Aggressive inherited and sporadic medullary thyroid carcinomas display similar oncogenic pathways

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Abstract

RET oncogene mutations are found in familial medullary thyroid carcinomas (MTC) and in one-third of sporadic cases. Oncogenic mechanisms involved in non-RET mutated sporadic MTC remain unclear. To study alterations associated with the development of both inherited and sporadic MTC, pangenomic DNA microarrays were used to analyze the transcriptome of 13 MTCs (four familial and nine sporadic). By using an ANOVA test, a list of 173 gene sequences with at least a twofold change expression was obtained. A subset of differentially expressed genes was controlled by real-time quantitative PCR and immunohistochemistry on a larger collection of MTCs. The expression pattern of those genes allowed us to distinguish two groups of sporadic tumors. The first group displays an expression profile similar to that expressed by inherited RET634 tumors. The second presents an expression profile close to that displayed by inherited RET918 tumors and includes tumors from patients with distant metastases. It is characterized by the overexpression of genes involved in proliferation and invasion (PTN, ESM1, and CEACAM6) or matrix remodeling (COL1A1, COL1A2, and FAP). Interestingly, RET918 tumors showed overexpression of the PTN gene, encoding pleiotrophin, a protein associated with metastasis. Using a MTC cell line, silencing of RET induced the inhibition of PTN gene expression. Overall, our results suggest that familial MTC and sporadic MTC could activate similar oncogenic pathways.

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Introduction

Medullary thyroid carcinoma (MTC) derives from thyroid C cells, a neuroendocrine cell that produces calcitonin and represents <1% of all thyroid cells (Leboulleux et al. 2004, Matias-Guiu et al. 2004, Hoff & Hoff 2007). MTC accounts for 5–10% of all thyroid cancers and occurs as either a sporadic form or in a familial context (25% of cases). Hereditary MTC is transmitted with an autosomal dominant pattern, either as an isolated form, familial MTC (FMTC), or as part of a multiple endocrine neoplasia type 2A (MEN2A) or type 2B (MEN2B). MEN2A comprises MTC, pheochromocytoma, and hyperparathyroidism. MEN2B consists of a MTC that is often aggressive, pheochromocytoma, ganglioneuromatosis, Marfan syndrome features, and skeletal abnormalities (Bachelot et al. 2002, Ball 2007).

The RET proto-oncogene is a cell-surface receptor tyrosine kinase for glial cell line-derived neurotrophic factor (GDNF) family ligands. RET transduces signals via its intracellular phosphorylated domains leading to the activation of several pathways (Ras/ERK, PI3K/Akt, etc.), resulting in cell growth, differentiation, neuron survival, and development (Manie et al. 2001, Kurokawa et al. 2003, Santoro et al. 2004). Mutations which activate RET are responsible for the
familial forms of MTC, MEN2A being mainly due to germline RET<sup>634</sup> mutation, and MEN2B being mainly due to germline RET<sup>918</sup> mutation (Ponder 1999). Mutations which activate RET, mainly the RET<sup>918</sup> mutation, are also detected in one-third of sporadic tumors (Donis-Keller et al. 1993, Manie et al. 2001). However, the development mechanisms of RET-mutated tumors as well as the oncogenic pathways involved in non-RET mutated sporadic MTC remain largely unknown.

The knowledge of induced genetic alterations in the various forms of MTC is essential for understanding the pathways involved in the development and progression of MTC with various phenotypes. It is also mandatory for the identification of molecular targets that could be used for new therapeutic strategies, particularly in the aggressive forms for which conventional therapies are poorly effective (Schlumberger et al. 2008). Few studies based on high-throughput microarray or differential display methods compared gene expression changes in MTC hereditary or sporadic forms (Watanabe et al. 2002, Jain et al. 2004, Musholt et al. 2005).

In this context, the present work was designed to explore gene expression changes associated with the development of both inherited and sporadic MTC, using 60-mer oligonucleotide microarrays. A subset of differentially expressed genes discriminated between RET<sup>634</sup>, RET<sup>918</sup> and sporadic tumors.

