−’.
$^f$A positive expression is indicated by ‘+’.

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Discussion

The lack of effective therapies for MTC may be changed drastically by the use of already available TKIs and the development of novel targeted drugs. Some of the TKIs have demonstrated remarkable clinical responses in MTC patients, and several new trials with these molecules are currently being conducted (Cohen et al. 2008, Schlumberger et al. 2009, Carr et al. 2010, Lam et al. 2010, Robinson et al. 2010, Wells et al. 2010, 2012, Ahmed et al. 2011, Hong et al. 2011, Kurzrock et al. 2011). However, there is a large inter-patient variability in TKI responses. The molecular basis for this variability is unknown, and the identification of biomarkers that could identify MTC patients who will probably benefit from these different drugs will improve clinical trial outcomes and ultimately progression-free survival and overall survival of patients with a disease that not long ago was untreatable. In this study, we used a large retrospective series of well-characterized MTC patients to define the expression of TKI target proteins in relation to specific clinical and tumor characteristics to provide data that could be used for rational selection of patients for TKI treatment.

So far, vandetanib and cabozantinib, which have markedly improved the progression-free survival of MTC patients (Schoffski et al. 2013, Thornton et al. 2012), are the only agents that have been approved for the treatment of this disease, but a wide variety of additional multi-targeted kinase inhibitors have entered clinical trials and several have shown clinical benefit in MTC patients. Promising results have been obtained with agents that primarily target angiogenesis and inhibit VEGF receptors at nanomolar concentrations. Because of the structural similarity between kinases, many of these molecules also have an effect on RET and other kinases, such as PDGFR, MET, and KIT, which could be all-important for the clinical responses observed. However, axitinib does not have an anti-RET activity and has displayed objective responses in MTC patients, suggesting that VEGFR might be as important as RET for targeted therapy (Cohen et al. 2008). Comparison of outcomes among the various phase II trials carried out so far is limited by variations in patient eligibility and response assessment. In addition, in most cases, genetic characteristics of the different MTC patients included (e.g. sporadic or familial and type of RET mutation) have not been reported.

In this study, we examined the expression of eight key proteins for TKI action (EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3) in 103 paraffin-embedded MTC samples characterized for RET mutations. We observed that these proteins were highly expressed in a subset of MTCs in a coordinated manner (Fig. 1, Supplementary Table 3). A previous publication that examined VEGF, VEGFR1, and VEGFR2 in 38 MTC samples has reported 95, 96, and 91% positive expression for these proteins respectively (Capp et al. 2010). In the present study, we adopted more strict criteria to define immunopositivity and, thus, the proportions of MTC samples exhibiting overexpression of these proteins were lower (32, 34, and 57% respectively). Applying criteria the same as those used by Capp et al., we obtained similar results for VEGF and VEGFR2, but observed a lower expression of VEGFR1 (see Supplementary Table 2). This difference could be due to the different VEGFR1 antibody used. The expression of some proteins was significantly different between primary tumor and metastatic samples (Table 2), but the number of metastatic samples included in this study was small, and we cannot rule out the possibility that additional changes would be detected in a larger series. In fact, a previous study by our group, including more metastatic cases and paired primary tumor/metastatic samples, had found a significant overexpression of VEGFR2 in the metastatic samples (Rodriguez-Antona et al. 2010). In the present study, we also found a higher expression of VEGFR2 in the metastatic samples than in the primary tumor samples (71 vs 55%), but it did not reach statistical significance. Additional histopathological information and clinical outcome data were not available to conduct additional analyses. Differences in the expression of the proteins were evident when comparing sporadic and familial MTC cases (Table 2), with a higher expression of KIT, PDGFRB, and VEGFR3 being observed in the familial cases. Most hereditary MTCs are caused by RET mutations affecting residue C634 and most sporadic cases have the M918T mutation (see Table 1), suggesting that the expression of TKI targets and RET mutations may be associated. In agreement with this, we found that MTC cases exhibiting VEGFR3 and KIT overexpression were mainly C634 RET-mutated cases; VEGFR1-positive cases were mainly M918T RET-mutated cases and cases without RET mutations; and MTC cases exhibiting PDGFRB overexpression were mainly tumors without RET mutations. VEGFR2, which is a target of several TKIs, exhibited similar staining among the different RET-mutated tumor cases, with the number of positive cases exceeding 40% in all cases (Table 3). When we examined the expression of KIT, PDGFRB, VEGFR1, and VEGFR3 in the tumor stroma, only KIT displayed a statistically significant difference in the C634 RET-mutated tumor cases, suggesting that for the
expression of the receptors, RET mutation is only relevant in the tumor cells. In general, and although a validation with an independent series is required to confirm these data, these results indicate that different TKI treatments could be more effective according to the specific RET mutation present in the MTC. It could be suggested that C634 RET-mutated cases (mainly hereditary), with a higher expression of VEGFR3, PDGFRB, and KIT, might benefit from drugs with a high affinity for these targets, while for the M918T RET-mutated cases (mostly sporadic), TKIs targeting RET and VEGFR2 might be more appropriate. In fact, a phase III clinical trial comparing vandetanib vs placebo has found that the response rate to this drug is greater in patients with sporadic tumors who had a M918T RET mutation (Wells et al. 2012). However, it is also important to note that intratumoral heterogeneity and changes in tumor molecular profile through the acquisition of new somatic mutations suggest that combinations of more than one TKI may be more effective than single-agent treatments and that changing to different TKIs over time might also be needed.

In conclusion, this study shows for the first time to our knowledge that a substantial number of MTCs exhibit high expression levels of kinases targeted by TKIs for which promising results have been obtained in recent clinical trials. Furthermore, the expression of these targets is associated with clinical and molecular characteristics of the MTCs, supporting the notion that these data could be used for the identification of patients most likely to benefit from specific TKIs, thus helping to design rational clinical trials and perform a molecular selection of treatments to ultimately improve the clinical response of MTC patients.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0316.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
C Rodríguez-Antona was responsible for study design, data analysis, data interpretation, manuscript writing, and final approval of the manuscript. I Muñoz-Repeto, L Inglada-Pérez, A A de Cubas, and M Cañamero were involved in data analysis, data interpretation, and final approval of the manuscript. V Mancikova and A Cascon were responsible for data collection, data interpretation, and final approval of the manuscript. A Maliszewska, L Sanchez, A Gómez, R Letón, C Álvarez-Escolá, and J Aller were responsible for data collection and final approval of the manuscript. L J Leandro-García and I Comino-Méndez were responsible for data interpretation and final approval of the manuscript. M Robledo was responsible for study design, data interpretation, manuscript writing, and final approval of the manuscript.

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