RET Receptor Expression in Thyroid Follicular Epithelial Cell-derived Tumors

Giuseppe Bunone, Mauro Uggeri, Piera Mondellini, Marco A. Pierotti, and Italia Bongarzone
Division of Experimental Oncology, Istituto Nazionale Tumori, 20133 Milan, Italy

Abstract

The RET proto-oncogene encodes a receptor tyrosine kinase for transforming growth factor-β-related neurotrophic factors, which include GDNF and neurturin. The expression of RET proto-oncogene was detected in several tissues, such as spleen, thymus, lymph nodes, salivary gland, and spinal cord, and in several neural crest-derived cell lines. RET expression in the thyroid gland was reported to be restricted to neural crest-derived C cells. The presence of RET mRNA or protein has not yet been reported in thyroid follicular cells. We previously demonstrated the expression of oncogenic rearranged versions of RET in papillary thyroid carcinomas: tumors derived from thyroid follicular cells. To assess the expression of the normal RET proto-oncogene in follicular cells, we analyzed its expression in a panel of neoplasias originating from thyroid follicular epithelial cells: papillary carcinomas and both follicular adenomas and carcinomas. We also demonstrated the presence of RET normal transcripts in two follicular thyroid carcinoma lymph node metastases. Moreover, we found the presence of the RET/ELE1 transcript, the reciprocal complementary form of the oncogenic fusion transcript ELE1/RET, in a papillary thyroid carcinoma specimen expressing the RET/PTC3 oncogene, thus demonstrating that the RET promoter is active in those cells after rearrangement. Finally, we show that in a papillary carcinoma-derived cell line expressing the proto-RET receptor and the related GFRα2 co-receptor, GDNF treatment induced RET tyrosine phosphorylation and subsequent signal transduction pathway, indicating that RET could be active in thyroid follicular cells.

Introduction

Thyroid neoplasias comprise a broad spectrum of lesions with different phenotypes and variable clinical behaviors. Thyroid adenomas are benign neoplasms rarely capable of malignant progression. PTC and FTC are the most common forms of thyroid cancer. Although originating from the same follicular cell, PTCs and FTCs are regarded as different biological entities (1). FTC, solitary and encapsulated, is an aggressive tumor that often gives rise to distant hematogenous metastases. The PTC is multifocal and associated with previous radiation exposure, and it frequently invades cervical lymph nodes. Anaplastic or undifferentiated thyroid carcinomas present a dramatic invasive potential and are almost invariably fatal. All of the above-mentioned neoplasias originate from the malignant degeneration of the thyroid follicular epithelium. Conversely, MTC develops from neural crest-derived C cells. These neoplasias usually present a poor outcome, spreading through both the lymphatic and hematoendothelium.

Specific gene alterations in the different types of thyroid tumors have been detected by molecular analyses. In particular, well-differentiated carcinomas of the papillary type are characterized by activation of the neurotrophin receptor tyrosine kinases, RET and NTRK1 proto-oncogenes (2). The other relevant oncogenic activation in differentiated thyroid carcinomas is that related to the presence of mutated RAS oncogene in follicular carcinomas, whereas RAS activation has been described to be a rare event in papillary carcinomas (3). During follicular carcinogenesis, RAS mutations appear to occur early. In fact, it is possible to detect RAS mutations in adenomas and even in multinodular goiters (2–6). The last relevant genetic alteration detected in thyroid tumors is represented by abnormalities in the TP53 tumor-suppressor gene. Many reports have described TP53 mutations in a fraction of poorly differentiated and in most undifferentiated or anaplastic thyroid carcinomas (7, 8).

As far as RET alterations are concerned, germline and somatic point mutations, dominantly activating the receptor tyrosine kinase activity, have been associated with three variants of both inherited multiple endocrine neoplasia type 2 (MEN 2A, MEN 2B, and FMTC) and sporadic MTC (9). In contrast, in a high percentage (35%) of PTCs, RET activation is due to oncogenic rearrangements of RET (2). These fusion proteins are generated after chromosome rearrangements in which the RET tyrosine kinase domain is fused to the NH2 terminus of different gene products designated “activating genes” (2). The most frequently involved are H4D10S170, R10x, and ELE1, respectively generating the RET/PTC1, RET/PTC2, and RET/PTC3 oncogenes (10–12). The fusion products express an intrinsic and constitutive tyrosine kinase activity. Therefore, RET represents a genetic element whose alterations (point mutations and structural rearrangements) are associated with the development of neoplasms originating from both the neural crest-derived C cells (MTC) and the follicular epithelium cells (PTC).

