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 neurotrophic 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 neurotrophic 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

Table 1 Data on patients, tumor specimens and results from *in situ* hybridization.

Case no.	I.D. no.	Sex ^a /Age at diagnosis	Diagnosis	Sample	RET mutation ^b	Survival ^c	Expression ^d				
							RET	GDNF	GFRalpha-1	NTN	GFRalpha-2
1	46	M/40	MTC	Primary	918	DfD	++	++	++	++	++
2	378	M/65	MTC	Primary	No	AwD	++	++	++	++	++
	378N			Normal	f		—	—	—	—	—
3	344	F/51	MTC	Primary	918	AwD	+	—	—	—	—
	847			Metastasis	918		++	++	++	+	+
4	1229	F/58	MTC	Primary	918	AwD	++	++	++	++	++
	1229N			Normal	No		—	—	—	—	—
5	1606	F/73	MTC	Primary	No	AwoD	++	++	++	++	++
6	1825	F/69	MTC	Primary	No	AwoD	++	++	++	++	++
7	1848	M/44	MTC	Metastasis	No	AwD	++	++	++	f	f
8	40	F/76	MTC	Primary	No	AwD	+	+	+	+	+
9	135	F/65	MTC	Primary	918	DfD	+	—	—	—	—
10	595	M/35	MTC	Primary	918	DfD	+	+	+	—	—
11	1072	M/61	MTC	Primary	918	AwD	+	+	—	—	—
12	1245	M/39	MTC	Metastasis	918	AwD	+	+	+	+	+
13	1500	M/76	MTC	Primary	918	AwD	+	+	+	—	—
14	1985	F/20	MTC	Primary	No	AwoD	++	+	+	++	++
15	2018	F/34	PTC ^e	Primary	f	AwoD	—	—	—	f	f
16	352N	M/43	FA ^e	Normal	f	AwoD	—	—	—	—	—

^aF = female, M = male. ^bNo = no RET mutation found. ^cDfD = dead from disease; AwoD = 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. ^ePTC = papillary thyroid carcinoma; FA = follicular adenoma. f = not analyzed.

somatic mutation in the 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 RET mutations in MTC have occasionally been described in other locations including codons 611, 634, 768, and 883 (11). In MTCs with 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 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 *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°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 *in situ* hybridization: 5'-GCCCGCACGA AGCTGCCGTT GGCCTGGACC GAGGTCTCGT TGGGC-3' complementary to nt 977–933

of *RET* (15); 5'-AACCAGGTTA TCATCTAAAA ACGA-CAGGTC ATCATCAAAG GCGATGGGT-3' for nt 437–389 of *GNDF* (16); 5'-GGCAGTCAGC GTAGTTTTCCTTTAGACAGC TGCTGACAGA CCTTGA-3' for nt 1102–1057 of *GFRalpha-1* (17); 5'-AGAAGCAGGC CCTCTCGACA CATCCAGATG GACAGCACGG-3' for nt 86–47 of *NTN* (4); and 5'-AACTTTACAA TCATACATTT TGCAGCCCTC CTTTTTTGAA-3' for nt 2277–2238 of *GFRalpha-2* (5). As a positive control 5'-CTACATGGCA ACTGTGAGGA GGGGAGATTC AGTGTGGTGG GGGAC-3' for nt 1193–1149 of glycerol aldehyde phosphate dehydrogenase (*GAPDH*) was used. The oligonucleotides were labeled at the 3' end with $\alpha^{35}\text{S}$ -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 $\times$ SSC (1 $\times$ SSC: 0.15 mol/l NaCl, 0.015 mol/l sodium citrate), 1 $\times$ Denhardt's solution (0.02% polyvinyl-pyrrolidone, 0.02% bovine serum albumin and 0.02% Ficoll), 1% N-lauroyl-sarcosine, 0.02 mol/l phosphate buffer (pH 7.0), 10% dextran sulfate, 500 $\mu\text{g}/\text{ml}$ heat-denatured salmon sperm DNA, 200 mmol/l dithiothreitol, and 37.5 $\mu\text{l}/\text{ml}$ 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 parafilm, 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 $\times$ 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 (Amersham, 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 RsaI, and the resulting restriction fragments were size-separated in a 6% polyacrylamide gel. If the *RET* codon 918 mutation is present, an RsaI 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 (Biotech 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*, *GNDF* 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'-TCAACATTTG CCCTCAGGAC TG-3'), RETr (5'-TGCAGG CCCC ATACAATTTG-3'), GDNFf (5'-TGTCGTGGCT GTCTGCCT-3'), GDNFr (5'-CATCGCAAGA GCCGCTGCAG-3'), GFRalpha-1f (5'-ATGTTCTCTG CGA CCCTGT-3'), GFRalpha-1r (5'-AGTTGGTCTC CTTG CCCGC-3'), NTNf (5'-AGGGCCTGCT TCTCAGCC-3'), NTNr (5'-AGTAGCGGAA CAGCACCGTC-3'), GFRalpha-2f (5'-ACGAGACCCCT CCGCTCTTTG-3') and GFRalpha-2r (5'-GGGAGGCTTC GTAGAACTCCTC-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 MgCl_2 , 200 $\mu\text{mol}/\text{l}$ dNTP, 0.01% gelatine, 1–2 U Amplitaq DNA polymerase and 1–2 U of each specific primer. The PCR products were subsequently 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 *GNDF*/*GFRalpha-1* and *NTN*/*GFRalpha-2* was studied by mRNA *in situ* hybridization on frozen tissue sections

