EXPERIMENTAL STUDY

Expression of RET and its ligand complexes, GDNF/GFRalpha-1 and NTN/GFRalpha-2, in medullary thyroid carcinomas

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Abstract

Objective: Mutations in the RET proto-oncogene are found in about one third of sporadic medullary thyroid carcinomas (MTCs), mostly affecting codon 918. Glial cell line derived neurotropic factor (GDNF) and its membrane-bound GDNF family receptor alpha (GFRalpha-1), as well as neurturin (NTN) and its membrane-bound receptor GFRalpha-2 form a complex with the RET product, a receptor tyrosine kinase, resulting in downstream signaling to the nucleus.

Design: To elucidate the role of these RET ligands in MTC tumorigenesis, their expression was determined in 15 MTC samples, one papillary thyroid carcinoma (PTC) and three normal thyroid tissue specimens.

Methods: The mRNA expression of RET, GDNF, GFRalpha-1, NTN and GFRalpha-2 was investigated by mRNA in situ hybridization, and confirmed by reverse transcription-PCR analysis.

Results: None of the five genes was expressed in the normal thyroids or in the PTC. All MTCs showed expression of RET, 13 expressed GDNF, 12 expressed GFRalpha-1 and 9 expressed NTN and GFRalpha-2. In 7 of the tumors RET, GDNF and GFRalpha-1 were expressed at high levels, and in five of these seven tumors NTN and GFRalpha-2 genes were also expressed at high levels. The high level of expression was preferentially seen in tumor cells adjacent to stroma and connective tissue. All MTCs without expression of the RET ligands harbored the RET codon 918 mutation.

Conclusions: The results suggest that this signaling pathway is important for MTC development, and that it may be activated by expression of the RET ligand complexes by the tumor cells themselves.

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Introduction

Medullary thyroid carcinoma (MTC) is a rare tumor comprising 5–10% of all carcinomas in the thyroid gland. The majority of MTC cases occur sporadically, however, approximately 25% of the patients have a familial form of the disease, such as multiple endocrine neoplasia type 2A and B (MEN 2A and B) and familial MTC (FMTC).

The RET proto-oncogene encodes for a tyrosine kinase receptor. The ligands, glial cell line derived neurotropic factor (GDNF) and neurturin (NTN), and their membrane bound receptors, GFRalpha-1 and GFRalpha-2, form a complex with RET. This results in the dimerization of RET, leading to the activation of its tyrosine kinase function, and allowing it to transduce downstream signaling to the nucleus by phosphorylation of tyrosine residues in signaling proteins (1). In vitro and in vivo studies have shown that GDNF can enhance the survival of various neurons by pre-venting apoptosis (2, 3). NTN and GFRalpha-2 are structurally highly homologous to GDNF and GFRalpha-1 respectively (4, 5). Recently, two additional ligand complexes have been described for RET. These are artemin and persephin, which activate RET by binding to the membrane bound receptors designated GFRalpha-3 and GFRalpha-4 respectively (6, 7). Thus, at least four different ligand systems are involved in RET signaling.

RET is expressed in both the familial and sporadic forms of MTC. Activating germline RET mutations have been shown to give rise to MEN 2A, MEN 2B, and FMTC and somatic RET mutations have been found in approximately one third of sporadic MTCs (8, 9). The
somatic mutation in the \textit{RET} gene commonly found in sporadic MTC is identical to the constitutional mutation found in MEN 2B at codon 918 in exon 16, where a methionine is substituted with a threonine. This is a highly conserved substrate-recognition site in the catalytic core of the tyrosine kinase domain, and the presence of this mutation in sporadic MTC has been suggested to correlate with a poor prognosis (10). In addition to the classical Met918Thr mutation, somatic \textit{RET} mutations in MTC have occasionally been described in other locations including codons 611, 634, 768, and 883 (11). In MTCs with \textit{RET} mutations, ligand independent receptor dimerization and/or substrate specificity shift may occur (11). Thus, in such situations the receptor bound ligand complex is probably not needed for activation of the signaling pathway. However, the ligands have also been shown to increase the transforming activity in cells with the codon 918 mutation, indicating that the RET ligands are important in the oncogenetic pathway (12, 13). Hence, in MTCs without detectable \textit{RET} mutation, a logical probability is that GDNF/GFRalpha-1 or NTN/GFRalpha-2 are somehow involved in the tumor development process. This involvement could theoretically comprise over-expression of the protein, stimulation of different pathways or constitutively activating point mutations.

