Expression Profiles Provide Insights into Early Malignant Potential and Skeletal Abnormalities in Multiple Endocrine Neoplasia Type 2B Syndrome Tumors

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ABSTRACT

Identifying the molecular basis for genotype-phenotype correlations in human diseases has direct implications for understanding the disease process and hence for the identification of potential therapeutic targets. To this end, we performed microarray expression analysis on benign (pheochromocytomas) and malignant (medullary thyroid carcinomas, MTCs) tumors from patients with multiple endocrine neoplasia (MEN) type 2A or 2B, related syndromes that result from distinctive mutations in the RET receptor tyrosine kinase. Comparisons of MEN 2B and MEN 2A MTCs revealed that genes involved in the process of epithelial to mesenchymal transition, many associated with the tumor growth factor β pathway, were up-regulated in MEN 2B MTCs. This MEN 2B MTC profile may explain the early onset of malignancy in MEN 2B compared with MEN 2A patients. Furthermore, chondromodulin-1, a known regulator of cartilage and bone growth, was expressed at high levels specifically in MEN 2B MTCs. Chondromodulin-1 mRNA and protein expression was localized to the malignant C cells, and its high expression was directly associated with the presence of skeletal abnormalities in MEN 2B patients. These findings provide molecular evidence that associate the previously unexplained skeletal abnormalities and early malignancy in MEN 2B compared with MEN 2A syndrome.

INTRODUCTION

The multiple endocrine neoplasia type 2 (MEN 2) syndromes are caused by dominant mutations in the RET receptor tyrosine kinase. The two major subtypes of this syndrome, MEN 2A and MEN 2B, manifest overlapping clinical features that include pheochromocytoma and medullary thyroid carcinoma (MTC; Ref. 1). Pheochromocytomas are tumors of adrenal chromaffin cells that are usually benign. MTCs are malignant tumors of the thyroid C cells. The clinical presentation of MTC is different in MEN 2A and MEN 2B. MTC occurs in late childhood/early adulthood in MEN 2A, whereas in MEN 2B, the tumor is present in infancy. The histological features of MTC are similar in both syndromes, however.

Surgery is the only effective treatment for MTC. Metastatic MTC is a major cause of mortality in these patients and is resistant to radiation, chemotherapy, and biological therapies. Aggressive screening of at-risk MEN 2 family members identifies gene carriers at an early age and allows thyroidectomy to be performed before metastatic disease occurs (2, 3). In a recent consensus, it was noted that MEN 2B MTCs are also more aggressive than MEN 2A MTCs (4). Thus, to minimize morbidity and mortality, it is recommended that patients with the MEN 2B mutation undergo thyroidectomy in infancy and those with MEN 2A by 5 years of age. Indeed, microinvasive MTC is often found in thyroids of MEN 2B patients even when they are resected during infancy. The molecular basis for the early malignant potential and supposed aggressiveness of MEN 2B MTCs compared with MEN 2A MTCs has been enigmatic.

There are also other clinical differences that distinguish the two syndromes. For example, hyperparathyroidism occurs in MEN 2A (~25%) but not in MEN 2B patients. MEN 2B patients develop mucosal neuromas, characteristic facial features, megacolon, and skeletal abnormalities (the so-called marfanoid habitus). The mechanisms underlying these phenotypic differences in the two syndromes are unknown.

The MEN 2A and MEN 2B syndromes are characterized by distinctive germ-line mutations in the RET tyrosine kinase. RET is a single pass transmembrane receptor that forms receptor complexes with the glycosylphosphatidyl inositol-anchored Glial cell line-derived neurotrophic factor family receptor α coreceptors to mediate signals of the Glial cell line-derived neurotrophic factor family of ligands (GFLs; Ref. 5). RET mutations in MEN 2A patients occur predominantly in the extracellular domain cysteine residues (codons 609, 611, 618, 620, 630, and 634). In contrast, all MEN 2B mutations are located in the intracellular domain with >95% at residue 918 (M918T). Clinical studies have demonstrated a strong correlation between age of onset of invasive disease and inherited RET mutation (MEN 2B or different MEN 2A mutations; Ref. 6). The molecular link of different RET mutations to the distinct clinical MEN 2 syndromes is unclear. However, experiments in vitro suggest that the M918T mutation alters RET substrate specificity such that it mimics that of the cytoplasmic tyrosine kinases (7). In contrast, MEN 2A-associated RET mutations result in ligand-independent receptor dimerization and constitutive activation of signaling pathways used during normal GFL-mediated RET signaling. Thus, it has been hypothesized that mutant RET in MEN 2B additionally activates aberrant signaling pathways that result in the expression of different target genes that are the reason for its distinct clinical phenotype from MEN 2A.