Figure 1 mRNA *in situ* expression analysis of *RET*, *GDNF*, *GFRalpha-1*, *NTN* and *GFRalpha-2* in medullary thyroid carcinoma. (a and b) Expression of *RET* in case no. 5 is seen in tumor cells adjacent to the stroma (outlined area in (a) is enlarged in (b)). (c) Expression of *RET* in case no. 13. (d) No expression of *RET* in normal thyroid tissue. (e-i) Two sets of consecutive sections of case no. 4 showing similar expression distribution for (e) *RET*, (f) *GDNF*, and (g) *GFRalpha-1*, and similar expression of (h) *NTN* and (i) *GFRalpha-2*. s, stroma; t, tumor. The original magnifications were (a) $\times 100$, (b and d) $\times 200$, (c, h, i) $\times 400$, (e-g) $\times 40$.

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 (++) (Table 1). 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

Figure 2 Expression of *RET*, *GDNF*, *GFRalpha-1*, *NTN* and *GFRalpha-2* demonstrated by RT-PCR in two MTCs (case nos 5 and 8), but not in the negative control sample (H_2O). The size of each amplified product is indicated in bp on the right.

(Fig. 1). In addition, the strong expression could also be observed within the tumor in areas where stroma and connective tissue were present. There was no difference in this expression pattern depending on whether the *in situ* hybridization was performed on specimens from primary tumors or metastases. In the three metastases analyzed, all five genes were found to be expressed, and in two of them, *RET*, *GDNF*, and *GFRalpha-1* were expressed at high levels (++) (Table 1). In addition, in case 3, *RET* and its ligands showed a stronger expression in the metastasis compared with the primary tumor (Table 1).

The fifteen MTC tumor specimens were screened for *RET* mutations (Table 1). All MTCs without expression of any of the *RET* ligands harbored a somatic codon 918 mutation, as did three of the seven tumors with strong expression, whilst four did not. No significant difference in the level of *RET* expression was detected between MTCs with and without the codon 918 mutation. Out of the nine mutation-positive MTCs, three showed a strong and six a moderate expression of *RET*, and in the six mutation-negative cases the *RET* expression was strong in five and moderate in one (Table 1).

The expression of mRNA from the five genes was verified by RT-PCR (Fig. 2). The samples tested confirmed the mRNA expression for all five genes. In addition, the positive *RET* expression was confirmed by restriction enzyme cleavage (data not shown), and as a negative control for the *RET* gene a series of PTC samples were used (data not shown). Products of the predicted size were seen for all genes in the tumors with positive mRNA *in situ* hybridization expression, but no product of *RET* was seen in any of the PTC samples.

Discussion

The role of *RET* in the genesis of MEN 2-related tumors is undisputed, as more than 90% of affected members of MEN 2 and FMTC families harbor germline mutations in this proto-oncogene. In addition, somatic *RET* mutations occur in sporadic MTCs in 25–60% of the cases,

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). 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 Edström, 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*,

NTN and *GFRalpha-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*, *GFRalpha-1*, *NTN* and *GFRalpha-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 *GFRalpha* receptors and induce cell survival independent of *RET* (29).

In summary, most MTCs express *RET* and its ligand complexes *GDNF* and *GFRalpha-1* as well as *NTN* and *GFRalpha-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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