BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

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

Agnieszka Maliszewska,^{*} Luis J. Leandro-Garcia,^{*} Esmeralda Castelblanco,[†] Anna Macià,[‡] Aguirre de Cubas,^{*} Gonzalo Gómez-López,[§] Lucía Inglada-Pérez,^{*¶} Cristina Álvarez-Escolá,^{||} Leticia De la Vega,^{*} Rocío Letón,^{*} Álvaro Gómez-Graña,^{*} Iñigo Landa,^{*} Alberto Cascón,^{*¶} Cristina Rodríguez-Antona,^{*¶} Salud Borrego,^{¶**} Mariangela Zane,^{††} Francesca Schiavi,^{‡‡} Isabella Merante-Boschin,^{††} Maria R. Pelizzo,^{‡‡} David G. Pisano,[‡] Giuseppe Opocher,^{††‡‡} Xavier Matias-Guiu,[†] Mario Encinas,^{*‡} and Mercedes Robledo^{*¶}

From the Hereditary Endocrine Cancer Group^{*} Human Cancer Genetics Program, and the Bioinformatics Unit,[§] Structural Biology Program, Spanish National Cancer Research Center (CNIO), Madrid, Spain; the Department of Pathology and Molecular Genetics,[†] Arnau de Vilanova University Hospital, Lleida, Spain; the Neuronal Signaling Unit,[‡] Department of Basic Medical Sciences, School of Medicine, University of Lleida and the Biomedical Research Institute of Lleida (IRBLleida), Lleida, Spain; the Center for Biomedical Network Research on Rare Diseases (CIBERER),[¶] Valencia, Spain; the Endocrinology Service,^{||} La Paz University Hospital, Madrid, Spain; the Genetics, Reproduction, and Fetal Medicine Clinical Management Unit,^{**} Institute of Biomedicine of Seville (IBIS), Virgen del Rocío University Hospital, the Spanish National Research Council (CSIC), and the University of Seville, Seville, Spain; the Department of Medicine DIMED,^{††} University of Padua, Padua, Italy; and the Familial Cancer Clinic and Endocrine Oncology,^{‡‡} Veneto Institute of Oncology, Padua, Italy

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Address correspondence to
Mercedes Robledo, Ph.D.,
Hereditary Endocrine Cancer
Group, Human Cancer Genetics
Program, Centro Nacional de
Investigaciones Oncológicas,
Melchor Fernández Almagro 3,
28029 Madrid, Spain, or to
Mario Encinas, Ph.D., Neuronal
Signaling Unit, Universitat de
Lleida/Institut de Recerca
Biomèdica de Lleida, Lleida,
Spain. E-mail: mrobledo@cnio.es
or mario.encinas@mex.udl.cat.

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 13⁷ 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

Reagent kit (MRC, Cincinnati, OH). RNA purity and integrity was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA of 19 cases was degraded [RNA integrity number (RIN) < 7] and discarded for hybridization assays, but considered in the RT-qPCR validation step. Total RNA from 23 independent FFPE MTCs was isolated according to the manufacturer's instructions using an RNeasy FFPE kit (Qiagen). Concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). These FFPE tumors were used for validation by RT-qPCR. After this first quality filter, 49 MTCs were used for hybridization assays and 42 (19 frozen and 23 FFPE) samples were used for validation purposes.

Total RNA from MZ-CRC-1 cells was extracted using an RNeasy kit (Qiagen).

cDNA Synthesis, Labeling, Hybridization, and Detection

For each of the 49 MTC tumors used for profiling, 500 ng of total RNA was amplified according to the manufacturer's instructions by double-strand cDNA synthesis, followed by T7-based *in vitro* transcription. Universal Human Reference RNA (Stratagene, La Jolla, CA) was used as a control for all samples. Amplified complementary RNA (cRNA) was labeled with cyanine 5-conjugated dUTP, whereas cRNA from Universal Human Reference RNA was labeled with cyanine 3-conjugated dUTP. The Agilent Whole Human Genome Array (4x44K) was used for competitive hybridization, and the slides were washed, dried, and scanned in an Agilent microarray scanner (Agilent Technologies).