MTC RET<sup>634</sup> displayed an expression profile similar to that of sporadic MTC from patients without metastases. MTC RET<sup>918</sup> and sporadic MTC from patients with distant metastases expressed a similar molecular signature characterized by the overexpression of genes involved in proliferation and invasion. These results suggest that MTC, occurring in either a familial or sporadic context, could activate similar oncogenic pathways independently of RET mutations.

**Materials and methods**

**Tumor samples**

Thyroid samples from 46 patients and their non-tumoral contralateral thyroid tissue were obtained from the Biobank at Institut Gustave-Roussy. They consisted of MTC and C cell hyperplasia (CCH). All tissue specimens were selected after histological analysis, classified according to World Health Organization recommendations (Matias-Guiu et al. 2004), and stored frozen in liquid nitrogen. The study was approved by the local human studies ethic committee. The histological and biological features as well as the clinical records of patients were obtained from the Pathological and Laboratory Medicine Department and the Nuclear Medicine Department at Institut Gustave-Roussy respectively.

**Cell line**

Thyroid tumor (TT) cell line, derived from a human MTC and carrying the RET<sup>634</sup> mutation, was obtained from the American Type Culture Collection (Zabel & Grzeszkowiak 1997). Cells were cultured in RPMI Medium (Gibco, Invitrogen) supplemented with antibiotics and 10% FCS (Invitrogen).

**RNA and DNA preparation**

Total RNA and DNA were isolated by Qiagen RNeasy micro KIT and Qiagen DNeasy tissue KIT respectively (Qiagen), according to the manufacturer’s protocols. Quality of RNA preparation, based on the 28S/18S rRNAs ratio, was assessed using the RNA 6000 Nano Lab-On-Chip, as developed on the Agilent 2100 Bioanalyzer device (Agilent Technologies, Palo Alto, CA, USA). All specimens included in this study displayed a ratio of 28S to 18S higher than 1.5. RNA and DNA samples were frozen in nuclease-free water.

**Genomic DNA sequencing**

Samples were screened for RET mutational status. The seven mostly mutated exons (8, 10, 11, 13, 14, 15, and 16) were amplified using standard PCR from 200 ng DNA, with the following conditions: denaturation at 97 °C for 15 min, 40 cycles of denaturation at 97 °C for 1 min, annealing at 68–72 °C for 30 s, and elongation at 72 °C for 1 min, final elongation at 72 °C for 10 min. Primers sequences are described in Table 1. All PCR products were visualized by electrophoresis on a 1% agarose gel, and sequencing used the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). Products were purified on a Sephadex G50 resin (Amersham Biosciences) and were analyzed on an automated sequencer 3730 DNA Analyzer (Applied Biosystems).

**Microarray analysis**

Thirteen MTC samples were used for microarray experiments on the basis of RNA quality and tumoral cell percentage in tissue sample (>80%). A pool composed of equal amounts of total RNA from each tissue sample was used as the RNA reference. Reverse transcription, linear amplification, cRNA labeling, and purification were performed using the Agilent Linear amplification kit (Agilent Technologies).
were scanned with an Agilent Technologies Scanner. After washing and drying procedures, microarray slides
PMTC. Selected genes had at least a twofold change, differentially expressed in the different groups of
60 nologies) were used for hybridization during 17 h at
microarrays of 44 000 sequences (Agilent Tech-
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der total RNA per tube in a final volume of 25
(Applied Biosystems). Quantitative PCR (Q-PCR)
tase (Invitrogen) in the presence of random primers
reverse transcribed by superscript II reverse transcrip-
one microgram of total RNA from each sample was
quantitative PCR
Reverse transcription and real-time
quantitative PCR