Recent studies using expression profiling have been instrumental in defining subtypes of tumors within previously described categories that are based on clinical or histological characteristics (8, 9). In some instances, tumor subtypes defined by expression fingerprints are also associated with specific genetic alterations, thus implying that these alterations underlie the changes in the expression profile and in tumor behavior. We hypothesized that the mutations present in MEN 2A and MEN 2B tumors, albeit within the same gene, might result in unique expression profiles that would identify the signaling pathways activated by oncogenic forms of RET and shed light on the disparate biology of these two closely related syndromes. To this end, we performed expression microarray analysis of tumors from patients with MEN 2A and MEN 2B harboring specific RET mutations. We found expression profiles that clearly distinguish tumors arising from extracellular RET mutations (MEN 2A) versus intracellular RET mutations (MEN 2B). Importantly, we found genes in which the expression is altered only in MTCs from MEN 2B patients, suggesting that
they may contribute to the early malignant potential of these tumors and the skeletal abnormalities often observed in these patients.

MATERIALS AND METHODS

Biological Materials. Fresh frozen tumor samples from MEN 2 patients (pheochromocytomas and MTCs) and non-MEN 2 patients (sporadic MTCs) were acquired from the Alvin J. Siteman Cancer Center Tissue Procurement Core Facility at Washington University (see supporting information Tables 1 and 4 for clinical data) and used in accordance with an Institutional Review Board-approved protocol. We selected tumor specimens in which >50% of cellularity consisted of tumor cells (10). All of the tissues selected had both tumor cell and stromal elements. All MEN 2 MTC patients in the microarray analysis had stage 3 or higher disease, whereas all of the pheochromocytomas were benign. A total of 40 different tumor samples from 34 MEN 2 patients was used for the final microarray analyses. The sample characteristics of these are reported in supporting information Table 1.

Other Clinical Features of MEN 2B Patients. All patients with MEN 2B had characteristic features of mucosal neuromas, mid-face hypergnathism, and megacolon. Skeletal abnormalities pertaining to marfanoid habitus were observed in a subset of patients as determined by clinical examination and medical records and included elongated long bones, scoliosis, slipped capitids femoral epiphysis, pectus, and joint deformities (Supplemental Table 4; Refs. 11–13). These abnormalities were not observed in MEN 2A or sporadic MTC patients.

RNA Isolation, Target Preparation, and Microarray Hybridization. Total RNA from 50-μm frozen sections of each tumor was prepared by standard methods. Ten μg of total RNA were used to prepare biotinylated cRNA targets, which were hybridized to Hu-U95Av2 oligonucleotide microarray GeneChips according to the manufacturer’s protocol (Affymetrix, Inc., Santa Clara, CA). The hybridization, washing, and scanning were performed on an Affymetrix Fluidics Station using standard procedures (14).