The RET proto-oncogene is expressed during the development of the lineage of neuroectodermal cells that give rise to thyroid C cells. However, the role of RET in the development of thyroid C cells is not clear. RET expression in thyroid follicular cells as well as its possible role in the differentiation or proliferation has not been yet reported. In particular, its expression in thyroid follicular cells is a vexing question. However, it is important to mention that the presence of the reciprocal product of ELE1/RET rearrangement, RET/ELE1 transcript, has been reported in thyroid tumors of children from Belarus after the Chernobyl reactor accident (13) and is considered to be a consequence of radiation exposure, which also transcriptionally activated the RET promoter. However, an alternative explanation implies that the RET promoter is active in a number of thyroid follicular cells.

Here we report the expression of proto-RET in sporadic and non-radiation-related thyroid follicular cell neoplasias, PTC, adenomas, and FTCs as well as in normal thyroid tissues. Moreover, we have found the RET/ELE1 transcript in a PTC specimen expressing the RET/PTC3 oncogene, demonstrating that in this case, the RET promoter is active after rearrangement. Finally, we show that in a papillary carcinoma-derived cell line expressing normal RET protein, GDNF treatment induced RET tyrosine phosphorylation and its subsequent signal transduction pathway. These data indicate that RET can
be expressed in follicular thyroid cells and that it can display a role in the differentiation/proliferation of these cells.

Materials and Methods

Cell Line. The human thyroid carcinoma cell line NPA (14) was grown in DMEM supplemented with 10% FCS. For PDGF or PGE2 stimulation, the cells were starved for 20 h in F12 medium and 0.5% FCS. Stimulation with 50 ng/ml PDGF (Alomone Labs) or 50 ng/ml PGE2 (Upstate Biotechnology) was performed for 10 min.

RNA Extraction and RT-PCR Analysis. Each frozen biopsy was mechanically disrupted in a Micro-Dismembrator II (B. Braun) containing liquid nitrogen. Total RNA was extracted by the Ultraspec II (Biotecx Laboratories) according to the manufacturer’s instructions. RT-PCR was performed as follows: 5 μg of total RNA were reverse-transcribed at 42°C for 50 min in the presence of 500 ng of random hexamers and Superscript II reverse transcriptase (Life Technologies, Inc.) in a final volume of 20 μl. Two μl of the cDNA reaction were then subjected to 30 PCR cycles (30 s at 95°C, and 1 min at 72°C) using the AmpliTaq Kit (Perkin-Elmer) and 0.4 μM of each specific primer. The following primers were used in RT-PCR experiments: RETTM1 (5'-CTGTCCTTTCCCCTC-3') and RETC2 (5'-TGGGCCCCCATACAAATTG-3') for the amplification of pro-RET only; RETC1 (5'-TGGGAACTCCCTGGAAGA-3') and RETC2 for the amplification of both the proto- or oncogenic version of RET being designed on the tyrosine kinase domain of RET; Aldo/F (5'-CGCAGAAAGGGCTTTTGGTGTA-3') and Aldo/R (5'-CAGCTCTCTTCTTGCTGGGGCTTGC-3') for the amplification of the aldolase A housekeeping gene; GDNFR1 (5'-AAGCATGTACAGACAGCTTCTGC-3') and GDNFR2 (5'-TGCTTCTCAGAGGACAGCCAC-3') for the amplification of GRFRα1; TRNRR2 (5'-CCAGGTTCTTGCCACACAG-3') and TRNRR2H3 (5'-AGCCAGAAGCTGTCGTCGTCGTTG-3') for the amplification of p110α; EST10 (5'-ACTGTCCT-3') and EST39 (5'-TGACCTGATCTTCTTGCCAC-3') for the amplification of ELE1/RET; and RET56 (5'-TGGCAAGAGTGGCCAGCCAC-3') for the amplification of RET56. PCR products were electrophoresed on a 3% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under UV light.

Genomic PCR Analysis. High-molecular weight DNA was extracted following standard procedures. ELE1/RET and RET/ELE1 fragments containing the breakpoints were amplified using the following primers: EST10 (5'-ACTGTCCTGCTCTTTGGAAACC-3') and RET39 (5'-TGAGCTAGTCTGCTGTG-3') for ELE1/RET; and RET56 (5'-TGCCGCTACGCTTACT-3') and EST4 (5'-CTTGAATACCTGGCCAGTT-3') for the amplification of RET/ELE1. PCR products were electrophoresed on a 3% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under UV light.

Sequencing. For sequencing reactions, a dye terminator cycle sequencing ready reaction kit (ABI Prism) was used. Reaction products were then analyzed using the ABI Prism 377 fluorescent DNA sequencer (Perkin-Elmer).