To further elucidate the role of RET and its ligand complexes in MTC tumorigenesis, the expression of these five genes was analyzed in a panel of such tumors by mRNA \textit{in situ} hybridization.

### Materials and methods

#### Patients

Fifteen thyroid tumor samples from 14 patients operated on for sporadic MTC were snap frozen in liquid nitrogen after removal and stored at $-70^\circ$C until analysis. The patients (7 women and 7 men) had an average age of 55 years at diagnosis (Table 1). Twelve primary tumors and three metastases (nos 3, 7 and 12 in Table 1) were analyzed. From one of the patients two specimens were used, one from the primary MTC and the other from a metastasis (no. 3 in Table 1). Specimens of normal thyroid glands from two patients operated on for MTC and one patient operated on for follicular thyroid adenoma (nos 2, 4 and 16 in Table 1), and tumor tissue from one patient operated on for papillary thyroid carcinoma (PTC; no. 15 in Table 1) were obtained and were used as control tissue. The tumors were classified as suggested by the World Health Organisation committee (14). Informed consent was obtained from all patients, and the study was approved by the Ethical Committee of the Karolinska Hospital.

#### Preparation of probes

The following synthetic oligonucleotides (Geneset, Paris, France) were used as probes for the \textit{in situ} hybridization: 5'-GCCGCGACGA AGCTGCGT TTGGCTGGACC GAGGTCTCGT TGGGC-3' complementary to nt 977–933

### Table 1 Data on patients, tumor specimens and results from \textit{in situ} hybridization.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>I.D. no.</th>
<th>Sex(^a)/Age at diagnosis</th>
<th>Diagnosis</th>
<th>Sample</th>
<th>\textit{RET} mutation(^b)</th>
<th>Survival(^c)</th>
<th>Expression(^d)</th>
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<tr>
<td>1</td>
<td>46</td>
<td>M/40</td>
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<td>Primary</td>
<td>918</td>
<td>DfD</td>
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<tr>
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<tr>
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<td></td>
<td></td>
<td>f</td>
<td>-- -- -- -- --</td>
</tr>
<tr>
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<td>MTC</td>
<td>Primary</td>
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<tr>
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<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>1848</td>
<td>M/44</td>
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<td>Metastasis</td>
<td>No</td>
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<td>++ ++ ++ ++ ++</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>F/76</td>
<td>MTC</td>
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<td>++ ++ ++ ++ ++</td>
</tr>
<tr>
<td>9</td>
<td>135</td>
<td>F/65</td>
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<td>Primary</td>
<td>918</td>
<td>AwD</td>
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<tr>
<td>13</td>
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</tr>
<tr>
<td>15</td>
<td>2018</td>
<td>F/34</td>
<td>PTC(^e)</td>
<td>Primary</td>
<td>f</td>
<td>AwD</td>
<td>-- -- -- -- --</td>
</tr>
<tr>
<td>16</td>
<td>352N</td>
<td>M/43</td>
<td>FA(^d)</td>
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<td></td>
<td>AwD</td>
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</table>