Normalization and Preprocessing

Two-channel arrays were hybridized and quantified using Feature Extraction software version 9.5 (Agilent Technologies). Microarray background subtraction was performed using the normexp method.¹⁶ The data set was normalized using loess within-array normalization and quantile between-array normalization. Differentially expressed genes were identified by applying linear models using the Bioconductor limma R package.¹⁷ Normalized data were preprocessed using the Gene Expression Profile Analysis Suite (GEPAS) server.¹⁸ Inconsistent replicates ($SD > 1$) were discarded, and consistent replicates were averaged. Finally, genes that exhibited flat patterns ($SD \geq 0.8$) across the set of samples were filtered and omitted in further comparisons.

Unsupervised and Supervised Analysis

Tumor samples were grouped according to their expression profiles by means of unsupervised clustering using GeneCluster 2.0.¹⁹ Unsupervised clustering was implemented by a self-organizing maps (SOM) algorithm. Only tumors exhibiting the 634 or the M918T mutation, along with tumors classified as WT, were used in further analysis.

Gene Expression Profiling and Gene Ontology Analysis

The Pomelo II toolset was used for *t*-testing,²⁰ and the T-Rex toolset from the GEPAS package was used for differential expression analysis.¹⁸ To account for multiple hypothesis testing, the estimated significance level (*P* value) was adjusted using Benjamini's false discovery rate (FDR) correction.²¹ Genes with $FDR < 0.05$ and with fold change > 2 were selected for the validation step.

Functional enrichment of gene ontologies (GOs) for differentially expressed genes was performed using the FatiGO tool²² implemented in Babelomics software version 3.2 (Institute of Computational Genomics-CIPF, Valencia, Spain). Fisher's exact test was performed to obtain significant over-representation of GO terms in the list of differentially expressed genes versus the rest of the genome. GO terms with $FDR < 0.05$ were considered significant.

Gene Set Enrichment Analysis (GSEA)²³ using Biocarta and KEGG annotations was applied. Gene expression values were ranked based on the *t*-statistic. Gene sets with $FDR < 0.25$ were considered enriched between classes under comparison.

Validation by RT-qPCR

For validation of microarray data, five genes significantly differentially expressed among profiling results were selected to be assessed by RT-qPCR: *LOXL2*, *PROM1*, *GAL*, *GFRA1*, and *DKK4*.

Out of 49 previously hybridized samples, 32 MTCs were used for validation, along with 42 available MTCs from an independent series (19 frozen MTC tissues not used in profiling and 23 FFPE tumors). After testing β -glucuronidase (*GUS*), 60S acidic ribosomal protein P0 (*RPLP0*), and peptidylprolyl isomerase A (*PPIA*) genes as endogenous references, only *GUS* was used to normalize the quantification of mRNA expression.

Reverse transcription was performed using 1 μ g total RNA and random hexamers (FFPE samples) or 0.5 μ g total RNA and oligo-dT (frozen specimens) and M-MLV Reverse Transcriptase (Promega, Madison, WI). PCR was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems; Life Technologies, Foster City, CA) using the Universal Probe Library set according to the manufacturer's recommendations. PCR reactions were performed in triplicate. The Kruskal-Wallis test (nonparametric analysis of variance) for multiple class comparisons and the nonparametric *U*-test for two-class comparisons were used to compare the genetic classes.

For MZ-CRC-1 cells, total RNA was reverse-transcribed using a TaqMan reverse transcription kit (Applied Biosystems; Life Technologies, Foster City, CA). The relative mRNA levels were measured by RT-qPCR using TaqMan probes (Hs01009250_m1 for *PROM1* and Hs99999905_m1 for *GAPDH*) (Life Technologies). *GAPDH* was used for data normalization. Quantification data were calculated by the $2^{-\Delta\Delta CT}$ method.