Western Blot Analysis, Immunoprecipitation, and Antibodies. Protein samples were prepared as described previously (15) and immunoprecipitated with affinity-purified antiphosphotyrosine polyclonal antiserum (Upstate Biotechnology). The antiphosphotyrosine immunoprecipitates were resolved by electrophoresis on 7.5% SDS polyacrylamide gels (PAGE). Proteins were transferred onto nitrocellulose filters and immunoblotted with the same antiphosphotyrosine antiserum, with anti-RET affinity-purified antibodies or the anti-Shc polyclonal antiserum (Upstate Biotechnology) essentially as described previously (15). Immunoreactive bands were visualized using horseradish peroxidase-conjugated antirabbit or antimouse antiserum and ECL detection reagents (Amersham).

Results

The RET proto-oncogene is expressed in a wide range of embryonal tissues and in parafollicular cells (C cells) of the thyroid gland, and usually is not detected in normal thyroid follicular cells. In this study we detected the expression of RET proto-oncogene by a RT-PCR method (13) using two primer combinations, RETTM1-RETC2 and RETC1-RETC2, respectively able to amplify the region of normal RET encompassing the RET/PTC fusion point and the region of RET that is 3'-terminal to the fusion point present in all RET and RET/PTC oncogenes. We analyzed 10 PTCs negative for the expression of RET (PTC1, 2, and -3) or NTRK1-derived oncogenes. As controls, we used normal thyroid tissues, a PTC expressing the RET/PTC3 oncogene, and a MTC sample. Fig. 1 shows the expression of the RET proto-oncogene in the above-mentioned specimens. We detected the expression of RET mRNA at different levels in all of the samples analyzed but not in the PTC expressing the RET/PTC3 oncogene. On the contrary, using primers specific for the RET tyrosine kinase domain, we detected a RT-PCR product in all of the analyzed specimens. It was possible that the observed expression of RET was due to the presence of parafollicular cells in the analyzed specimens. This was true for the normal thyroid tissues, but in the PTC samples the presence of C cells should be irrelevant.

To address the presence of RET mRNA in thyroid follicular tumors, we analyzed the expression of RET proto-oncogene in a panel of thyroid neoplasias and relative lymph node metastases originating from these cells. Using primers specific for RET only (RETTM1-RETC2), we analyzed the expression of RET mRNA in three adenomas (benign neoplasias of follicular cells) and in four FTCs (Fig. 2). These results indicated that, although at low levels, RET proto-oncogene was expressed in these follicular thyroid cells. RET expression was also detected in two FTC lymph node metastases (Fig. 2, 248m and 266m).

To confirm the possibility that the RET promoter was active in thyroid follicular cells, we looked for expression of the reciprocal product of RET rearrangement in a PTC sample expressing the RET/PTC3 oncogene. Genomic DNA of tumor samples expressing the RET/PTC3 oncogene was further investigated by PCR to find the breakpoint region. We previously have described the chromosomal mechanism generating the oncogenic version of ELE1/RET as an
inversion within chromosome 10, band q11.2, where the genes ELE1 and RET are located (16). In Fig. 3A is shown the PCR products of both genomic rearrangements between the ELE1 and RET genes. We also analyzed the genomic sequence around the breakpoint region by direct sequencing. Using specific primers for the RET and ELE1 genes, we detected the expression of ELE1/RET (RET/PTC3) and of the reciprocal RET/ELE1 transcript by RT-PCR (Fig. 3B). We also sequenced the breakpoint regions of both the transforming and the reciprocal products of rearrangement. The RET-specific primer was located at the 5' end of the fusion point, and the ELE1-specific primer was on its 3' end. Fig. 3B shows the products of this RT-PCR, demonstrating the expression of both the transforming and reciprocal products of ELE1/RET rearrangement. Moreover, direct sequencing of the fragment confirmed the specificity of the RT-PCR products.

To study the biochemical activity of RET tyrosine kinase receptor in those thyroid follicular cells expressing the receptor, we first analyzed the expression of the RET gene, and GFRα1 and GFRα2 co-receptors in the PTC cell line NPA. We found the expression of RET mRNA by RT-PCR together with that of GFRα2, a glycosylphosphatidylinositol-anchored co-receptor for RET signaling. On the other hand, we did not find expression of GFRα1 (Fig. 4A). To demonstrate that the RET proto-oncogene product expressed on thyroid follicular cells was a functional receptor, we further investigated the expression of RET protein and its activation by GDNF. We immunoprecipitated RET protein from NPA cells before and after treatment with GDNF. We detected the expression of RET receptor in NPA cells and GDNF-induced RET tyrosine phosphorylation (Fig. 4B). We also investigated the ability of RET tyrosine kinase receptor to activate the signal transduction pathway upon GDNF binding. In Fig. 4C is shown Shc phosphorylation after GDNF treatment in these cells. Similar results were obtained upon PDGF stimulation. Taken together, these data demonstrated the presence of a functional RET receptor in follicular thyroid cells.