Table 1 RET primer sequences

<table>
<thead>
<tr>
<th>RET exon</th>
<th>Primers</th>
</tr>
</thead>
</table>
| 8       | F: 5’ CTCCATCCGTGGGCAGCTCAG 3’  
R: 5’ GGCCCCAGGACCCGGTTTC 3’ |
| 10      | F: 5’ CCTTGGGACACTGGCCTGAAATATG 3’  
R: 5’ GCTGTTAGGACCTTCTGAGGCT 3’ |
| 11      | F: 5’ CAGAGCATGACGGCTGACCCAGT 3’  
R: 5’ CCCCTCACAGGAGGCTCTGTC 3’ |
| 13      | F: 5’ GGAGAAGCCTCAACAGCAGCTG 3’  
R: 5’ CAGAGACATTTGAAAAGGGAAAGA 3’ |
| 14      | F: 5’ GGCTTCAAGGTCTGCGCTCTACA 3’  
R: 5’ GCAGGGGCTATGGGCTAGGTGT 3’ |
| 15      | F: 5’ CCCGCCCAGGTCTCACCA 3’  
R: 5’ TCTTTCAGGGCTGCTCCAAGG 3’ |
| 16      | F: 5’ TGTCTACAGCACTCCTGTCTG 3’  
R: 5’ GCGTCGTGGCCCAAATACTACA 3’ |

500 ng aliquots of total RNA were used to generate antisense cRNA labeled with Cyanine 3 (Cy3)-CTP or cyanine 5 (Cy5)-CTP (Perkin-Elmer NEN, Boston, MA, USA), as previously described (Lacroix et al. 2005). Custom-designed 60-mer oligonucleotide microarrays of 44 000 sequences (Agilent Technologies) were used for hybridization during 17 h at 60 °C, in a dye-swap procedure (Lacroix et al. 2005). After washing and drying procedures, microarray slides were scanned with an Agilent Technologies Scanner.

Bioinformatic analysis

Image analysis, quantification of fluorescence intensities, and normalization of data using the local background subtraction option were performed with Feature Extraction software (Agilent Technologies). Microarray data analysis was performed using Resolver software (Rosetta Inpharmatics, Kirkland, WA, USA). One-way ANOVA test based on RET634 versus RET918 mutation was used to analyze genes differentially expressed in the different groups of MTC. Selected genes had at least a twofold change, with a P value ≥ 10⁻³. All data obtained from microarray analysis have been submitted to Array Express at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/).

Reverse transcription and real-time quantitative PCR

One microgram of total RNA from each sample was reverse transcribed by superscript II reverse transcription (Invitrogen) in the presence of random primers (Applied Biosystems). Quantitative PCR (Q-PCR) was performed on an equivalent amount of 10 ng per total RNA per tube in a final volume of 25 μl (Lacroix et al. 2005). The reference pool, corresponding to all samples included in the Q-PCR experiment, was used as a calibrator. Oligonucleotide primers and TaqMan probes specific for each amplified gene were designed to be intron spanning. CALCA, EPOR, endothelial cell specific molecule 1 (ESM1), ACTB, RPLPO, and 18S were designed using the Primer-Express computer software (Applied Biosystems) and purchased from MWG Biotech (Courtaboeuf, France). Primers and probes for PTN, GEM, POMC, ITGAV, KAZALD1, and IRS2 genes were obtained from Assays-On-Demand (Applied Biosystems).

Tissue array and immunohistochemistry

Tissue microarrays (TMAs) including all 46 samples were constructed using a tissue-arrayer device (Alphelys, Plaisir, France) with a 1 mm needle. Quadruplicate samples, for a total of 368 spots, were prepared from both the tumor and the non-tumoral thyroid tissue taken at a distance from the tumor (Lacroix et al. 2005).

Immunohistochemistry was performed on formalin-fixed paraffin-embedded 5 μm sections of the three TMA, with the DAKO LSAB System procedure (DAKO, Carpinteria, CA, USA), as previously described (Lacroix et al. 2005). Sections were incubated for 30 min at room temperature with antibodies against calcitonin, thyroglobulin (Dako Cytomation Norden, Glostrup, Denmark), pleiotrophi
(Shiota Biotechnology Inc., Santa Cruz, CA, USA), ESM1 (Endotis Pharma, Romainville, France), proopiomelanocortin (“clone 3-E7”, Bachem, San Carlos, CA, USA), and Cyclin D1 (NeoMarkers, Fremont, CA, USA), according to the manufacturer’s recommendations. Negative controls were obtained by performing the same procedure on tissue sections without the primary antibody incubation step.