Microarray Data Processing and Analysis. Analysis was performed on 13 MEN 2A MTCs, 10 MEN 2B MTCs, 9 MEN 2A pheochromocytomas, and 8 MEN 2B pheochromocytomas. We used Affymetrix Microarray Analysis Suite (MAS 5.0) to generate globally normalized signal intensities from the scanned images. For comparison across samples, the microarrays were scaled to a global intensity of 500 units and a normalization factor of 1 (available online). The scaled data were imported into dCHIP (v1.3) for filtering, hierarchical clustering, and linear discriminant analysis (see below; Ref. 15). In cases where the same tumor target was hybridized on duplicate chips (supporting information in Table 1), average signal intensities were used with the help of the replicate separators in dCHIP. Before analysis, the data were filtered to eliminate genes that did not vary or were not expressed. Genes were included that met the following filtering criteria: (a) variant filter (SD/mean) across samples >0.5; (b) called present in >25% of the samples; and (c) expression level >100 in 25% of the tumors. After filtering, a list of 879 probe sets remained in MEN 2A versus MEN 2B pheochromocytoma comparison and 1162 in MEN 2A versus MEN 2B MTC comparison. For identifying differentially expressed genes among the different tumor types, we used Significance Analysis Microarrays (SAMs) software using two class unpaired analysis with 3000 permutations, >1.5-fold change, and false discovery rate and delta values as specified (supporting information in Table 2; Ref. 16). This algorithm calculates the difference between the means of two classes divided by the sum of their SDs. It then performs random permutations of the data set and assesses the false discovery rate, rank orders the genes according to score “d,” and provides a statistical significance q value (corrected for multiple comparisons). The delta threshold values in SAM were selected to yield biologically significant genes at a low false-positive rate as determined by repeated permutations at different delta values and counting the number of significant genes (17). Relationships among the genes were identified by centroid linkage hierarchical clustering analysis using 1 − r as the similarity metric (r is the Pearson correlation) on differentially expressed genes identified by SAM. We have obtained similar results when expression values were calculated by dCHIP software instead of by the Affymetrix MAS 5.0 (data not shown). The genes were centered to have median 0 and SD of 1 before clustering. To evaluate the class prediction properties of the MEN 2 MTCs from the differentially expressed genes identified by SAM (Fig. 2A), we used the statistical software R2 and performed linear discriminant analysis (LDA; executable from the dCHIP software). The LDA maximizes the ratio of between-class variance to within-class variance to obtain maximum class separability (18). We used the gene list in Fig. 2A, right, to perform the LDA on the 13 MEN 2A MTCs and 10 MEN 2B MTCs (see Supplemental Table 3).

Quantitative Reverse Transcription-PCR (RT-PCR) Analysis. One μg of total RNA was used to prepare cDNA, and 10 ng of cDNA were used to perform quantitative RT-PCR on a Model 7700 instrument (Applied Biosystems, Foster City, CA). We used Sybr Green 1 fluorescence (Molecular Probes) to detect amplicons (typically 100–150 nucleotides long). Standard curves for target genes were developed to determine relative levels of expression across samples as described previously (10). We normalized individual samples to glyceraldehyde-3-phosphate dehydrogenase mRNA. Primer Express software v2.0 (Applied Biosystems) was used to design primers and sequences are shown in supporting information Table 5.

In Situ Hybridization and Immunohistochemistry. We generated sense and antisense human chondromodulin-1 (CHM1) riboprobes [nucleotides 558–1005 cloned in pcDNA3 (Invitrogen)] labeled with dUTP, using T3 and T7 polymerases, respectively. In situ hybridization was performed on 14-μm fresh frozen tumor samples, and the signals were visualized using alkaline phosphatase as described previously (19). Immunohistochemistry was performed on 6-μm sections of formalin-fixed, paraffin-embedded tumor samples using antigen retrieval in 1 m M EDTA (pH 8.0) for 20 min (20). We used CHM1 antisera at 1:400 (21), secondary antirabbit biotinylated antibody at 1:500 and streptavidin-horseradish peroxidase at 1:1000 (Jackson ImmunoResearch Laboratories). The signals were visualized using 3,3′-diaminobenzidine (Sigma, St. Louis, MO).

RESULTS AND DISCUSSION

Genes Associated with Highly Invasive Carcinomas Distinguish MEN 2B from MEN 2A MTCs. The molecular targets that represent the phenotypic differences among the MEN 2 syndromes are largely unknown. To this end, we sought to identify expression profiles that distinguish analogous tumors from MEN 2A and MEN 2B patients (MEN 2A MTC versus MEN 2B MTCs and MEN 2A pheochromocytomas versus MEN 2B pheochromocytomas), although they result from mutations within the same gene. Because the MEN 2B-associated RET mutation is thought to alter substrate specificity, we hypothesized that the expression profiles may help clarify the molecular basis for the differences seen in the MEN 2A and MEN 2B syndromes and shed light on aberrations caused by these mutations in the RET signaling pathway. We were particularly interested in determining why the MEN 2B mutation results in earlier onset of MTCs and why these patients develop skeletal abnormalities.

