Exome sequencing reveals mutant genes with low penetrance involved in MEN2A-associated tumorigenesis

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Abstract
Activating rearranged during transfection (RET) mutations function as the initiating causative mutation for multiple endocrine neoplasia type 2A (MEN2A). However, no conclusive findings regarding the non-RET genetic events have been reported. This is the first study, to our knowledge, examining genomic alterations in matched MEN2A-associated tumors. We performed exome sequencing and SNP array analysis of matched MEN2A tumors and germline DNA. Somatic alterations were validated in an independent set of patients using Sanger sequencing. Genes of functional interest were further evaluated. The germline RET mutation was found in all MEN2A-component tumors. Thirty-two somatic mutations were identified in the nine MEN2A-associated tumors, of which 28 (87.5%) were point mutations and 4 (12.5%) were small insertions, duplications, or deletions. We sequenced all the mutations as well as coding sequence regions of the 12 genes in an independent sample set including 35 medullary thyroid cancers (20 MEN2A) and 34 PCCs (22 MEN2A), but found no recurrent mutations. Recurrent alterations were found in 13 genes with either mutations or alterations in copy number, including an EIF4G1 mutation (p. E1147V). Mutation of EIF4G1 led to increased cell proliferation and RET/MAPK phosphorylation, while knockdown of EIF4G1 led to reduced cell proliferation and RET/MAPK phosphorylation in TT, MZ-CRC1, and PC-12 cells. We found fewer somatic mutations in endocrine tumors compared with non-endocrine tumors. RET was the primary driver in MEN2A-associated tumors. However, low-frequency alterations such as EIF4G1 might participate in MEN2A-associated tumorigenesis, possibly by regulating the activity of the RET pathway.

Introduction
Multiple endocrine neoplasia type 2A (MEN2A), originally named Sipple's syndrome, consists of tumors derived from parafollicular C cells (medullary thyroid cancer (MTC)), adrenal medulla (pheochromocytoma (PCC)), and less commonly parathyroid (parathyroid hyperplasia or adenomas (PHPT)) (Sipple 1961). Although parafollicular cells,
adrenal medulla cells, and parathyroid cells all originate from neural crest, the tumor components demonstrate both malignant and benign behavior. MTC is malignant and the primary cause of mortality in MEN2A, while PCC is rarely malignant, with PHPT only presenting as hyperplastic lesions. Moreover, heterogeneity of phenotypes including age of onset, aggressiveness of MTC, and the presence or absence of other endocrine neoplasms has been observed between and within families.

In the early 1990s, direct DNA analysis found mutations in the rearranged during transfection (RET) proto-oncogene as the genetic cause of MEN2A (Mulligan et al. 1993, Asai et al. 1995, Santoro et al. 1995). RET encodes a receptor kinase that was originally described as activated by binding of glial-cell-line-derived neurotrophic factor (GDNF) and GDNF family receptors. RET mutations lead to ligand-independent constitutive activation, autophosphorylation, and aberrant stimulation of downstream signaling (Schlee et al. 2006). Genotype and phenotype correlations have been reported in MEN2A patients, and these correlations have led to stratified risk and specific management guidelines developed by the American Thyroid Association (ATA; Kloos et al. 2009). For example, patients with RET codon 634 mutations are classified as ATA level C, showing higher MTC aggressiveness, with prophylactic thyroidectomy recommended before 5 years of age, and should be screened for PCC and PHPT beginning at 8 years of age. These results indicated a dominant role for the RET proto-oncogene in MEN2A tumorigenesis.