Cell transfection

TT cells were transfected using the Nucleofector transfection KIT (Amaza, Cologne, Germany) on the Nucleofector transfecter (Amaza) with recommended program L-029 following the manufacturer’s protocol. About 6 μg of RET siRNA (Dharmacon, Lafayette, CO, USA) or stealth siRNA (Invitrogen) were used to transfect 2.5 × 10⁶ cells. Control cells were treated in the same conditions in the absence of siRNA. After transfection, cells were cultured in a 6 cm² plate with complete medium at different times (24, 48, 72, and 96 h). Cells were collected after trypsinization/PBS wash and centrifugation (× 500 g/5 min), and pellets disrupted by adding RNA extraction buffer, RLT (Qiagen).

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Results

RET mutational status

Among the 46 C cell tumors, 41 were classified as MTC, 1 as a mixed MTC and follicular thyroid carcinoma, 1 as a micro MTC, and 3 as a CCH. Twenty-one patients displayed a germline RET mutation: RET\textsuperscript{634} mutation in 11 samples, including nine MTC (germline RET\textsuperscript{634} MTC), the mixed  

carcinoma, and the micro MTC; RET\textsuperscript{918} mutation (germline RET\textsuperscript{918} MTC) in three MTC. Other germline mutations involved codons 611, 618, 620, and 790 of RET (Table 2). Among the 25 sporadic MTC, nine exhibited somatic RET\textsuperscript{918} mutation and were designated as sporadic RET\textsuperscript{918} MTC. No other RET mutations were identified in the 16 remaining sporadic tumors that were designed as sporadic MTC RET\textsuperscript{wt} (Table 2).

Table 2: Clinical and histological features of tumor samples

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Type</th>
<th>Age at diagnosis</th>
<th>Sex</th>
<th>RET mutation</th>
<th>Origin/syndrome</th>
<th>TNM stage</th>
<th>Metastases localization</th>
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<tbody>
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<td>MTC</td>
<td>Primary</td>
<td>19</td>
<td>F</td>
<td>C634S TGC (T/A)</td>
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<td>T1(m)N0M0</td>
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<td>MEN2A</td>
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<td>MTC</td>
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<td>59</td>
<td>F</td>
<td>WT</td>
<td>Sporadic</td>
<td>T1N0Mx</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>MTC</td>
<td>Node metastases</td>
<td>61</td>
<td>M</td>
<td>WT</td>
<td>Sporadic</td>
<td>TxN1M1</td>
<td>Lung</td>
</tr>
<tr>
<td>45</td>
<td>MTC</td>
<td>Primary</td>
<td>69</td>
<td>M</td>
<td>WT</td>
<td>Sporadic</td>
<td>T1(m)N1bMx</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>CCH</td>
<td>Primary</td>
<td>53</td>
<td>M</td>
<td>WT</td>
<td>Sporadic</td>
<td>T1(m)N0M0</td>
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Gene expression profile associated with germline and sporadic MTC

Microarray experiments were performed on 13 MTC, including four hereditary and nine sporadic MTC. Two hereditary tumors displayed RET\textsuperscript{634} mutation and two had RET\textsuperscript{918} mutation; two sporadic MTC presented a RET\textsuperscript{918} mutation and the seven others did not have any identifiable RET mutation (RET\textsuperscript{wt}). All samples were characterized for their RNA quality (ratio RNA 18S/28S = 1.5–2) and for their high tumor cell population (> 80%).

A total of 173 gene sequences were identified as differentially expressed between RET\textsuperscript{634} and RET\textsuperscript{918} MTC by ANOVA test with a fold change level higher than 2 and a \( P \) value \( \leq 10^{-3} \) (Fig. 1). Among these 173 gene sequences, 17 correspond to replicate sequences and 20 correspond to genes with unknown function. Classification of the 13 MTC, with or without RET mutation, and based on those 173 sequences, divided the tumors in two groups: the first group included the two germline RET\textsuperscript{634} MTC and three sporadic RET\textsuperscript{wt} MTC, whereas the second group included the two germline RET\textsuperscript{918} MTC, two sporadic RET\textsuperscript{918} MTC, and four sporadic RET\textsuperscript{wt} MTC. Interestingly, the sporadic RET\textsuperscript{wt} MTC in each group differed according to the clinical features of patients. Indeed, in the first group, the three sporadic RET\textsuperscript{wt} MTC arose from patients without lymph node or distant metastasis (Table 2). In contrast, the four sporadic RET\textsuperscript{wt} MTC, which segregated with sporadic and germline RET\textsuperscript{918} MTC, were from patients bearing lymph nodes and distant metastases (Table 2).

