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

Jie Cai^{1,*}, Lin Li^{2,*}, Lei Ye^{1,*}, Xiaohua Jiang¹, Liyun Shen¹, Zhibo Gao², Weiyuan Fang¹, Fengjiao Huang¹, Tingwei Su¹, Yulin Zhou¹, Weiqing Wang¹ and Guang Ning^{1,3}

¹School of Medicine, Shanghai Clinical Center for Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiao Tong University, #197 Ruijin 2nd Road, Shanghai 200025 People's Republic of China

²BGI-Shenzhen, Shenzhen 518083, China

³Laboratory for Endocrine and Metabolic Diseases of Institute of Health Science, Shanghai Jiao Tong University School of Medicine and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, #227 South Chongqing Road, Shanghai 200025, People's Republic of China

*J Cai, L Li, and L Ye contributed equally to this work

Correspondence
should be addressed
to G Ning
Email
gning@sibs.ac.cn

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.

Key Words

- MEN2A
- *RET*
- *EIF4G1*
- exome sequencing

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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 tumorigenesis (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 cat. 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 to 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.C634W 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 (passages 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 QuikChange II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). siRNAs targeting human and rat *EIF4G1* and nonspecific negative controls were synthesized by GenePharma 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). In the validation set, an additional 69 patients were included (Supplementary Table 2, see section on supplementary data given at the end of this article).

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

	Patient 1	Patient 2	Patient 3	Patient 4
Gender	F	M	M	F
Age at surgery (MTC/PCC) year	49/50	33/33	56/27	35/35
<i>RET</i> mutation	p.C634S (TGC>TCC)	p.C634R (TGC>CGC)	p.C634Y (TGC-TAC)	p.C634Y (TGC-TAC)
Pathological findings	MTC/PCC	MTC/PCC	MTC/PCC/HPT	MTC/PCC
TNM (MTC)	T1N0M0	T1N0M0	T1N0M0	T2N1M0
Tumor diameter (MTC) (cm)	0.8	1.8	0.8	2.5
Ct (before/after ^a)	334.34/92.98 ^A	540.4/27.92 ^A	160/2 ^A	2636/13.1 ^B
CEA (before/after ^a)	53.84/49.36 ^C	15.87/14.86 ^D	2.17/1.87 ^D	NA
Tumor diameter (PCC) (cm)	3	5.5	10	3
MN (before/after ^a)	872.5/53.6	980.3/206.3	330.3/190.4	NA
NMN (before/after ^a)	450.8/38.2	639.4/102.4	17014/230.5	NA
Duration of follow-up (months)	78	42	51	34

F, female; M, male; MTC, medullary thyroid cancer; PCC, pheochromocytoma; HPT, hyperparathyroidism; TNM, Tumor–Node–Metastases staging score; Ct, calcitonin (^Areference range: 0.1–10 pg/ml; and ^Breference range: <300 pg/ml); MN, metanephrine (reference range: 14–90 pg/ml); NMN, normetanephrine (reference range: 19–121 pg/ml); CEA, carcinoembryonic antigen (^Creference range: <5 ng/ml; ^Dreference range: <10 ng/ml); NA, not available.

^aCt and CEA, before and 1 day after MTC surgery; MN and NMN, before and 1 day after PCC surgery.

Table 2 Annotation information for somatic mutations including *RET* in individual tumors of the discovery set