\(^{a}\)F = female, M = male. \(^{b}\)No = no \textit{RET} mutation found. \(^{c}\)DfD = dead from disease; AwD = alive without disease; AwD = alive with disease. \(^{d}\)-- = no cells showing expression; ++ = minority of cells showing strong expression; +++ = majority of cells showing strong expression; the assessment was made from the area with maximum of expression. \(^{e}\)PTC = papillary thyroid carcinoma; FA = follicular adenoma. f = not analyzed.
of RET (15); 5′-AACCAGGTTA TCATCTAAAAC AGCAGGTTTGGGAAGGCAATCCAGATG GACAGCACGG-3′ for nt 437–389 of GDNF (16); 5′-GGCACTTCCG TGATTTTCCCC TTATGACACG TGCTGAGCA CCTTGAAGTTG CTGTT-3′ for nt 1102–1057 of GFRalpha-1 (17); 5′-AACAAGCAAGC CCACTCGACA CATCCAGATG GACAGCACGG-3′ for nt 86–47 of NTN (4); and 5′-AAGCTTCACTCATACTTT TGCCAGGGCCCT CTGTTTTTGA-3′ for nt 2277–2238 of GFRalpha-2 (5). As a positive control 5′-CTACACGGCA CTGCTGAGGA GGGAGATTC AGTGTGGTGG GGGAC-3′ was used. The oligonucleotides were labeled at the 3′ end with α35S-dATP (NEN, Life Science Products, Boston, MA, USA) using terminal deoxynucleotidyl transferase (Amersham Life Science, Tokyo, Japan). The labeled probes were purified using Nensorb-20 columns (Du Pont, Wilmington, DE, USA).

mRNA in situ hybridization

Cryostat sections 6 µm thick were cut and thaw-mounted on SuperFrostPlus slides (Menzel-Gläser, Braunschweig, Germany). mRNA in situ hybridization was performed as follows. Hybridization solution (50% formamide, 4× SSC (1× SSC: 0.15 mol/l NaCl, 0.015 mol/l sodium citrate), 1× Denhardt’s solution (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin and 0.02% Ficol), 1% N-lauroyl-sarcosine, 0.02 mol/l phosphate buffer (pH 7.0), 10% dextran sulfate, 500 µg/ml heat-denatured salmon sperm DNA, 200 mmol/l dithiothreitol, and 37.5 µl/ml of the labeled probes) were mixed and placed in a hybridization oven for 45 min. The solution was spread out on the sections, covered with paraffin, placed in a humidified box and incubated for 16–18 h at 42°C. After hybridization, the sections were sequentially rinsed five times (15 min in 1× SSC at 60°C), left at room temperature for 45 min, and then dehydrated with ethanol in increasing concentrations. After air drying, the sections were exposed to Hyperfilm beta-max X-ray film (Amershams, CEA AB, Uppsala, Sweden) for 4–6 days. The sections were then dipped in Kodak NTB-2 emulsion, exposed for 1–4 weeks, developed and finally counterstained with hematoxylin-eosin. The entire procedure was carried out at least twice on consecutive sections. All sections were separately evaluated in both light and dark field microscopy by two independent investigators. Assessment of the in situ hybridization results were as follows: − = no cells showing expression; + = minority of cells showing strong expression; ++ = majority of cells showing strong expression. The assessment was consistently made from the area showing maximum expression.

Screening for RET mutations

All MTC specimens except nos 6, 12 and 14 (Table 1) had previously been screened for RET mutations in exons 10, 11 and 16 (18), while MTC specimens nos 6, 12 and 14 were screened for specific mutation in the RET codon 918 in this study. Two rounds of PCR were carried out using three different primers for the RET sequence as described previously (19). Twenty microliters of the second PCR product were then incubated overnight with 2 µl Rsal, and the resulting restriction fragments were size-separated in a 6% polyacrylamide gel. If the RET codon 918 mutation is present, an Rsal restriction site is introduced in the second PCR. Thus the mutation can be resolved as an aberrant restriction pattern on the polyacrylamide gel.