In the first group, major up-regulated genes were those involved in cell survival signaling such as \textit{GEM}, encoding a small GTPase of RAS-related GTP-binding proteins family, \textit{NR4A1}, and \textit{NR4A2}, encoding for nuclear receptor subfamily 4, group A, members 1 and 2 respectively. Other up-regulated genes were genes coding for activating transcription factors (\textit{BHLHB3},

\textbf{Figure 1} Heat map representing the genes identified as differentially expressed between RET\textsuperscript{634}/RET\textsuperscript{918} MTC in the 13 medullary thyroid carcinoma samples. The clustering of the 173 gene sequences is based on the RET\textsuperscript{634}/RET\textsuperscript{918} set of genes determined by the ANOVA test, with criteria of fold change \( \geq 2 \) and \( P \) value \( \leq 10^{-3} \). Red or green color scales represent respectively up- and down-regulated genes in comparison with the reference. Each line corresponds to a gene and each column corresponds to a MTC sample (G634, tumors from patient with germline RET\textsuperscript{634} mutation; G918, tumors from patient with germline RET\textsuperscript{918} mutation; SWT, tumors from patient without RET mutation).
signaling (ITGAV, CAV1, EGR1, and EGR3; Table 3). In the second group, which contained both germline and sporadic RET<sup>918</sup> MTC and RET<sup>wt</sup> MTC with distant metastasis, up-regulated genes were associated with cell migration and proliferation, such as ESM1, SPOCK1 (testican), RASGEF1A (a small GTP-binding proteins of the Ras super family), and matrix remodeling such as COL1A1 and COL1A2, encoding for collagen type 1 α-1 and -2, Cadherin 11 (CDH11), the fibroblast activation protein FAP, and FBLN1, an extracellular matrix protein. The POMC gene, encoding proopiomelanocortin, a pro ACTH–endorphin molecule synthesized by the anterior pituitary gland, displayed a significant over expression. Finally, in the germline RET<sup>918</sup> MTC group, PTN gene coding for pleiotrophin, a neurite outgrowth-promoting factor, and KAZALD1, a KAZAL-type protease inhibitor domain, also designated BONO1 for bone and odontoblast-expressed gene 1, were significantly up-regulated (Table 3).

Functional pathway analysis was performed using Ingenuity software (Ingenuity Systems, Redwood City, CA, USA). The most significant pathway was represented by the integrin signaling pathway, which was overexpressed in the MTC RET<sup>634</sup> and the sporadic RET<sup>wt</sup> MTC without metastases.

### Quantitative gene expression analysis

Q-PCR was carried out to confirm changes in the expression of 11 genes selected on the basis of increased fold change. This analysis was done on a series of 22 MTC, including the 13 MTC analyzed in the microarray study, and nine additional samples comprising five germline MTC displaying RET mutations at position RET<sup>634</sup> (n = 2), RET<sup>611</sup> (n = 1), RET<sup>620</sup> (n = 1), RET<sup>918</sup> (n = 1), and four sporadic RET<sup>wt</sup> MTC, two obtained from patients with metastases and the other two from patients without metastasis. The TT cell line was also included in this study. The values were normalized using housekeeping genes (RPLPO and 18S) and compared with a pool of all samples.