Sample	Gene	Transcript	Mut effect	cds_mutation	aa_mutation	Chr	Chrom_start	Chrom_end	Genome mutation	Sift prediction	PolyPhen2
M1	<i>CYB5D1</i>	NM_144607	Nonsynonymous SNV	c.612C>A	p.D204E	17	7762855	7762855	C>A	1	0.001
M1	<i>EPB42</i>	NM_000119	Nonsynonymous SNV	c.178C>T	p.R60C	15	43508574	43508574	G>A	0	0.999
M1	<i>MLL</i>	NM_001197104	Stopgain SNV	c.439C>T	p.Q147X	11	118339496	118339496	C>T	0	0.735246
M1	<i>ZDHH8</i>	NM_001185024	Nonsynonymous SNV	c.907C>G	p.L303V	22	20128752	20128752	C>G	0.04	0.031
M1	<i>RET</i>	NM_020630	Nonsynonymous SNV	c.1901G>C	p.C634S	10	43609949	43609949	G>C	0.02	0.999
M2	<i>MNAT1</i>	NM_001177963	Frameshift insertion	c.225_226insA	p.R75fs	14	61263070	61263070	->A	0.05	0.99
M2	<i>NDNF</i>	NM_024574	Nonsynonymous SNV	c.653A>T	p.E218V	4	121958473	121958473	T>A	0	0.99
M2	<i>RET</i>	NM_020630	Nonsynonymous SNV	c.1900T>C	p.C634R	10	43609948	43609948	T>C	0	0.99
M3	<i>RET</i>	NM_020630	Nonsynonymous SNV	c.1901G>A	p.C634Y	10	43609949	43609949	G>A	0	0.999
M4	<i>BEX5</i>	NM_001012978	Nonsynonymous SNV	c.131C>T	p.P44L	X	101409107	101409107	G>A	0.01	0.146
M4	<i>BHLHB9</i>	NM_001142530	Nonsynonymous SNV	c.523G>A	p.G1175R	X	102004446	102004446	G>A	0	0.987
M4	<i>EIF4G1</i>	NM_004953	Nonsynonymous SNV	c.3440A>T	p.E1147V	3	184046490	184046490	A>T	0	0.998
M4	<i>KRBA1</i>	NM_032534	Frameshift deletion	c.2665_2666del	p.R89_889del	7	149430709	149430710	GT>-	0	0.406
M4	<i>KRT38</i>	NM_006771	Nonsynonymous SNV	c.1060C>T	p.R354C	17	39594526	39594526	G>A	0	0.994
M4	<i>OR3A1</i>	NM_002550	Nonsynonymous SNV	c.725C>A	p.S242Y	17	3195152	3195152	G>T	0	0.994
M4	<i>TRIP12</i>	NM_004238	Frameshift insertion	c.5763_5764ins-AG	p.S1921fs	2	230633351	230633351	->CT	0.01	0.085
M4	<i>TROVE2</i>	NM_001042369	Nonsynonymous SNV	c.199G>T	p.D67Y	1	193038383	193038383	G>T	0	0.999
M4	<i>RET</i>	NM_020630	Nonsynonymous SNV	c.1901G>A	p.C634Y	10	43609949	43609949	G>A	0	0.999
P1	<i>A2M</i>	NM_000014	Nonsynonymous SNV	c.4334C>A	p.A1445D	12	9221368	9221368	G>T	0	0.999
P1	<i>ANO2</i>	NM_020373	Nonsynonymous SNV	c.2548T>C	p.F850L	12	5685073	5685073	A>G	0	0.962
P1	<i>APEX2</i>	NM_014481	Nonsynonymous SNV	c.910C>A	p.H304N	X	55033221	55033221	C>A	0	0.964
P1	<i>GRAP</i>	NM_006613	Nonsynonymous SNV	c.547G>A	p.D183N	17	18925379	18925379	C>T	0	1
P2	<i>CDC42BPG</i>	NM_017525	Nonsynonymous SNV	c.T695C	p.V232A	11	64606686	64606686	A>G	0	1
P2	<i>DYNCTH1</i>	NM_001376	Nonsynonymous SNV	c.12704C>T	p.P4235L	14	102510630	102510630	C>T	0	1
P2	<i>GPR172B</i>	NM_001104577	Nonsynonymous SNV	c.1070T>C	p.M357T	17	4936620	4936620	A>G	0	1
P2	<i>RET</i>	NM_020630	Nonsynonymous SNV	c.1900T>C	p.C634R	10	43609948	43609948	T>C	0	0.99
P3	<i>ATRX</i>	NM_138270	Stopgain SNV	c.675G>A	p.W225X	X	76939959	76939959	C>T	0	0.734879
P3	<i>IFITM1</i>	NM_003641	Nonsynonymous SNV	c.278G>T	p.G93V	11	315013	315013	G>T	0	0.934
P3	<i>PLCH2</i>	NM_014638	Nonframeshift insertion	c.2349_2350ins-GTGGGGGCC	p.E783delins-EVGA	1	2430086	2430086	->GTGG-GGGC	0.19	0.023
P3	<i>SHC3</i>	NM_016848	Nonsynonymous SNV	c.973C>G	p.L325V	9	91661899	91661899	G>C	0	0.999
P3	<i>RET</i>	NM_020630	Nonsynonymous SNV	c.1901G>A	p.C634Y	10	43609949	43609949	G>A	0.21	0.697
P4	<i>CLSTN3</i>	NM_014718	Nonsynonymous SNV	c.1405G>A	p.D469N	12	7293919	7293919	G>A	0.01	1
P4	<i>DNAH10</i>	NM_207437	Nonsynonymous SNV	c.10901C>T	p.A3634V	12	124403245	124403245	C>T	0	0.16
P4	<i>PAFAH2</i>	NM_000437	Nonsynonymous SNV	c.116C>A	p.P39H	1	26316067	26316067	G>T	0	0.19
P4	<i>WDR36</i>	NM_139281	Nonsynonymous SNV	c.1457A>C	p.K486T	5	110443101	110443101	A>C	0	0.987
P4	<i>ZNF415</i>	NM_001136038	Nonsynonymous SNV	c.781C>T	p.R261C	19	53612517	53612517	G>A	0	0.999
P4	<i>RET</i>	NM_020630	Nonsynonymous SNV	c.1901G>A	p.C634Y	10	43609949	43609949	G>A	0	0.730152
PT	<i>KCNAB2</i>	NM_001199863	Stopgain SNV	c.450C>A	p.Y150X	1	6155445	6155445	C>A	0	0.015
PT	<i>UBR4</i>	NM_020765	Nonsynonymous SNV	c.2449A>G	p.I817V	1	19504143	19504143	T>C	0.02	0.015