Cell Culture and Nucleofection

MZ-CRC-1 and TT cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% pyruvate, 1% nonessential amino acids, 100 U/mL penicillin and streptomycin at 37°C in 5% CO₂ in air. For nucleofection, 2×10^6 cells per condition were incubated with 2 µmol/L synthetic siRNAs (Sigma-Aldrich, St. Louis, MO) directed against human PROM1 in a solution containing 100 mmol/L phosphate buffer, 100 mmol/L KCl, and 10 mmol/L MgCl₂. Cells were nucleofected using the A30 program of an Amaxa Nucleofector II device (Lonza, Walkersville, MD). Immediately after nucleofection, cells were resuspended in medium and seeded onto six-well plates.

Apoptosis Assay

Three days after transfection, the number of apoptotic nuclei was analyzed by Hoechst staining as described previously.²⁴ Cell-permeant Hoechst dye 33342 was directly added to cells at a final concentration of 0.5 mg/mL and incubation was continued for 15 minutes at 37°C. Cells were then photographed under a fluorescence microscope (IX70; Olympus, Tokyo, Japan). Cells with condensed and/or fragmented chromatin typical of apoptosis were scored in triplicate as percentage of total nuclei. For each condition, 500 to 1000 cells were counted.

Lentivirus Construction and Infection

An expression cassette consisting of the cytomegalovirus (CMV) promoter followed by the human Proliferin 1 open reading frame was cloned into a modified pDSL_hpUG vector (10326371; ATCC, Manassas, VA) lacking the ubiquitin c-GFP cassette. Briefly, the Proliferin 1 open reading frame was first cloned into pcDNA3 (Invitrogen; Life Technologies, Carlsbad, CA) by standard techniques. Using such construct as a template, a PCR product consisting of the CMV promoter followed by the Proliferin 1 open reading frame and flanked by attB sites was generated. Finally, this product was sequentially cloned into pDONR221 entry vector (Invitrogen; Life Technologies) and then moved to the final destination vector via gateway recombination technology.

Lentiviral particles were produced as follows. Briefly, 293T cells were cotransfected by the calcium phosphate method with the above plasmid plus plasmids coding for the envelope and the packaging systems (VSV-G and Δ8.9, respectively). The day after transfection, the culture medium was replaced with fresh medium and left for an additional 2 to 3 days to allow lentiviruses to be produced and released. Supernatants were then harvested, filtered through a 0.45-µm filter, and directly applied to TT cells. Typically, cells were infected overnight and experiments were performed 4 to 5 days after infection.

RT-qPCR

Total RNA from MZ-CRC-1 cells was extracted with an RNeasy kit (Qiagen) and reverse-transcribed using a TaqMan Reverse Transcription Kit (Applied Biosystems; Life Technologies). The relative mRNA levels of CD133 were measured by RT-qPCR using TaqMan probes (Hs01009250_m1 for CD133 and Hs99999905_m1 for GAPDH (Life Technologies)). GAPDH was used for data normalization. Quantification data were calculated by the $2^{-\Delta\Delta CT}$ method.

BrdU Incorporation

Cells were incubated with 3 ng/mL 5'-bromodeoxyuridine (BrdU; Sigma-Aldrich) for 20 minutes and fixed with 4% paraformaldehyde. After DNA denaturing with 2 mol/L HCl for 30 minutes and neutralization with 0.1 mol/L Na₂B₄O₇ (pH 8.5) for 2 minutes, cells were blocked in PBS solution containing 5% horse serum, 5% fetal bovine serum, 0.2% glycine, and 0.1% Triton X-100 for 1 hour. Subsequently, cells were subjected to indirect immunofluorescence with a mouse anti-BrdU monoclonal antibody (Dako, Carpinteria, CA) and Alexa Fluor 594-conjugated anti-mouse secondary antibody (Invitrogen; Life Technologies). Nuclei were counterstained with 5 µg/mL Hoechst dye 33258, and cells were visualized using an epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Cell Viability

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5'-diphenyltetrazolium bromide (MTT) assay. TT and MZ-CRC-Z cells were plated on 96-well dishes at 2×10^4 cells per well. Next day, cells were treated with the Ret inhibitor RPI-1, which has been shown to induce apoptosis in MTC-derived cell lines.^{25,26} After 48 hours of treatment, cells were incubated for 35 to 45 minutes with 0.5 mg/mL of MTT reagent (Sigma-Aldrich), washed, and solubilized with dimethyl sulfoxide. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry

Immunohistochemical staining was performed on both of the MTC tissue microarrays available. TMA blocks were sectioned at a thickness of 3 µm and dried for 1 hour at 60°C. Deparaffinization, rehydration, and epitope retrieval were achieved by heat treatment in a PT Link module (Dako, Glostrup, Denmark) at pH 9. Before staining of the sections, endogenous peroxidase was blocked. Primary antibodies used were anti-PROM1 (1:500, ab19898; Abcam, Cambridge, MA) and anti-GFRA1 (1:500, C-20; Santa Cruz Biotechnology, Santa Cruz, CA); incubation with primary antibodies was performed according to the manufacturer's instructions. After incubation with anti-PROM1, the reaction

was visualized with EnVision FLEX system (Dako) using diaminobenzidine chromogen as a substrate; anti-GFRA1 binding was visualized with an LSAB+ kit (Dako) using diaminobenzidine chromogen as a substrate. Sections were counterstained with hematoxylin. Appropriate positive and negative controls were also tested.

Immunoreexpression was graded semiquantitatively by considering the percentage and intensity of the staining. A histological score was obtained for each sample, on a scale from 0 (no immunoreaction) to 300 (maximum immunoreactivity). Interpretation was performed as described previously.²⁷ Because each TMA included two cores from each case, immunohistochemical evaluation was performed after examination of all samples.

Statistical Analysis

Immunoreexpression was analyzed in two ways, as a continuous variable and as a binary variable using the median as the cutoff. For the first analysis, a *U*-test was used; for the second analysis, difference in expression was tested with a χ^2 test or Fisher's exact test, as appropriate. Statistical analyses were performed using SPSS software package version 17.0 (IBM SPSS, Chicago, IL). Nominal two-sided *P* values of <0.05 were considered statistically significant.

Results

Molecular Genetic Characteristics of the MTC Tumors

Tumors were classified according to their specific *RET* mutation. Among the 49 tumors used for profiling, 7 carried a germline *RET* mutation in codon 634, 14 carried the M918T *RET* mutation (1 germline and 13 somatic mutations), and 20 were classified as WT. The remaining eight MTCs had six different germline mutations and two different somatic mutations in *RET* (Supplemental Table S1). These last eight tumors were not considered in clustering analysis.

Data Reveal Differential Expression Profiles in Inherited and Sporadic MTC

Unsupervised hierarchical clustering, performed using standard correlation as a measure of similarity across all hybridized samples, showed a relatively low heterogeneity distributed over two principal branches (Figure 1). An enrichment of familial MTC cases with various germline *RET* mutations was observed in the second branch of the cluster, whereas the first branch was enriched for sporadic MTC with somatic *RET*^{M918T} mutation and for WT cases. This observation led us to do a supervised analysis based on the known mutations of each available tumor. Differential expression analysis was performed to compare MTC^{M918T} versus MTC^{WT} and MTC^{M918T} versus MTC⁶³⁴ (Figures 2 and 3), from which we obtained a list of 360 and 18 significant genes (GEO accession number GSE32662), respectively.

Based on threshold-free methodology using Gene Set Enrichment Analysis software version 2.08 (GSEA; Broad Institute, Cambridge, MA), signaling pathways were identified as significantly differently expressed (FDR < 25%) for comparison of MTC^{M918T} versus MTC⁶³⁴ and MTC^{M918T} versus MTC^{WT} (Supplemental Table S3). The M918T cluster was correlated with several pathways related to malignant tumor behavior, such as the p53, caspase, cytokine, Wnt, NFκB, MAPK, IL1R, JAK/Stat, and Notch pathways.

Gene Ontology (GO) analyses revealed significant biological processes in the M918T group associated with malignant behavior (Supplemental Table S4). Hierarchical clustering analysis of the most relevant FatiGO outputs for biological processes pointed to the importance of cell adhesion, migration, and proliferation ontologies (data not shown).

GSEA analysis (Supplemental Table S3) and FatiGO analysis (Supplemental Table S5) revealed similar significant pathways associated with the M918T group.