Among the most up-regulated genes in the germline RET<sup>634</sup> MTC group, GEM, BHLHB3, and ITGAV genes were found to be increased in the Q-PCR analysis (Fig. 2). Upregulation of NR4A1 and PCDH11y genes was also confirmed (data not shown). Interestingly, two genes, EPOR and IRS2, which displayed less than a twofold change with \( P \) value \( \leq 10^{-3} \), were found to be mainly increased in RET<sup>634</sup> MTC. In the group defined by both germline and sporadic RET<sup>918</sup> MTC and sporadic metastatic RET<sup>wt</sup> MTC, the expression of PTN, ESM1, KAZALD1, and POMC genes was confirmed as up-regulated. It was particularly striking that PTN and ESM1 genes were highly increased in sporadic RET<sup>918</sup> MTC. The POMC gene expression was also significantly up-regulated in both sporadic RET<sup>918</sup> MTC and metastatic RET<sup>wt</sup> MTC. Finally, the TT cell line exhibited a particular gene expression profile with high levels of GEM, EPOR, PTN, and KAZALD1 genes expression, in contrast to ESM1 and POMC genes whose expression was not detected.

### Immunohistochemistry

Tissue array allowed an extensive immunohistochemical analysis of C cell tumors (n = 46) and their non-tumoral contralateral tissue, including tissue specimens tested in the microarray experiments. In order to control the histotype and to estimate the percentage of tumor cells, all samples were immunostained for calcitonin and thyroglobulin. Pleiotrophin, proopiomelanocortin, ESM1, and cyclin D1 protein expressions were then examined and results are presented in Fig. 3.

Pleiotrophin immunostaining showed a characteristic cytoplasmic localization that was predominantly observed in both germline and sporadic RET<sup>918</sup> MTC and in metastatic RET<sup>wt</sup> MTC. Staining was inconstantly observed in germline RET<sup>634</sup> MTC and was slight or absent in other tumors as well as in

<table>
<thead>
<tr>
<th>Group of tumors</th>
<th>Genes</th>
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<tbody>
<tr>
<td>1</td>
<td>GEM, NR4A1 and NR4A2, PCDH11y</td>
</tr>
<tr>
<td>Germline RET&lt;sup&gt;634&lt;/sup&gt; MTC</td>
<td>ITGAV, CAV1, BHLHB3, ATF3, JUNB, NTRK2, IER2, NCOA7, COL17A1, MAPK10, EGR1 and EGR3</td>
</tr>
<tr>
<td>Germline RET&lt;sup&gt;918&lt;/sup&gt; MTC</td>
<td>PTN, KAZALD1, LAMB2</td>
</tr>
<tr>
<td>2</td>
<td>ESM1, POMC, CEACAM6 and CEACAM7, GHRL, COL1A1 and COL1A2, FAP, CDH11, RASGEF1A, FBLN1, SPOCK, CIT</td>
</tr>
<tr>
<td>Germline and sporadic RET&lt;sup&gt;918&lt;/sup&gt; MTC and sporadic RET&lt;sup&gt;wt&lt;/sup&gt; MTC (with metastases)</td>
<td></td>
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non-tumoral contralateral tissues. ESM1 protein staining was particularly strong in both sporadic RET$^{918}$ and RET$^{wt}$ metastatic MTC, and it was weak in some germline RET$^{634}$ and RET$^{918}$ MTC. Proopiomelanocortin displayed a cytoplasmic and granular staining that was observed in both germline and sporadic RET$^{918}$ MTC and in metastatic RET$^{wt}$ MTC. Proopiomelanocortin staining was also observed in germline RET$^{634}$ MTC and in nonmetastatic RET$^{wt}$ MTC. Finally, Cyclin D1 immunostaining was positive in all MTC and negative in CCH tissues.

**siRNA RET transfection**

To assess the consequences of RET inhibition on the expression of the selected genes, siRNA RET transfection was performed on the TT cell line. The expression of RET mRNA, as analyzed by Q-PCR, was abolished from 24 to 96 h posttransfection; RET gene expression level was then <10% of the level observed for the control and siRNA control-transfected groups (Fig. 4A).

