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Differential Gene Expression of Medullary Thyroid Carcinoma Reveals Specific Markers Associated with Genetic Conditions

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Medullary thyroid carcinoma accounts for 2% to 5% of thyroid malignancies, of which 75% are sporadic and the remaining 25% are hereditary and related to multiple endocrine neoplasia type 2 syndrome. Despite a genotype-phenotype correlation with specific germline *RET* mutations, knowledge of pathways specifically associated with each mutation and with non-*RET*-mutated sporadic MTC remains lacking. Gene expression patterns have provided a tool for identifying molecular events related to specific tumor types and to different clinical features that could help identify novel therapeutic targets. Using transcriptional profiling of 49 frozen MTC specimens classified as *RET* mutation, we identified *PROM1*, *LOXL2*, *GFRA1*, and *DKK4* as related to RET^{M918T} and *GAL* as related to RET^{634} mutation. An independent series of 19 frozen and 23 formalin-fixed, paraffin-embedded (FFPE) MTCs was used for validation by RT-qPCR. Two tissue microarrays containing 69 MTCs were available for IHC assays. According to pathway enrichment analysis and gene ontology biological processes, genes associated with the MTC^{M918T} group were involved mainly in proliferative, cell adhesion, and general malignant metastatic effects and with Wnt, Notch, NFκB, JAK/Stat, and MAPK signaling pathways. Assays based on silencing of PROM1 by siRNAs performed in the MZ-CRC-1 cell line, harboring RET^{M918T}, caused an increase in apoptotic nuclei, suggesting that PROM1 is necessary for survival of these cells. This is the first report of *PROM1* overexpression among primary tumors. *(Am J Pathol 2013, 182: 350—362; http://dx.doi.org/10.1016/j.ajpath.2012.10.025)*

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Medullary thyroid carcinoma (MTC) is a rare malignancy derived from calcitonin-producing parafollicular thyroid cells (thyroid C cells); it constitutes 5% to 10% of all thyroid neoplasias.[1] Approximately 25% of all MTCs are hereditary, as part of multiple endocrine neoplasia type 2 (MEN2; OMIM #171400), a rare cancer syndrome (1:30,000) that follows an autosomal dominant inheritance pattern and exhibits a variable clinical expression.

MEN2 is subdivided into MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC), according to the patient's clinical features. Specific activating point mutations in the *RET* proto-oncogene are responsible for the three forms of MEN2. *RET* encodes a receptor tyrosine kinase (RTK) implicated in neural crest tissue development and differentiation,[2] that activates a variety of signaling pathways. Ret signaling takes place mainly through autophosphorylation of Ret tyrosine residues, which trigger the recruitment of several intercellular adapters upon phosphorylation, leading to the activation of several cascades.

Individuals with MEN2A commonly carry *RET* mutations in codon 634 encoding cysteine,[3] and less frequently in the cysteine-encoding codons 609, 611, 618, 620, and 630.[4,5] Those with a mutated codon 634 have a high risk of lymph node metastasis and a more than 40% increased risk of developing the disease by age 20.[6] Individuals with FMTC carry mainly *RET* mutations affecting highly conserved cysteine regions, such as codons 609, 611, 618, and 620, as well as other infrequent mutations in residues of the intracellular domain, such as in exons 13[7] and 15.[8] RET^{M918T} mutation in exon 16 correlates with aggressive disease[9–11] and is found as a germline mutation in the majority of MEN2B cases (94%) and as a somatic mutation in 30% to 50% of sporadic MTC cases.[11] This mutation leads to constitutive activation of RET and transforming potential in a ligand-independent manner.[12]

MTC progresses slowly and metastasizes to cervical and mediastinal nodal groups in up to 50% of cases. It also metastasizes to distant organs such as lungs, liver, and bones in 20% of cases. Total thyroidectomy remains the only effective treatment, and only prior to metastasis; once metastatic disease has occurred, there is no effective therapy available.[13]

Although there is a well-established genotype-phenotype correlation for MEN2 patients, the mechanisms by which *RET* mutations cause tumors, the development of sporadic MTC in the absence of *RET* mutations, and the specific oncogenic pathways involved require further study. Our main aim was to identify signaling pathways specifically related to familial and sporadic MTC using transcriptional profiling.

Materials and Methods

Human MTC Tissue Samples and Cell Lines

Tumors ($n = 68$) from unrelated patients diagnosed with MTC were collected at the time of surgery from hospitals in Spain through the Spanish National Tumor Bank Network and from Italy through the Surgical Pathology Unit, Department of Medical and Surgical Sciences, University of Padua. Tissue samples were frozen in liquid nitrogen, embedded in optimal cutting temperature compound (Tissue-Tek OCT; Sakura Finetek, Torrance, CA), and stored at −80°C until they were used for RNA extraction and hybridization onto arrays, or for *RET* mutational screening (if germline genetic information on the patient was absent). All tissues were evaluated by pathologists using H&E staining. Only samples with a high percentage (>80%) of tumor cells were included in the study, and cases with high amyloid content were excluded. Additional independent FFPE MTCs ($n = 23$) were available to validate expression profiling through quantitative RT-PCR (RT-qPCR). After consent of each patient was obtained, clinical information was collected by questionnaire. Genetic and general characteristics of the cases are listed in Supplemental Table S1.

Two tissue microarrays (TMAs) were available for further analysis (Supplemental Table S2). The first, TMA-1, contained 54 independent molecularly characterized FFPE MTC tissues; of these, 12 carried a germline *RET* mutation in codon 634, 9 had a somatic M918T mutation, and 22 were classified as WT because no mutations were found. [Other MTCs were not included in the analysis as they carried different mutations (3 MTCs with the C618F mutation, 1 MTC with C618R, 1 MTC with S891A, 1 MTC with C620F, 1 MTC with A883F), or the cores included in the TMA were not useful for immunostaining evaluation (2 negative MTC and 2 with mutation at 634 residue). These four tumors are referred as to "not used" in Supplemental Table 2. This TMA also contained four other thyroid cancer types and two normal tissues used as controls, as well as normal thyroid tissue of 13 MTC cases included in the TMA. TMA-2 was constructed with cores from the corresponding paraffin-embedded material from 13 frozen samples used for profiling purposes, along with two MTCs that were not hybridized, two thyroid cancers of different subtype, and 12 normal thyroid tissues from the corresponding MTCs. This TMA contained MTCs from patients carrying a germline *RET* mutation in codon 634 and somatic *RET* mutations affecting codon 918, as well as MTCs from patients classified as WT.

MZ-CRC-1 cells are derived from a metastatic MTC and harbor the RET^{M918T} mutation.[14,15] The TT cell line has a codon 634 mutation in the *RET* gene.

Molecular Characterization of Tumors

Screening of *RET* was performed by direct sequencing of exons 10, 11, and 13 to 16 in DNA extracted from peripheral blood samples (when available) and, if negative, from tumor specimens. Genomic tumor DNA was obtained according to the manufacturer's instructions using a DNeasy kit (Qiagen, Valencia, CA).

RNA Isolation

Total RNA from 68 frozen MTC tissues was obtained according to the manufacturer's instructions using a TRI