Biomarkers of Specific Genetic Conditions

After application of the filtering criteria, five genes appeared to be biomarkers of different genetic conditions: *PROM1*, *GFRA1*, *LOXL2*, *GAL*, and *DKK4*. These were selected for further validation at the mRNA level.

GAL was the only statistically significant gene found to be a specific marker for the germline *RET* mutation in codon 634. The expression of the other four genes was validated by RT-qPCR as up-regulated markers in MTC^{M918T}. Because of limited availability of RNA, it was possible to validate *PROM1* and *LOXL2* only in FFPE MTC samples. These results are summarized in Figure 4 for the independent frozen series and in Figure 5 for the FFPE series.

Although they were not selected for further validation, other genes differentially expressed among the three different genetic conditions considered here have been reported to be involved in cancer of tissues derived from the same neural crest origin as MTC, and also in the RTK signaling pathway. These previous findings were therefore considered to be a cross-validation of our results. Among these markers, the up-regulated genes associated with the M918T group were *ESM1*, *NPR1*, *PITX2*, *RTN1*, and *SHC1*.

ESM1 (encoding endothelial cell-specific molecule 1) is associated with cell migration and proliferation; its overexpression has been described previously in sporadic *RET*^{M918T} MTC cases.²⁸ *NPR1* (encoding atrial natriuretic peptide receptor 1/guanylate cyclase A) regulates the activation of ERK1/2,²⁹ as well as the Ras/Raf/ERK and Wnt/β-catenin pathways. *PITX2* (encoding paired-like homeodomain transcription factor 2) is involved in tumorigenesis and in Wnt/β-catenin signaling; it is expressed in neural crest cells.³⁰ *RTN1* (encoding reticulon-1), overexpression of which leads to apoptosis of neuroblastoma cell lines, is involved in secretion or membrane trafficking in neuroendocrine cells as well as in tumorigenesis; it is

Figure 1 Unsupervised clustering of hybridized frozen MTC series. Partial heat map shows two principal branches after unsupervised clustering was applied to the 49 MTCs. The first branch was enriched with MTC^{M918T} cases and the second with familial MTC cases, mostly with *RET*⁶³⁴ mutation. Relative underexpression is shown as shades of green and relative overexpression is shown as shades of red. Cases are identified by MTC case number and a mutation extension: S_918 for sporadic MTC with somatic *RET*^{M918T} mutation, F_634 for familial MTC with *RET*⁶³⁴ mutation, and S_WT for sporadic MTC with no *RET* mutation.

expressed in neurons and neuroendocrine cells.³¹ Fibroblasts transformed by *RET*^{C634R} display constitutive RET-mediated phosphorylation of SHC, which activates the RAS/MAPK signaling pathway.³²

Immunohistochemistry

We performed immunohistochemistry for GFRA1 and PROM1 (Supplemental Figure S1). Results were evaluated according to uniform pre-established criteria.

Differences at the mRNA level were not observed at the protein level. GFRA1 protein levels were not significantly

different among the groups. PROM1 showed higher staining in MTC^{M918T} than in MTC⁶³⁴ ($P = 0.059$), in accord with mRNA detection.

Silencing PROM1 by siRNA Induces Apoptosis

We used the MZ-CRC-1 cell line nucleofected with two different synthetic siRNAs against human PROM1 for *in vitro* validation. Knockdown efficiency was assessed in parallel dishes by RT-qPCR, and the number of apoptotic nuclei was scored as percentage of total nuclei. Both siRNAs caused an increase in the number of apoptotic

Figure 2 Differential expression analysis of MTC^{M918T} versus MTC^{WT} using the GEPAS package. The first 100 genes with FDR < 0.15 are shown. Genes are listed in descending order by *t*-statistic. Sigma units denote standard deviation.