However, there is increasing evidence indicating the involvement of additional genes or second events in MEN2A-associated tumorgenesis (Benn et al. 2000, Huang et al. 2000, Diaz-Cano et al. 2001, Koch et al. 2002). These include the variability in tumor phenotype among individuals, even in patients from the same family; the effect of genetic background on the penetrance and aggressiveness of MTC in RET transgenic mouse models (Michiels et al. 1997); the lack of correlation between effectiveness of tyrosine kinase inhibitors (TKIs) in MTC patients; and RET mutation status (Kurzrock et al. 2011). Several efforts have been made to uncover these non-RET events. Low penetrance genes that interact with the RET pathway were reported to be associated with a greater risk of developing MTC (Ruiz et al. 2007). Frequent allelic loss including regions 1p, 4q, 7q36.1, 12p13.31, 13q12.11, 19p, and 22q has been found by array-comparative genomic hybridization (CGH) profiling in MTC tumors (Ye et al. 2008, Flicker et al. 2012). Concurrent RAS mutation was found exclusively in non-RET mutated MTCs via exome sequencing (Agrawal et al. 2013).

Other MTC-associated loci that have been reported include P18, Sprouty1, NRAS, and others (Takahashi et al. 2006, Van Veelen et al. 2008, Macià et al. 2012). However, there have been no conclusive findings defining non-RET genetic events in MEN2A-associated tumors.

To comprehensively reveal the genetic architecture of these neural crest-derived tumors, we conducted exome sequencing and SNP array analysis on matched MEN2A tumors (MTC, PCC, and PHPT) and peripheral blood DNA from four patients.

Materials and methods

Patients and sample collection

The board of medical ethics of Ruijin Hospital approved the study and all patients gave their informed written consent. The diagnosis and management of MEN2A was determined on the basis of the 2009 ATA guidelines. Germline RET mutation analysis (exons 8, 10, 11, 13, 14, 15, and 16) was performed according to the method described in a previous report (Zhou et al. 2007). A total of 45 MEN2A families, 125 MEN2A patients, and 65 sporadic MTC were diagnosed in Ruijin Hospital from 2001 to 2013. This study included four MEN2A patients in the discovery set for exome sequencing (pedigrees in Supplementary Fig. 1, see section on supplementary data given at the end of this article). An additional 18 MEN2A family members (9 RET-positive gene carriers and 9 RET-negative members from three unrelated families) and 69 patients (42 MEN2A and 27 sporadic patients) were included in the validation set. None of the patients received chemotherapy or radiotherapy before surgery. Surgical specimens were snap-frozen in liquid nitrogen until use.

DNA sample preparation

For the tumor tissues to be analyzed, the diagnosis of MEN2A-associated tumors was confirmed by two independent pathologists. The tumor cells were dissected by laser capture using a Leica AS LMD 6500 microscope following the manufacturer’s instructions. DNA extraction was performed using the QIAamp DNA Micro Kit (Qiagen vat. no. 56304). DNA quality and quantity was analyzed by NanoDrop 1000 and agarose-gel electrophoresis.

Exome sequencing, data mining, and validation

The DNA libraries were enriched using the NimbleGenEZ 44M human exome array and sequenced using an Illumina HiSeq2000. The data were post-processed using
Picard and Samtools and then analyzed with Varscan2 and GATK. The variants were annotated using ANNOVAR (detailed methods are described in the Supplementary Methods, see section on supplementary data given at the end of this article). The germline variants were filtered to identify somatic variants in individual tumors. The common variants between tumor type and individuals were further analyzed. Finally, dbsNP 132, dbsNP 135, and YH were used as filters to identify novel germline variations that associated with MEN2A. All somatic single nucleotide variations (SNVs) were validated by Sanger DNA sequencing, both in the discovery set and in an independent sample set including both inherited and sporadic MTC/PCC. The genes that satisfied one of the following criteria were selected for coding sequence (CDS) region sequencing in the validation set: i) genes with differences in expression between tumor and normal control based on the Oncomine database; ii) genes with somatic mutation in MEN2A-associated tissues (MTC/PCC/PHPT) or cell lines (TT/MZ-CRC1) based on the COSMIC database; and iii) mutations found in other endocrine-derived malignant tumors. A total of 12 genes were selected for CDS screening (primers are listed in Supplementary Table 1, see section on supplementary data given at the end of this article).