After siRNA RET transfection, ACTB gene expression remained unchanged, whereas expression of CALCA gene was reduced by 50 and 90% at 48 and 96 h respectively (Fig. 4B). PTN gene expression was reduced to 60 and 30% of its basal level after 48 and 96 h posttransfection respectively. Similarly, the expression level of the GEM mRNA was reduced by 55% at 96 h posttransfection (Fig. 4B). CCND1 gene expression showed a 40% decrease after inhibition of RET until 96 h posttransfection (data not shown). Several other genes were investigated: at 96 h posttransfection, ITGAV gene showed a 40% reduction of its expression, while the expression of EPOR (Fig. 4B), IRS2, and BHLHB3 (data not shown) genes remain unchanged.
Figure 3 Immunostaining of calcitonin, cyclin D1, pleiotrophin, ESM1, and proopiomelanocortin proteins in medullary thyroid carcinomas. A tissue microarray including 46 tissue samples was constructed as described in Materials and methods. Immunostaining is presented at magnification of (A) ×25 for a MTC sample (right) and its contralateral non-tumoral tissue (left), and (B) ×100 for the corresponding MTC sample. Calcitonin immunostaining in tumor cells was found to be cytoplasmic and heterogeneous; most of tumors cells were strongly stained and a subpopulation showed a weak staining. A strong positivity was observed for Cyclin D1 protein in the nuclei of tumor cells. A strong and heterogeneous cytoplasmic staining was observed with the pleiotrophin antibody, particularly on a subpopulation displaying an intense staining. Finally, ESM1 and proopiomelanocortin staining was located in the cytoplasm, showing a particular granular staining for proopiomelanocortin. No staining was detected in contralateral non-tumoral thyroid tissue.
Tumor mutations in the RET oncogene are found in FMTC and in 25–30% of sporadic MTC. However, other genes and mechanisms involved in the tumoral MTC process are unclear, and genetic alterations occurring in most sporadic MTC remain unknown. MTC is a rare disease with a slow progression rate, but prognosis may be poor for aggressive forms, including the MEN2B syndrome that is due to RET $^918$ mutation (Bachelot et al. 2002, Ball 2007). High-throughput methods, particularly those based on analysis of the transcriptome, proved to be useful for understanding oncogenic processes in other tumor tissues, but few studies have been dedicated to the genomic profiling of MTC (Watanabe et al. 2002, Jain et al. 2004, Musholt et al. 2005). In order to explore genomic alterations associated with the development of both inherited and sporadic MTC, a series of MTC from patients carrying either a MEN2A mutation (RET $^{634}$), a MEN2B mutation (RET $^{918}$), or a sporadic MTC (RET$^{918}$ and RET$^{wt}$) were analyzed by microarrays. It is noteworthy that there are no benign tumors arising from C cells and that normal C cell or even hyperplastic C cell population represents only a small percentage of thyroid tissue cells, and for this reason, a normal thyroid tissue cannot be used as a representative reference for normal C cells. Moreover, MTC being a rare disease, it is difficult to gather a large sample collection for genomic study, thus data obtained by microarrays experiments were then analyzed by Q-PCR and immunohistochemistry on a larger series of MTC.

Cluster analysis of all samples discriminated between germline MTCs that resulted from either RET$^{634}$ or RET$^{918}$ mutation. Independently of their inherited or sporadic status, MTC bearing the RET$^{918}$ mutation clusterized in the same group. Interestingly, sporadic RET$^{wt}$ MTC from patients with distant metastases segregated with RET$^{918}$ MTC, whereas sporadic RET$^{wt}$ MTC from patients free of distant and cervical lymph node metastases were classified in the same group as RET$^{634}$ MTC. This observation suggests that, in MTC, genomic profiling may distinguish aggressive from less aggressive tumors.

MTC bearing either the RET$^{634}$ mutation or a sporadic RET$^{wt}$ showed overexpression of genes related to proliferation and cell survival, as compared with MTC bearing the RET$^{918}$ mutation. Some of them are mediated by RET and GDNF family intracellular signaling, such as the IRS2 pathway (Hennige et al. 2000) leading to downstream factor activation of signal transducers and activators of a transcription, focal adhesion molecule (FAK), and Fyn, a Src-like kinase (Sariola & Saarma 2003, Panta et al. 2004, Plaza Menacho et al. 2005). Furthermore, vitronectin receptor integrin (ITGAV) and caveolin (CAV1), two factors involved in the FAK and Fyn stimulation pathways, leading to Ras/MAPK pathway activation and cell proliferation, were also found to be overexpressed (Wary et al. 1998, Panta et al. 2004, Plaza Menacho et al. 2005). This observation suggests that these molecules could be involved in tumor growth and progression; in agreement with a recent report showing that calcitonin stimulates prostate cancer cells through vitronectin receptor integrin signaling (Thomas et al. 2007).