Reverse transcription (RT)-PCR

In order to verify the specificity of the mRNA in situ hybridization, RT-PCR was performed for all five genes, and the RET product was then further confirmed by EcoRI cleavage. mRNA was isolated from case nos 5 and 8 using Ultraspec-II RNA isolation system (Biotex Laboratories Inc., Houston, TX, USA). cDNA was synthesized from the isolated mRNA with random primers using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The template mixtures were incubated at 37°C for 1 h. The primers used in the amplification of RET, GDNF and GFRalpha-1 exons have been described previously (20, 21). Primers for NTN and GFRalpha-2 were selected from published sequences and chosen to span exon borders for each gene (f, forward; r, reverse): RETf (5′-TCAACATTTGG CCTCTCAGGAC TG-3′), RETr (5′-TGAGCAGCCC ATACAATTTG-3′), GDNff (5′-TTTGTTGGCT GTCTGCTC-3′), GDNfr (5′-CATCGCAAGA GCCGTCGAC-3′), GFRalpha-IIf (5′-ATGTTCTCTG CGAACCTTTG-3′), GFRalpha-Irf (5′-ATGTTCTCTG CTTGGCCG-3′), GFRalpha-IIr (5′-AGGGCGTCTG TCTAGCCG-3′), GFRalpha-Ifr (5′-AGGGCGTCTG AGCGCCGG-3′), GFRalpha-IIr (5′-AGGGCGTCTG AGCGCCGG-3′), GFRalpha-IIr (5′-AGGGCGTCTG AGCGCCGG-3′), GFRalpha-IIr (5′-AGGGCGTCTG AGCGCCGG-3′), GFRalpha-IIr (5′-AGGGCGTCTG AGCGCCGG-3′).

PCR amplification was performed for each gene in a total volume of 50 µl containing 1 µl of the synthesized cDNA, 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 0.75–1.5 mmol/l MgCl2, 200 µmol/l dNTP, 0.01% gelatine, 1–2 U Amplitaq DNA polymerase and 1–2 U of each specific primer. The PCR products were then electrophoresed on 1.4% agarose gels.

Restriction enzyme cleavage of the RET product was performed by incubating 20 µl of the RET PCR product with 20 µl buffer and 2 µl EcoRI restriction enzyme overnight. Twenty microliters of each digestion were electrophoresed on a 1.4% agarose gel.

Results

The expression of RET and its ligand complexes GDNF/GFRalpha-1 and NTN/GFRalpha-2 was studied by mRNA in situ hybridization on frozen tissue sections
from 15 MTCs, three normal thyroids and one papillary thyroid carcinoma. The results are illustrated in Fig. 1 and detailed for each case in Table 1 together with the clinical information.

No expression above background level was seen for any of the five genes in the normal thyroid tissues or in the papillary thyroid carcinoma tissue (Table 1). 

mRNA expression from the positive control gene GAPDH was seen in all normal and tumor specimens (data not shown). RET expression was detected in all MTCs, and for seven of the cases the expression was moderate (+) whilst in eight tumors it was strong (++). In addition, thirteen of the MTCs showed expression of GDNF, twelve of GFRalpha-1, and nine of NTN and GFRalpha-2 (Table 1). In seven of the eight tumors with a strong RET expression a similarly strong expression of GDNF and GFRalpha-1 was evident (Table 1). Moreover, in five of these, strong expression of NTN and GFRalpha-2 was also detected (Table 1; note that one of these MTCs, no. 7, was not analyzed for NTN and GFRalpha-2).

In all the cases where strong expression (+++) of RET, GDNF, GFRalpha-1, NTN or GFRalpha-2 was observed, the signals were found in tumor cells typically located adjacent to the stroma, usually peripheral in the tumor.
of any of the RET primary tumor (Table 1). In addition, in case 3, RET mutations (Table 1). All MTCs without expression and six a moderate expression of MTCs with and without the codon 918 mutation. Out of expression, whilst four did not. No significant difference mutation, as did three of the seven tumors with strong expression, which have no documented RET mutation, and also for sporadic MTCs and other MEN 2-related tumors (11). So far, GDNF is the only RET ligand that has been analyzed in this respect, but GDNF mutations could not be demonstrated in such families, nor in sporadic MTCs or pheochromocytomas (22, 23). Interestingly, pheochromocytomas express RET, but not GDNF/GFralpha-1 or NTN/GFralpha-2 (E Edstro¨m, unpublished observations), suggesting that for this tumor type, artemin or persephin may be the important RET ligand.

In this study, we have shown that a significant proportion of sporadic MTCs express not only RET, but also GDNF, GFralpha-1, NTN and GFralpha-2 mRNA. At least half of the tumors in this series showed a very strong expression of the five genes, and in these cases the expression was preferentially detected in tumor cells adjacent to the stroma.