SNP array analysis

All the DNA samples included in the discovery set, except MTC of patients 1 and 3 (because of sample limitation) were additionally analyzed using an Affymetrix Genome-Wide Human SNP 6.0 Array. Briefly, 250 ng of sample DNA was digested with NspI and StyI enzymes, followed by adaptor-ligation and amplification. The amplified DNA was then fragmented, labeled, and hybridized to the array. The arrays were then washed, scanned, and analyzed using AGCC Software (Affymetrix, Santa Clara, CA, USA). Sample data were compared with HapMap 270 control DNA using the Hidden Markov Model in Genotyping Console 3.0.2 (Affymetrix). The UCSC Genome Browser Build hg18/NCBI Map Viewer Build 36.3 was used to obtain information on the genomic segments involved in the copy number variations (CNV) detected and the corresponding list of genes.

Cell culture, transfection, and infection

TT cells (harboring the RET p.M918T heterozygous mutation), a human MTC cell line derived from a MEN2A patient, were from the American Type Culture Collection (ATCC) (CRL1803). MZ-CRC1 cells (harboring the RET p.M918T heterozygous mutation) derived from a malignant pleural effusion from a patient with a metastatic MTC were kindly provided by Robert Gagel (MD Anderson Cancer Center, Houston, TX, USA). PC-12 cells, derived from rat pheochromocytoma, were from ATCC (CRL 1721). TT cells (passage 16–23) were maintained in F-12K Nutrient Mixture, Kaighn’s Modification (1×) supplemented with 10% FCS (Invitrogen). MZ-CRC1 cells (passage 5–12) were maintained in DMEM (Gibco, BRL) supplemented with 10% FCM (Invitrogen). PC-12 cells (passage 10–14) were maintained in 85% RPMI-1640 medium (Gibco, BRL) supplemented with 10% heat-inactivated horse serum (Gibco, BRL) and 5% FCM (Invitrogen). cDNA ORF clones of human EIF4G1 and GRAP with a C-terminal Myc-DDK epitope tags were obtained from OriGene Technologies (Rockville, MD, USA) (RC212877 and RC208908).

Mutations were induced into plasmids using the Quik-Change II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). siRNAs targeting human and rat EIF4G1 and nonspecific negative controls were synthesized by Gene-Pharma Co. Ltd (Shanghai, China). The siRNA transfection was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. Both real-time PCR and western blotting were performed to test the inhibitory efficiency of siRNA. The lentivirus particles containing EIF4G1 (p. E1147V) or WT EIF4G1 plasmids were used to infect TT (MOI: 6), MZ-CRC1 (MOI: 8), and PC-12 (MOI: 10) cells separately. The infected cells were selected for viral integration using 1.5 µg/ml (TT), 1 µg/ml (MZ-CRC1), and 1 µg/ml (PC-12) puromycin as appropriate.

Cell proliferation assay

Cell proliferation was determined using the Cell Counting Kit-8 Assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, 6 days after overexpression of EIF4G1 and 72 h after siRNA treatment, cells were incubated with the CCK8 reagent for 2 h. The relative cell number was determined by measuring light absorbance at a wavelength of 450 nm.

Western blotting

Western blots were generated by a standard method with protein lysates prepared using RIPA protein extract buffer (Santa Cruz Biotechnologies). The antibodies included the following: anti-RET was purchased from Santa Cruz; anti-phosphorylated RET (Tyr905), anti-ERK1/2, anti-phosphorylated ERK1/2 (Thy202/Tyr204), anti-AKT, anti-phosphorylated AKT (Ser473), anti-GAPDH, anti-α-tubulin,
anti-DDK, and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Individual protein detection was performed using ECL reagents (Amersham Pharmacia).