The MTC group containing the more aggressive tumors was characterized by the upregulation of several genes, particularly the PTN and ESM1 genes. PTN gene, encoding pleiotrophin, a heparin-binding neurite outgrowth-promoting factor, is involved in mitogenic signaling. Its expression, through stimulation of the stromal cell microenvironment,
accelerates tumor progression (Chang et al. 2007), stimulates angiogenesis, and predisposes to local spread and metastasis. Increased expression of PTN gene was reported in a variety of human tumors, and it has been suggested to be a potential target for new treatments (Klomp et al. 2002, Kadomatsu & Muramatsu 2004, Malerczyk et al. 2005). Pleiotrophin signaling increases tyrosine phosphorylation of beta catenin (Meng et al. 2000). Interestingly, a novel mechanism of RET-mediated function highlights the role of increased phosphorylated beta-catenin in the development and aggressivity of medullary thyroid cancer (Gujral et al. 2008). In this context, our observation showing that targeting RET with siRNA results in a rapid decrease in PTN gene expression would deserve further investigation.

Genes encoding for factors involved in matrix remodeling and cell adhesion, such as ESM1, COLIA1, COLIA2, CDH11, and FAP, were found to be up-regulated. COLIA1 and COLIA2 genes, as well as CEACAM6 gene, are overexpressed in the aggressive group, and were reported to be potentially involved in invasion and metastasis (Oue et al. 2004). Furthermore, COLIA2 and CEACAM6 have been previously reported in MEN2B patients and identified as overexpressed in an aggressive sporadic tumor (Jain et al. 2004). However, these results are not in agreement with those observed in a NIH 3T3 fibroblast model, transfected with RET mutants, and this discrepancy can be attributed to the in vivo versus in vitro design of the experiments (Watanabe et al. 2002, Jain et al. 2004). Interestingly, the KAZALDI or BONO1 gene, encoding a molecule belonging to the IGFBP superfamily involved in the proliferation of osteoblasts during bone formation and regeneration (Shibata et al. 2004), was found to be one of the most up-regulated genes, particularly in one patient presenting skeletal abnormalities. These clinical features are encountered in some MEN2B patients and are characterized by the secretion of another developmental bone regulator, the chondromodulin-1 protein (Jain et al. 2004). It would be interesting to investigate how these two molecules are potentially involved in the skeletal changes observed in MEN2B patients. Finally, proopiomelanocortin, a precursor for several opioid peptides, including ACTH, lipotropin, endorphin, α- and β-MSH, was overexpressed at both gene and protein levels. It has been demonstrated that measuring POMC mRNA by in situ hybridization is very helpful in confirming MTC as the source of ectopic ACTH production (Smallridge et al. 2003). Cushing’s syndrome, due to ectopic ACTH secretion, is rare in MTC and usually occurs in patients with aggressive disease and large metastases (Barbosa et al. 2005).

TT cell, a human MTC cell line bearing a RET634 mutation, showed gene expression attributes of both groups of tumors, as defined by the molecular signatures. This suggests that TT cells have acquired additional genetic alterations during in vitro growth, and display peculiar phenotypic and genetic features different from those present in the initial explanted tumor cells. Using this model for silencing the RET pathway indicated that CALCA, PTN, and GEM genes are likely to be regulated either directly or indirectly by the RET634 mutant.

Our results show that the oncogenic process in wild-type RET MTC can be close to that observed in mutated RET MTC. MTC with MEN2B RET mutation and aggressive MTC RET WT show gene expression related to invasion and metastasis pathways, further studies are needed to understand their mechanism of action in this pathology, to evaluate their use in diagnosis on fine needle aspiration biopsies or as prognostic markers and therapeutic targeting in MTC (Russo et al. 1999). The presence of genomic profiles typical of aggressive tumors, even in the absence of known RET mutations, when confirmed in larger series of samples, may allow the use of these genetic markers for the prognosis of RET-negative samples.

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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