Discussion

The expression of the RET proto-oncogene was detected in several tissues, including spleen, thymus, lymph node, salivary gland, and spinal cord, and in several neural crest-derived cell lines. On the other hand, the expression of this tyrosine kinase receptor in the thyroid gland was reported to be restricted to parafollicular cells. In fact, the presence of RET mRNA or protein has yet to be fully documented in thyroid follicular cells.

Here we have shown RET proto-oncogene expression in 10 PTC specimens selected for being negative for the expression of the main oncogenic versions of RET reported to occur in these tumors (2). It is possible that the RET expression was due to neural crest-derived C cells contaminating the analyzed tissue specimens. Although C cells should not be present in these PTC specimens, as documented by a careful pathological examination, to ensure that their presence did not interfere with our study, we analyzed RET expression on an additional panel of neoplasias originating from thyroid follicular cell carcinomas, follicular adenomas, and two related lymph node metastases. We found RET proto-oncogene expressed in both adenomas and FTCs and in two FTC lymph node metastases. Moreover, we have shown that GDNF treatment induced RET tyrosine phosphorylation and activated
the subsequent signal transduction pathway in a papillary carcinoma-derived cell line expressing RET and the related glycosylphosphatidylinositol-linked GFRα2 co-receptor. These results thus indicate that RET expression may play a role in follicular thyroid cells.

Finally, we showed that the RET promoter is still active after rearrangement with the ELE1 gene in a non-radiation-induced PTC. We previously have demonstrated that at a genomic level, the fusion sequence RET/ELE1 is reciprocal to the transforming ELE1/RET rearrangement (12). Subsequently, Klugbauer et al. (13) identified tumor samples with the RET/PTC3 rearrangement that also expressed the reciprocal RET/ELE1 transcript. These authors proposed that the RET promoter might be activated by radiation exposure, thus triggering the expression of RET/ELE1 transcripts. We have now found the reciprocal product of the ELE1/RET rearrangement expressed in a tumor sample from a patient with a non-radiation-related cancer, thus implying that the RET promoter region is active independent of radiation exposure. In this case, both the structural features of this tumor and the location of the two metastatic specimens should rule out a significant contribution of type C cells.

The combination of our in vivo and in vitro results strongly supports the concept that the thyroid follicular component can express a functional RET receptor, which may be activated in the presence of specific ligands in the thyroid microenvironment. Because C cells express the RET receptor, the concept that RET ligands are present in this microenvironment is highly plausible.

In recent years, it has been shown that there are some interconnections between follicular- and parafollicular-type C cells. The microenvironment provided by MTC cells has the capacity to stimulate the proliferation of follicular cells, resulting in hyperplastic and adenomatous follicles, and as suggested recently, the latter can ultimately acquire a fully developed neoplastic phenotype (either follicular or papillary; Refs. 17, 18). The opposite situation has also been described: C-cell hyperplasia was recognized in some patients with Hashimoto thyroiditis as well as in thyroid adjacent to follicular and papillary neoplasms (17, 18). A large amount of evidence supports the concept that RET oncogenic activation is important for both follicular and parafollicular cell components. In fact, RET is involved in the tumorigenesis of almost all of the hereditary MTCs and in a proportion (~50%) of sporadic MTCs. The importance of RET oncogenic rearrangements in sporadic and radiation-induced papillary thyroid tumorigenesis has been fully demonstrated. Approximately 35% of sporadic and >60% of radiation-induced tumors carry an oncogenic version of the RET gene. Biochemical studies of the RET/PTC oncoprotein signal transduction pathways have demonstrated that they recruit cytoplasmic proteins containing SH2 domains such as phospholipase Cγ (19) phosphatidylinositol 3-kinase, the GTPase-activating protein Ras, Src kinase, and the adapter proteins Shc and Grb2 (20–22). It has been also demonstrated that activated RET constructs stimulate JNK activation in different cell lines (23). Additional results also support the concept that RET oncogenic activation is an early event in thyroid carcinogenesis and that further or concomitant molecular events could determine neoplastic progression.

We propose that RET stimulation can constitute a factor contributing to the transformation of both follicular and parafollicular cells. Further efforts must be aimed to better define the role of RET in these cells and to clarify the question of their histogenetic origin (17). Finally, an interesting question still to be answered is why the same gene with different mechanisms is involved in tumorigenesis of both follicular and parafollicular thyroid cell components.

Acknowledgments

We thank Cristina Mazzadi for secretarial assistance.

References

RET Receptor Expression in Thyroid Follicular Epithelial Cell-derived Tumors

Giuseppe Bunone, Mauro Uggeri, Piera Mondellini, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/11/2845

Cited articles
This article cites 20 articles, 4 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/11/2845.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/11/2845.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.