Results
Features of MEN2A patients in exome sequencing studies
The discovery group chosen for exome sequencing included four MEN2A patients with available matched tumors for analysis. Patient data are summarized in Table 1. The four patients were probands from four unrelated Chinese Han families. MTC and PCC were identified in all four patients, with parathyroid adenoma occurring only in patient 3. MTC was diagnosed before PCC in three individuals. Patient 3 was the exception, with MTC detected 29 years after the PCC had been diagnosed. The levels of serum calcitonin and plasma MNs (NMN, normetanephrine; MN, metanephrine) were highly elevated at diagnosis and decreased markedly after surgery. All patients underwent total thyroidectomy because of elevated levels of serum calcitonin. Patient 3 was diagnosed as MEN2A before MTC developed, but he refused to have prophylactic thyroidectomy. Patients 1 and 4 underwent right adrenalectomy; patients 2 and 3 underwent bilateral adrenalectomy. All four kindreds displayed RET codon 634 mutations representing three common nucleotide substitutions. Additional six family members were confirmed to be carriers of germline RET mutations (Supplementary Fig. 1).

Exome sequencing identifies the established RET germline variations
As expected, exome sequencing detected the same germline codon 634 RET oncogene mutation as identified by Sanger-sequencing performed in the original pedigree analysis. Three additional RET germline variations were also identified by exome sequencing (Supplementary Table 3, see section on supplementary data given at the end of this article), including A45A, A432A, and L769L. Patients 1, 3, and 4 exhibited all three variations while patient 2 only exhibited the A432A variation. Notably, both RET germline variants were present in all tumor samples.

Somatic events revealed by exomic sequencing in MEN2A-associated tumors
To ensure accurate detection of somatic events, our average sequencing read length was 86.7 bp with an on-target read depth of 143 (Supplementary Table 4, see section on supplementary data given at the end of this article). All the mutations were manually reviewed to remove sequencing/alignment errors. Noncoding region (UTR3, UTR5, intronic/exonic noncoding RNA) variations were not further analyzed. Mutations localized to protein coding regions were confirmed by Sanger sequencing. There were 32 unique somatic mutations identified in the nine MEN2A-associated tumors, of which 28 (87.5%) were point mutations and 4 (12.5%) were small insertions, duplications, or deletions, ranging from 1 to 9 basepairs in length. Among the point mutations, three created premature stop codons, while three of the four complex mutations were predicted to cause reading frameshifts (Table 2 and

Table 1  Clinical characteristic of the four MEN2A patients in the discovery set

<table>
<thead>
<tr>
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<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
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<td>M</td>
<td>M</td>
<td>F</td>
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<td>Age at surgery (MTC/PCC) year</td>
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<td>33/33</td>
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<td>MTC/PCC</td>
<td>MTC/PCC/HPT</td>
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<td>TNM (MTC)</td>
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<td>T1N0M0</td>
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<td>Tumor diameter (MTC) (cm)</td>
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<td>1.8</td>
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<td>Ct (before/after*)</td>
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<td>540.4/27.92A</td>
<td>15.87/14.86B</td>
<td>2.17/1.87D</td>
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<td>CEA (before/after*)</td>
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<td>9.80/2.063</td>
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<td>MN (before/after*)</td>
<td>872.5/53.6</td>
<td>980.3/206.3</td>
<td>17014/230.5</td>
<td>2636/13.1B</td>
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<td>NMN (before/after*)</td>
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<td>Duration of follow-up (months)</td>
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<td>42</td>
<td>51</td>
<td>34</td>
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F, female; M, male; MTC, medullary thyroid cancer; PCC, pheochromocytoma; HPT, hyperparathyroidism; TNM, Tumor–Node–Metastases staging score; Ct, calcitonin (*reference range: 0.1–10 pg/ml; and *reference range: < 300 pg/ml); and MN, metanephrine (reference range: 14–90 pg/ml); NMN, normetanephrine (reference range: 19–121 pg/ml); CEA, carcinoembryonic antigen (*reference range: < 5 ng/ml; *reference range: < 10 ng/ml); NA, not available.