All MTCs showed at least some expression of RET, in contrast to the papillary thyroid carcinoma and the normal thyroid tissue samples. This might be explained by a lack of C-cells in the analyzed sections, as C-cells normally mostly occur in a centrally located area of the thyroid lobe. In two MTCs no expression of GDNF was demonstrated, three cases lacked GFralpha-1 expression and for NTN and its ligand GFralpha-2 no expression was detected in six of the cases. These MTCs harbored a somatic codon 918 mutation, which has been shown to be sufficient to trigger tumor development without the involvement of ligand stimuli (24). However, some tumors with strong expression of all five genes also had the 918 mutation. While signaling through wildtype RET is mediated by ligand complexes, signaling through mutated RET in cells from neuroepithelial origin give rise to phosphorylation and transformation independent of ligand stimulation. Neuroepithelial cells with a typical MEN 2A mutation in RET codon 634 show a higher biological activity than cells with the MEN 2B mutation in codon 918. Nevertheless, when a ligand is added to MEN 2B cells, the activation of RET increases plentyfold and both types of mutated cells are able to induce cell differentiation, in contrast to cells with wildtype RET signaling (12, 13). Previous studies have shown that a heterogeneous distribution of the 918 mutation may occur within MTC tumors. Thus, large parts of some MTCs in which a codon 918 mutation has been detected may consist of cells with wildtype RET (25). It would be of interest to determine whether the expressions of GDNF, GFralpha-1, mainly affecting codon 918. No mutations in the RET gene have previously been described in parathyroid tumors. However, in the second most common MEN 2-related tumor, pheochromocytoma, somatic mutations of RET have been observed in a subset of tumors. Therefore, a logical step should be to examine elements of the RET ligand complexes, to see if activation of this pathway at a different point has occurred, and as such has contributed to the development of tumors. This is especially important for those MEN 2 families which have no documented RET mutation, and also for sporadic MTCs and other MEN 2-related tumors (11).
NTN and GFRα-2 in the tumors with a 918 mutation in this study occur in tumor areas without a 918 mutation. This could be achieved by microdissection of areas with high expression, followed by PCR amplification and RsaI restriction analysis of the 918 mutation.

The distribution of RET expression and its ligand complexes is striking: in seven of the specimens the expression was found preferentially in tumor cells peripheral in the tumor formations, adjacent to stroma and connective tissue (Fig. 1). These results are in contrast to the findings by immunohistochemical analysis, where RET protein expression was seen in the same MTC subpopulations that also harbored a RET mutation (26). This discrepancy may have several possible explanations, of which the most likely is that the mutated RET leads to increased stability or over-expression of the protein, but not necessarily to increased RNA levels.

A similar distribution pattern has been shown in neuroblastomas, a tumor also derived from the neural crest, where the RET expression was seen only in stroma rich areas (27). This peripheral location of RET expression may be explained by a growth advantage scenario: i.e., the expression can reflect a more proliferative state in these cells, or allow greater invasive properties. The centrally located cells may therefore subsequently undergo a downregulation of this specific expression. The similar expression patterns of GDNF, GFRα-1, NTN and GFRα-2 could reflect a paracrine or autocrine distribution of these proteins, allowing the peripheral tumor cells maximum growth stimulation. Autocrine and paracrine loops have been suggested to stimulate tyrosine receptor kinases and induce oncogenic activity in different tumors, including stimulation of growth factors (28). However, growth advantage in the cells with high expression of the RET complex may be reflected by other mechanisms. For instance, in vitro studies have shown that GDNF may prevent cell death in dopaminergic neurons, and in vivo can interrupt the apoptotic program in motor neurons (2, 3). Furthermore, recently GDNF has been shown to bind to GFRα receptors and induce cell survival independent of RET (29).

In summary, most MTCs express RET and its ligand complexes GDNF and GFRα-1 as well as NTN and GFRα-2 in a specific pattern. The findings suggest that this signaling pathway is an important step in MTC development. In the future, interference somewhere along this pathway could be important in developing new treatment modalities for patients with these tumors.

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Expression of RET and its ligands in MTCs

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