We took advantage of the known mutation status of the MEN 2 tumors and performed supervised analysis to find the genotype-associated expression profiles. We first performed a two-class distinction SAM on the 13 MEN 2A and 10 MEN 2B MTCs in our sample set. We identified 118 probe sets that were differentially regulated in MEN 2B versus MEN 2A MTCs, 98 that were up-regulated in MEN 2B, and 20 that were up-regulated in MEN 2A samples (supporting information in Table 2). To determine whether a subset of these genes has potential use in predicting tumor genotype (i.e., MEN 2A versus MEN 2B), we performed LDA on all 23 MTCs (see “Materials and Methods”). We found that the top five most discriminating genes from the SAM analysis were sufficient to correctly predict the classification of all MEN 2A and MEN 2B MTCs in our sample set using LDA (Fig. 1A, Supplemental Table 3). We also used hierarchical clustering of this data in an attempt to distinguish primary from metastatic tumor samples; however, our data set was not useful in discriminating between these lesions (unpublished observations).
To determine whether the differentially expressed genes could provide clues as to the underlying biology of these tumors, we performed hierarchical clustering on the 118 probe sets representing 107 unique genes. These 107 genes represent two main gene clusters, corresponding to either MEN 2B or MEN 2A MTC-specific genes (Fig. 1B). To confirm the veracity of the microarray profiles, we examined the expression of a number of genes that were differentially expressed in MEN 2A versus MEN 2B MTCs using quantitative RT-PCR (qRT-PCR). We performed this experiment on all of the tumor samples used for our studies and found that 10 of 14 genes tested by qRT-PCR were in agreement with the microarray analysis (Table 1). In the qRT-PCR analysis we used the same false discovery rate that was used in the microarray analysis for identifying discriminating genes (false discovery rate = 12%, supporting information Table 2).

Among the MEN 2B MTC-specific gene cluster, of particular note is a subcluster enriched in genes associated with matrix remodeling and the epithelial to mesenchymal transition (collagen 1α2, COL1A2; thrombospondins, THBS1 and THBS2; laminins, LAMA4 and LB2A6; and fibronectin, FN1; Fig. 1B). These and other gene prod-
products in the MEN 2B cluster have been previously associated with an increased metastatic potential in a variety of other tumors, including breast, prostate, and bladder carcinomas (22–24). For example, chemokine receptor 4, which is up-regulated in breast cancer, was expressed at higher levels in MEN 2B MTCs than in MEN 2A MTCs (25). In addition, a number of these genes in the MEN 2B cluster also modulate tumor growth factor β signaling. The tumor growth factor β signaling pathway is important for the epithelial to mesenchymal transition, thus indicating a potential role for this pathway in the early malignant transformation phenotype of MEN 2B MTCs (26). Many of these genes, i.e., COL1A2 and FN1, are thought to be expressed by stromal cells in other tumors, suggesting that reciprocal interactions between these cells and the malignant C cells may contribute to the early onset and increased aggressiveness of MEN 2B MTCs (23, 27).

Alternatively, the behavior of these tumors may be affected by synergism between RET and tumor growth factor β activities because they both signal via the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways (1, 26, 28). Thus, the aberrant signaling from the RET intracellular domain mutation in MEN 2B MTCs results in specific expression profiles that highlight several pathways (e.g., epithelial to mesenchymal transition, metastasis genes) potentially contributing to the early onset MEN 2B phenotype.

We also identified proenkephalin as one of the genes that was up-regulated in MEN 2B MTCs (13-fold, Supplemental Table 2). Proenkephalin expression has been previously identified in neuroendocrine cells, including chromaffin and C cells, and has been shown to alter gastrointestinal problems in MEN 2B patients.

Sporadic MTCs Show Molecular Attributes of Both MEN 2A and MEN 2B MTCs. Sporadic MTCs occur in late adulthood and ~40% are found to harbor the M918T mutation in RET (11). We therefore asked whether the sporadic MTCs are molecularly similar to the MEN 2B MTCs by comparing their patterns of gene expression.
aggressive behavior in other neoplasias (e.g., epithelial to mesenchymal transition cluster genes: COL1A2 and CSPG2, chemokine receptor 4). Our analysis revealed that sporadic MTCs show attributes of both MEN 2A and MEN 2B MTCs (Fig. 2A). For example, some genes expressed at high levels in MEN 2B (e.g., RGS2) or MEN 2A (e.g., UCHL1) were also highly expressed in sporadic MTCs ($P < 0.02$ and 0.07, respectively). Whereas other genes expressed predominantly in MEN 2B MTCs were not expressed significantly in sporadic MTCs (e.g., CHM1, $P < 0.001$; CXCR4, $P < 0.07$ and COL1A2, $P < 0.1$). A similar observation was made with genes expressed primarily in MEN 2A MTCs (e.g., PNMA2, $P < 0.005$).