Figure 4 RT-qPCR with TaqMan probes of *LOXL2*, *PROM1*, *GAL*, *GFRA1*, and *DKK4* expression in an independent frozen MTC validation series. Asterisks indicate values below the lowest detected C_T value. ANOVA, analysis of variance.

nuclei (Figure 6), suggesting that *PROM1* is necessary for the survival of these cells. We next analyzed *PROM1* expression levels in two MTC-derived cell lines bearing a MEN2B *RET* mutation (MZ-CRC), and a MEN2A *RET* mutation (TT). In accord with our results in MTC specimens, MZ-CRC expressed *PROM1* mRNA levels approximately fivefold higher than those found in TT cells. To check whether overexpression of *PROM1* in TT cells could confer a growth advantage to TT cells, we overexpressed *PROM1* in these cells by means of lentiviral infection. Overexpression of *PROM1* did not affect the proliferation rate of TT cells as measured by BrdU uptake,

but did afford protection against cell death caused by the *RET* inhibitor RPI-1²⁵ (Figure 6). Expression of *PROM1* thus appears to influence cell death, rather than proliferation.

Discussion

Survival of MTC patients is relatively low if their carcinomas metastasize.³³ Surgery is the primary treatment for MTC, and alternatives are lacking for tumors with poor response to either conventional chemotherapy or radiotherapy.³³ Thus,

Figure 5 RT-qPCR with TaqMan probes of *LOXL2* and *PROM1* expression in an independent FFPE MTC validation series. Asterisks indicate values below the lowest detected C_T value.

Figure 6 **A:** Knockdown efficacy of two different siRNAs against human PROM1. MZ-CRC-1 cells were nucleofected with either buffer or siRNAs against PROM1 (CD133.1 and CD133.2). Three days later, PROM1 expression was assessed by RT-qPCR (**top left**). Silencing of PROM1 causes apoptotic death of MZ-CRC-1 cells. Cells were nucleofected as above; 3 days later, the number of nuclei showing apoptotic morphology was scored (**bottom left**). Representative micrographs of Hoechst 33342-stained nuclei of cells (**right**). Original magnification, $\times 100$. **B:** mRNA levels of PROM1 of MZ-CRC-1 and TT cell lines. **C:** mRNA levels of PROM1 after lentiviral infection with empty vector or PROM1. **D:** BrdU uptake of TT cells transduced with lentivirus. **E:** Cell viability of TT-derived cell lines after incubation with the RET inhibitor RPI-1. * $P < 0.01$ two-tailed Student's *t*-test.

there is a pressing need to identify potential therapeutic targets. Although a genotype-phenotype correlation has been established for MEN2 cases, and it is known that clinical behavior differs according to specific germline and somatic *RET* mutations, the signaling pathways involved in the clinical behavior are largely unknown. It is therefore important to identify pathways related to non-*RET*-dependent sporadic MTC and to the most frequent pathogenic *RET* mutations, to reveal prognostic markers. Although this need is well recognized, several difficulties have contributed to a lack of studies on MTC tumors. First, comparisons of primary tumors versus normal C cells are not possible, because of the very low parafollicular cell representation ($\sim 1\%$) in the thyroid cell mass.³⁴ In addition to these difficulties, it is a challenge to

obtain a sufficiently large series of frozen MTC tissues for genomic study, because of the rarity of MTC. This explains why so few studies on MTC profiling have been reported.^{28,35–38} The present study, based on one of the largest series of primary MTCs described to date, was focused on finding differences among expression profiles according to the genetic condition of the MTC samples.

Biomarkers and Pathways Are Specific to Each Genetic Class of MTCs

In accord with our finding in MTC⁶³⁴, *GAL* overexpression has been observed in PC12 rat pheochromocytoma cell lines.³⁹ *GAL* is involved in neuropeptide signaling,

tumorigenesis, nervous system development, and the regulation of cell proliferation; it is expressed in neuroblastoma and pheochromocytoma cancer cell lines, and it activates MAPK and ERK signaling.^{39–42} The present findings suggest that *GAL* overexpression may be involved in the development of the phenotype exhibited by MEN2A patients carrying a germline *RET*⁶³⁴ mutation; pheochromocytoma develops in at least 50% of these cases.^{3,43}

GDNF family receptor α -1 (encoded by *GFRA1*), associated with MTC^{M918T}, is a known coreceptor of RET that mediates its activation through the binding of RET ligands. These include ligands that are widely expressed and promote the survival of neurons during development. *GFRA1* has been previously reported as a genetic risk factor associated with susceptibility to developing sporadic MTC in a case-control study,⁴⁴ and our results support its role in development of MTC.