* Ct and CEA, before and 1 day after MTC surgery; MN and NMN, before and 1 day after PCC surgery.
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<th>Chr end</th>
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<th>aa_mutation</th>
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M, medullary thyroid carcinoma; P, pheochromocytoma; PT, parathyroid.
Endocrine-Related Cancer

Supplementary Table 5, see section on supplementary data given at the end of this article). Table 2 summarizes all annotation information of somatic mutations including RET in individual tumors. It is interesting to note that patient 3, who presented initially with PCC and late-onset MTC had no detectable coding region mutations in the MTC tumor. The low number of mutations found in MEN2A tumors surprised us. To determine whether our findings were representative of other tumor types, we examined the available literature and databases. Figure 1 shows the frequency of somatic mutations in MEN2A-associated tumors (e.g., MTC and PCC from our study), sporadic MTC, sporadic parathyroid adenoma, and epithelial-derived cancers including pancreatic carcinoma, glioblastoma, colorectal carcinoma, and breast cancer (Supplementary Table 6, see section on supplementary data given at the end of this article) from previous studies (Sjöblom et al. 2006, Wood et al. 2007, Jones et al. 2008, Parsons et al. 2008, Cromer et al. 2012, Newey et al. 2012, Agrawal et al. 2013, Lawrence et al. 2013, 2014). Endocrine tumors, hereditary or sporadic, showed significantly fewer mutations per tumor than non-endocrine tumors (7.58 versus 147.7 mutation per tumor, P<0.01).

![Figure 1](http://erc.endocrinology-journals.org)

**Figure 1**

Somatic mutations in MEN2A components and other tumors. APA, adrenal aldosterone-producing adenomas; PT, parathyroid adenoma (arrows, data derived from our study, the remainder including ‘MTC1’ were obtained from Sjöblom et al. (2006) (including ‘Colorectal cancer’), Wood et al. (2007), Jones et al. (2008), Parsons et al. (2008), Newey et al. (2012), Cromer et al. (2012), Agrawal et al. (2013) and Lawrence et al. (2013, 2014) (including ‘Colorectal cancer’)). **P<0.01.

Recurrent somatic events revealed by exomic sequencing and SNP array

With the exception of RET, exome sequencing failed to identify another gene as being commonly mutated in any tumor samples. We further sequenced the 32 unique SNVs in an independent sample set (35 MTCs (20 MEN2A) and 34 PCCs (22 MEN2A)) and found no recurrent mutation. Based upon Oncomine and the COSMIC database, we selected 12 genes with potential biological function (A2M, BEX5, BHLHB9, EIF4G1, GRAP, MLL (KMT2A), MNAT1, OR3A1, TRIP12, TROVE2, UBR4 and ZDHHC8) and sequenced their CDS regions in the validation set. Again, no recurrent disease causing mutations (Supplementary Table 2) was found.

To evaluate gene copy number changes, we interrogated seven of the nine tumor samples using the Affymetrix SNP Array 6.0 (Supplementary Fig. 2, see section on supplementary data given at the end of this article). A total of 27 copy number alterations were observed in MTC, while 425 copy number alterations were identified in PCC tumors (Supplementary Tables 7 and 8, see section on supplementary data given at the end of this article). Figure 2 summarizes copy number alterations in the loci where the 32 mutant genes are located. Of the 32 mutated genes identified, there was CNV for 11 genes supporting a loss-of-function mechanism, and amplification of a single gene, TROVE2, supporting a possible gain of function. A single gene, KRBA1, showed mutation, amplification, and deletion. For two PCC there was evidence of concurrent gene mutation and loss (PLCH2 and GRAP) supporting a tumor suppressor function for these genes. Genes with either mutations or copy number alterations in multiple tumors included: CYBSD1, DYNC1H1, EIF4G1, GRAP, GPR172B (SLC52A1), IFITM1, KRBA1, KCNAB2, OR3A1, PAFAH2, PLCH2, TROVE2, and UBR4.