We first assessed the expression of genes that were capable of discriminating between MEN 2A and MEN 2B MTCs on 11 different sporadic MTC tumors (9 patients) (Supplemental Table 4). The genes selected were the most informative discriminating genes validated from the qRT-PCR results (Table 1) and include those associated with

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**Table 2. CHM1 expression is associated with skeletal abnormalities in MEN 2B MTC patients**

<table>
<thead>
<tr>
<th>CHM1 +</th>
<th>CHM1 −</th>
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<tbody>
<tr>
<td>MTCs</td>
<td></td>
</tr>
<tr>
<td>MEN 2B</td>
<td>11</td>
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<tr>
<td>Non-MEN 2B</td>
<td>2</td>
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<tr>
<td>Association of MEN 2B MTC with CHM1 ($P &lt; 0.002$, Fisher’s exact test)</td>
<td>19</td>
</tr>
<tr>
<td>Skeletal abnormality</td>
<td></td>
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<tr>
<td>Present</td>
<td>9</td>
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<tr>
<td>Absent</td>
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<tr>
<td>Association of skeletal abnormalities with CHM1 ($P &lt; 0.001$, Fisher’s exact test)</td>
<td>5</td>
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</tbody>
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* A “+” denotes present, and a “−” denotes absent. An “*” represents indeterminate status for skeletal abnormalities as these patients are prepubertal. Skeletal abnormality data is only from MEN 2B MTC patients as non-MEN 2B MTC patients did not have any skeletal abnormalities. The non-MEN 2B patients includes data from 12 MEN 2A and 9 sporadic MTC patients.

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expression promotes cartilage deposition but inhibits bone deposition, whereas studies of CHM1 mutant mice show that its deficiency results in increased bone deposition (33, 34). These observations suggest that aberrant CHM1 expression by MEN 2B MTCs could cause abnormalities in the growth plates of developing bones and lead to the observed skeletal abnormalities in MEN 2B patients. We first verified that MEN 2B MTCs express high levels of CHM1 using qRT-PCR (Fig. 3A). These results show that CHM1 was highly expressed in 8 of 10 MEN 2B MTCs but in only 1 of 13 MEN 2A MTCs and 1 of 11 sporadic MTCs (non-MEN 2-associated MTCs). The high CHM1 expression found in MTCs was localized to the malignant thyroid C cells in these tumors using in situ hybridization and immunohistochemistry (Fig. 3B, left, and 3C, a and b). MTCs with reduced CHM1 mRNA expression by microarray and qRT-PCR analyses showed only background staining for CHM1 (Fig. 3B, right, and 3C, c–f). Although the high CHM1 mRNA expression in MTCs was unexpected, it should be noted that calcitonin, another protein involved in bone metabolism, is also produced by thyroid C cells and has been used diagnostically in monitoring MEN 2 patients. Whether high CHM1 gene transcription is a direct result of signaling alterations secondary to the RET intracellular mutation in these patients or whether it is an indirect consequence of a change in C cell environment brought on by the alterations in extracellular matrix observed in these tumors remains to be determined.