DKK4 (encoding Dickkopf-related protein 4), another gene overexpressed in MTC^{M918T}, is significantly up-regulated in colorectal cancer, and this up-regulation reflects activation of the Wnt canonical pathway.⁴⁵ Furthermore, *KREMEN2* (encoding kringle containing transmembrane protein 2) cooperates with *DKK4* to regulate Wnt signaling.⁴⁶ *KREMEN2* was up-regulated similar to *DKK4* in M918T tumors included in the present study. Deregulation of the Wnt pathway results in the development of cancer and other diseases, and Wnt/ β -catenin signaling and JNK noncanonical pathways are implicated in RET signaling, which is activated in MTC. Deregulation of the Wnt pathway specifically associated with *RET*^{M918T} MTC could open new perspectives for treatment.

The most noteworthy results among the M918T group were the overexpression of *LOXL2* (GEO accession number GSE32662) and *PROM1*. *LOXL2* (encoding lysyl oxidase-like protein 2) activates Snail/E-cadherin and Src kinase/focal adhesion kinase (Src/FAK) pathways and is associated with tumor cell invasion. *LOXL2* is also linked to epithelial-mesenchymal transition, which leads to metastasis and lower survival rates in epithelial and neuroendocrine tumors.^{47–50} Increased expression of genes associated with epithelial-mesenchymal transition has been previously observed in MEN2B MTCs.³⁸

PROM1 was the gene most significantly overexpressed among the M918T MTCs included in our series of primary tumors. *PROM1* was recently reported by Zhu et al⁵¹ to be a cancer stem cell marker in MZ-CRC-1 cell lines harboring a *RET*^{M918T} mutation. Because MTCs bearing the *RET*^{M918T} mutation are resistant to treatment, a high expression level of *PROM1* might be related to resistance to therapy. In this regard, high expression of *PROM1* is associated with neural crest-derived stem cells, and slowly differentiating cancer stem cells have been proven to be resistant to irradiation and cytotoxic drugs.⁵¹ To our knowledge, the present study is the first study based on primary tumors that points to *PROM1* overexpression associated with the M918T mutation. Another study recently showed an association of poor

prognosis with a high expression of *PROM1* in medulloblastoma tumors, suggesting that it might be a potential prognostic marker.⁵²

Immunohistochemical assessment of GFRA1 and PROM1 in the 71 FFPE MTCs revealed a tendency toward association of the M918T group with PROM1 overexpression. Furthermore, after silencing of *PROM1* with two different siRNAs, we observed decreased levels of *PROM1* mRNA which led to an increased number of apoptotic nuclei. This significant correlation between silencing of *PROM1* and apoptosis, taken together with our profiling data, led us to conclude that *PROM1* is a specific biomarker for the *RET*^{M918T} mutation and that its targeting seems feasible and could lead to apoptotic cell death. In this regard, GO biological function analyses using FatiGO and Babelomics revealed that important processes such as antiapoptotic functions, proliferation, migration, cell cycle, and adhesion were highly associated with the M918T group. Pathway enrichment analysis of the M918T group using GSEA software pointed to signaling cascades involved mainly in tumor invasion and metastasis (NF κ B, interleukin, cytokine, p38 MAPK, Wnt, JAK/Stat, Notch, MAPK, or cell adhesion), which agrees with what would be expected on the grounds of the clinical behavior of M918T-related MTCs. Among these pathways, the Notch pathway is known to play an important role in the normal development of neuroendocrine cells, including the calcitonin-producing C cells of the thyroid.⁵³

In summary, we have identified specific biomarkers related to different genetic conditions by means of expression analysis. The overexpression of *LOXL2* (involved in the cell adhesion pathway and the epithelial-mesenchymal transition process) and of *DKK4* (involved in the Wnt pathway), along with the fact that a decrease in *PROM1* expression was associated with an increased number of apoptotic nuclei, opens new therapeutic possibilities for improved management of MTC.

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Supplemental Data

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