Functional analysis of EIF4G1 and GRAP

Among the 13 genes with either SNVs or copy number alterations, we further investigated the functional roles of EIF4G1 and GRAP in MTC and PCC cell lines because of their previously described association with malignancy (Feng et al. 1996, Silvera et al. 2009, Tu et al. 2010, Patel et al. 2012).

For EIF4G1, patient 4 harbored a mutation (p.E1147V) in the MTC tumor, while patients 1 and 3 showed copy number reductions (average copy number 1.32) in PCC tumors. Forced expression of mutant but not WT EIF4G1 was found to significantly increase the proliferation of TT cell lines with the endogenous RET mutation p.C634W.
With the change in proliferation, we observed concomitant increases in phosphorylation of RET (p905) and ERK (p202/p204) (Fig. 3). We observed similar results in PC-12 cells. Notably, both WT and mutant EIF4G1 significantly increase proliferation of PC-12 cells and phosphorylation of RET (Fig. 3). Knockdown EIF4G1 with siRNA decreased phosphorylation of RET (p905) and ERK (p202/p204) as well as proliferation of TT cells (Fig. 3). Similar results were observed for MZ-CRC1 cells harboring the RET p.M918T mutation and rat pheochromocytoma-derived PC-12 cells.

We also tested GRAP, as one of its functions is to couple regulatory signals of tyrosine kinase receptors with the RAS signaling pathway (Feng et al. 1996). Two PCC tumors showed GRAP alterations. Patient 1 harbored a point mutation in GRAP (p.D183N) as well as a reduction in GRAP alterations (Fig. 3).
in DNA copy number (copy number 1.31), while patient 3 showed DNA copy number loss (copy number 1.32; Fig. 2). To test whether the mutation in GRAP (p.D183N) might compromise its function, we induced ectopic expression of both the WT and the mutant GRAP constructs in the TT and PC-12 cell lines which do not endogenously express the gene. No difference in cell proliferation or phosphorylation of RET receptor was observed between the transfected cell lines (Supplementary Fig. 3, see section on supplementary data given at the end of this article).

Discussion

In this study we profiled genetic alterations in matched MEN2A-associated tumors using exome sequencing and high-density SNP array analysis. In total 32 nonsynonymous mutations were found in nine MEN2A tumors, which was markedly less than the numbers reported for non-endocrine epithelial tumors. We failed to find recurrent mutations even in an expanded validation set, confirming the critical role of RET in tumor initiation and progression. Of the 32 mutated genes identified, there were CNVs for 13 genes. An oncogenic role of mutant EIF4G1 (p. E1147V) was confirmed in two MTC cell lines.

Genomic instability, commonly associated with malignancy, plays a clear role in the acquisition of mutations because aggressive malignant tumors nearly uniformly harbor more genetic alterations than benign tumors of the same origin. As neuroendocrine tumors have traditionally been classified as slow-growing and less aggressive, we compared our results with published exome sequencing data (Sjöblom et al. 2006, Wood et al. 2007, Jones et al. 2008, Parsons et al. 2008, Cromer et al. 2012, Newey et al. 2012, Agrawal et al. 2013, Lawrence et al. 2013, 2014) for tumors with different derivations. We found that MEN2A-component tumors and non-MEN2A endocrine tumors showed significantly fewer mutations than epithelial-cell-derived tumors (Supplementary Table 6). The reduced rate of acquisition of genomic abnormalities might partially contribute to the benign behavior of MEN2A tumors. Moreover, the fact that no somatic mutation was detected in the MTC of patient 3 is indicative of an early stage of tumorigenesis. This is consistent with the clinical findings that patient 3 was diagnosed as MTC during routine MEN2 follow-up and resected as microMTC (0.8 cm in diameter). The earliest reported age of detection of MTC for a RET p.C634Y carrier was 10 months (Chabre et al. 2007), almost 60 years younger than patient 3 (57 years old). This heterogeneity observed here might be explained by genetic susceptibility or somatic non-RET events associated with MTC tumorigenesis. Another patient with C634Y mutations in our discovery set, patient 4, who had their MTC detected at 35 years of age, had the greatest number of exomic mutations detected and largest tumor size (2.5 cm in diameter).