M, medullary thyroid carcinoma; P, pheochromocytoma; PT, parathyroid.

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 previously 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

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^a'). ** $P < 0.01$.

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: *CYB5D1*, *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

Figure 2

Copy number variation (CNV) of the loci harboring the somatic non-silent mutations revealed by exome sequencing in MEN2A components. SNV, yellow; INDEL, green; CNV loss, blue; CNV amplification, red. M, MTC;

P, PCC; PT, parathyroid; 1, 2, 3, 4 indicate patients 1, 2, 3, 4 respectively. Copy number variation of M1 and M3 was not analyzed because of sample limitation.

(Fig. 3). 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

Figure 3

Functional analysis of *EIF4G1* in TT and MZ-CRC1 cell lines. CON, control; WT, WT *EIF4G1*; Mut, mutant *EIF4G1* (p.E1147V); NC, negative control; siRNA, siRNA targeting *EIF4G1*. Experiment were conducted independently three times (** $P < 0.01$ and * $P < 0.05$). GAPDH is used as an internal control for TT and MZ-CRC1 cells, α -tubulin for PC-12 cells. (A) Cell proliferation assay with CCK8. Mutant *EIF4G1* (p. E1147V) increased cell proliferation 15% ($P < 0.01$), 8.6% ($P < 0.05$), and 10.4% ($P < 0.01$) compared with WT *EIF4G1* in TT, MZ-CRC1, and PC-12 cell lines respectively. (B) Activation status of the RET pathway. Results of western blotting analysis indicated that mutant *EIF4G1* (p.E1147V) increased phosphorylation of *RET* (905) and

ERK compared with WT in TT, MZ-CRC1, and PC-12 cell lines. Increased phosphorylation of AKT was observed in MZ-CRC1 and PC-12 cells. (C) Cell proliferation assay with CCK8. Knockdown of *EIF4G1* by siRNA decreased cell proliferation 15% ($P < 0.05$), 17% ($P < 0.01$), and 9.3% ($P < 0.01$) compared with negative control in TT, MZ-CRC1, and PC-12 cell lines respectively. (D) Inactivation status of the RET pathway. Results of western blotting analysis indicated that silencing *EIF4G1* decreased phosphorylation of *RET* (905), AKT, and ERK compared with negative controls in both TT and MZ-CRC1 cell lines.

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 *EGFR*, 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 (Ramírez-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 pheochromocytoma 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 pheochromocytoma-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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