The high levels of the secreted developmental bone regulator, CHM1, found specifically in MEN 2B MTC tumor cells, encouraged us to examine its association with skeletal abnormalities in MEN 2B patients. Additional samples of archived MTC tumor tissue from MEN 2 patients were analyzed for CHM1 expression using immunohistochemistry, and these findings were correlated with the clinical characteristics of these patients (Fig. 3C, Table 2, and Supplemental Table 4). The results show that all MEN 2B MTC patients with skeletal abnormalities had high CHM1 expression and a high tumor burden at diagnosis, as determined by calcitonin levels (Fig. 3C, a and b, Table 2, and Supplemental Table 4). Interestingly, there were two MEN 2B patients with high CHM1 expression but no reported skeletal abnormalities (Table 2 and Supplemental Table 4, patients MTC28B6 and MTC28B13). Both of these patients had a low tumor burden at diagnosis and are still prepubertal; thus, we consider their skeletal status as indeterminate at this time. However, it is possible that they may never develop skeletal abnormalities because their tumor was removed at a very early age. Skeletal abnormalities were not observed in any patient (with MEN 2A, MEN 2B or sporadic MTCs) in the absence of CHM1 expression (Fig. 3C, c and d, Table 2, and Supplemental Table 4). In this category, there is again one prepubertal patient whose tumor was removed in infancy; thus, we again labeled this patient’s skeletal status indeterminate (marked by an “*” in Table 2). During our analysis, we identified one MEN 2A and one sporadic MTC patient (of 21 non-MEN 2B MTC patients) that had high CHM1 expression without skeletal abnormalities (Table 2 and Supplemental Table 4, patients 12 and 20). The absence of skeletal abnormalities in these patients with high CHM1 expression may be due to the adult onset of MTCs, well after skeletal development has been completed. Alternatively, the development of skeletal changes may require additional contributions from other aberrantly regulated genes that are not present in these non-MEN 2B patients. This explanation is consistent with the observation that some MEN 2B patients appear to develop congenital skeletal abnormalities before clinically evident MTC is apparent. However, it is also possible that these patients develop these abnormalities because they harbor microinvasive disease at birth (often detected in MEN 2B infants undergoing prophylactic thyroidectomy), leading to high levels of CHM1. Although not feasible in this study, it will be interesting to correlate the age-dependent systemic levels of CHM1 in MEN 2 patients with their skeletal status.

Interestingly, none of the other discriminating genes had an expression profile similar to CHM1 with respect to skeletal abnormalities (Fig. 1B and unpublished observations). Taken together, we conclude that among the MTCs examined, only MEN 2B MTCs are associated with high CHM1 expression (P < 0.001, Fisher’s exact test), and sustained CHM1 expression in MEN 2B MTC patients is highly associated with skeletal abnormalities (P < 0.002, Fisher’s exact test; Table 2). These results support the role of high CHM1 expression by malignant thyroid C cells in the development of MEN 2B-associated skeletal abnormalities, thus providing a likely molecular basis for this well-known but previously enigmatic feature of the MEN 2 syndromes.

Recently, expression profiling of NIH 3T3 mouse fibroblasts transfected with RET mutants associated with MEN 2A and MEN 2B has been reported (35). The genes we identified in this study of MEN 2 MTC tumors did not overlap with those identified in their experiments. This presumably reflects the in vivo versus in vitro design of these studies. It may also reflect the different cell types (mouse fibroblasts versus human neuroendocrine tumor cells) or temporal events. In their study, they identified STC1, a possible regulator of skeletal growth, as a gene specifically induced in fibroblasts expressing RET M918T. We did not find STC1 to be differentially expressed in MEN 2B versus MEN 2A MTCs at a statistically significant level using multiple microarray algorithms (Fig. 1B and data not shown). They did find by immunohistochemistry that STC1 was strongly expressed in 2 MEN 2B MTCs but not in sporadic MTCs; thus, it will be interesting to determine whether STC1, as with CHM1, is associated with skeletal abnormalities in MEN 2B patients.

New insights into cancer biology are often discerned from the study of rare inherited cancers. To this end, we have focused on identifying gene profiles that underlie the observed phenotype/genotype differences in MEN 2 syndromes. These have provided insights at various levels into the pathogenesis of the associated tumors. For example, the metastasis genes that are up-regulated in MTCs provide a starting point for alternative therapies for these resistant tumors and may also be applicable to the more common sporadic MTCs. This data also provides insights into the molecular basis of the MEN 2B MTC phenotype and raises the possibility that aberrant high level expression of chondromodulin by MTCs in MEN 2B patients may contribute to some, if not all, associated skeletal abnormalities. Most cancer microarray studies have either compared expression profiles of histopathologically distinct tumor types or have examined histologically similar tumors from patients with germ-line mutations in different genes (e.g., BRCA1 and BRCA2 in breast tumors; Refs. 36–38). In this study, we have shown that expression profiling can also distinguish histologically similar tumors that arise because of different germ-line mutations in the same gene, thus demonstrating the robustness of this approach in studying the effects of even subtle genetic alterations on disease phenotype.

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