A long-standing question has been whether activated RET is the only driver during MEN2A tumorigenesis. Evidence favoring this view is based largely on the significant correlation between RET genotype and MEN2A phenotype, as well as the clinical benefits from the timing of prophylactic thyroidectomy based on this correlation (Machens et al. 2003, Skinner et al. 2005, Chabre et al. 2007). Inhibition of RET kinase has also showed anti-tumor effects both in MTC mice model and MEN2A patients (Santoro et al. 1995, Michiels et al. 1997). In fact, the concept of RET ‘oncogene addition’ led to the development and FDA approval of first vandetanib (Wells et al. 2011), and ultimately XL184, as the first-line treatment for patients with advanced MTC (Kurzrock et al. 2011, Solomon & Rischin 2012, Chau & Haddad 2013). However, the response to treatment, and eventual MTC regression, cannot be correlated with RET mutation status (Carlomagno et al. 2004, Kurzrock et al. 2011, Solomon et al. 2012, Chau et al. 2013), suggestive of non-RET targets mediating the anticancer effects. A recent study employing a Ret-kinase-driven Drosophila model of MEN2A found that inhibition of Ret plus Raf, Src, and S6K was required for optimal targeting of the proliferative pathway (Dar et al. 2012). The efforts to find non-RET driver genes in MTC started decades ago. Loss of p18 showed a synergistic effect with oncogenic RET in development of MTC (Van Veelen et al. 2008). Overexpression of EGF, and VEGFR2 was associated with metastatic phenotype of MTC (Ciampi et al. 2013). Deficiency of NRAS led to metastasis and invasion of MTC in Rb1+/− mice as a result of elevated RAS homolog family A (RhoA) activity (Takahashi et al. 2006). Frequent somatic RAS mutations have been recently found in sporadic RET-mutation negative MTC (Agrawal et al. 2013, Ciampi et al. 2013). We also applied a genomic CGH approach that found loss of 11q23.3 uniquely altered in RET-negative MTC tumors (Ye et al. 2008).

Next-generation sequencing has brought huge opportunities for exploring and understanding the genetic mechanisms of human diseases, especially cancer. We therefore applied exome sequencing to MEN2A-associated tumors, trying to obtain evidence of key mediators of tumorigenesis beyond RET and to determine whether these pathways were shared between MTC, PCC, and PT. We failed to find any single gene that was as commonly altered.
as RET. When results regarding sequencing-derived variants and array-derived copy number alterations were combined, we found 13 genes with recurrent alterations. Notably, all recurrences involved multiple PCC tumors. While none of the MTC tumors displayed recurrent mutations, this observation is tempered by the fact that two samples were excluded from SNP array analysis because of sample limitations. We chose to examine the oncogenic potential of EIF4G1, which regulates the initiation of translation of mRNAs encoding mitochondrial, cell survival, and cell-growth-associated genes in response to different stresses (Ramirez-Valle et al. 2008, Silvera et al. 2009). Overexpression of EIF4G1 was observed in breast cancer and nasopharyngeal carcinoma, and treatment with rapamycin mediated downregulation of the gene in breast cancer (Silvera et al. 2009, Tu et al. 2010). In two human MTC cell lines and a rat pheochromacytoma cell line, we found that ectopic expression of mutant EIF4G1 increases cell proliferation in a background of WT gene expression. Concomitant changes in phosphorylated RET and ERK indicated that this gene may provide an additional growth advantage beyond that due to activated RET alone in inherited MTC tumorigenesis. Knockdown of EIF4G1 reversed the above changes. Interestingly, phosphorylation of AKT was observed in MZ-CRC1 cells but not TT cells, indicative of differences in susceptibility to mutant EIF4G1 between different RET mutations. We also sequenced the CDS region of EIF4G1 gene in TT and MZ-CRC1 cells. The TT cells harbor the p.G1043R mutation and two SNPs in EIF4G1 (rs2178403 p.M236V and rs2230571 p.H1140H). Rs2178403 was also detected in our validation samples (MTC 17/35, PCC 7/34). The relatively high frequency of rs2178403 may indicate a potential role of EIF4G1 in MTC tumorigenesis. We investigated the association between the clinicopathological features and rs2178403. We found that age of surgery was younger in those with rs2178403 than those without (38.11 ± 12.55 versus 44.00 ± 9.56, P = 0.024). No significant association was observed with TNM stage, survival, tumor-onset sequence, and tumor metastasis. However, a larger sample size is needed to confirm this finding.

With the sequential occurrence of multiple tumors in one individual, MEN2A provides an ideal model for studying the time and accumulated genetic alterations required for the formation of tumors. Using methods analogous to those employed in a previous study (Yachida et al. 2010), we analyzed the timing of tumor evolution by comparison of proliferation rate, determined by Ki67 labeling, and passenger mutations estimated by SIFT and POLYPHEN2. Tumor formation time for PCC was significantly longer than that for MTC (20.26 years versus 7.98 years), explaining the differences in tumor onset and penetrance of MEN2A-related MTC and PCC (average age at diagnosis: 20 years versus 38 years; penetrance: 90% versus 57%, MTC versus PCC) (Rosai et al. 1992, Kloos et al. 2009).

Despite the enormous amount of information obtained by exomic sequencing, we recognize that our study is not without limitations. First, to help focus our studies we only included protein-coding regions in our analysis. The small size of our sample population meant that it was inherently more difficult to assign functional significance to mutations present in noncoding regions and synonymous mutations. Also despite an average target coverage depth of nearly 150, we did not feel confident about making determinations of gene copy number based on exomic sequencing alone. An SNP array analysis demonstrated that a majority of the mutated genes identified were additional targets of copy number loss, but unfortunately our dataset was incomplete due to sample limitation. Finally, it will be important to expand our functional studies to other genes and cell lines. The TT and MZ-CRC1 cell lines have served as a model for inheritance of MTC for several decades and clearly have acquired genetic changes that potentially limit their use. PC-12 is a rat pheochromacytoma-derived cell line and there is currently no human cell line available. Given that all the MTC alterations were observed as unique events, there remains a need to uncover the tumorigenesis pathways that differentially affect MTC and PCC.

In conclusion, we applied next-generation sequencing to evaluate multiple tumors from four MEN2A patients and explored their relevance in an independent cohort. We discovered 13 genes with either single-nucleotide variations or copy-number alterations, although none of them occurred as commonly as the RET mutation in either germline DNA or somatic tumor DNA. One of them, EIF4G1, played an oncogenic role in cell proliferation and phosphorylation of RET/ERK in TT, MZ-CRC1, and PC-12 cell lines. Perhaps the most intriguing finding is that we observed significantly fewer mutations in endocrine tumors versus non-endocrine tumors, and further failed to uncover a common tumorigenic pathway in MTC and PCC. Thus, while activated RET serves as a primary initiator of tumorigenesis, it does not appear to drive the process of mutagenesis. Instead, as results from our previous CGH studies indicate (Ye et al. 2008), genetic losses and haploinsufficiency may be the critical mediators of the process. To obtain support for this concept, future studies will need to more closely examine the roles of the non-coding area of the cancer genome, as well as epigenetic...
mechanisms and microenvironmental factors that may contribute to MEN2A tumorigenesis.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0225.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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