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Preliminary whole-exome sequencing reveals mutations that imply common tumorigenicity pathways in multiple endocrine neoplasia type 1 patients

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Abstract

Background—Whole-exome sequencing studies have not established definitive somatic mutation patterns among patients with sporadic hyperparathyroidism (HPT). No sequencing has evaluated multiple endocrine neoplasia type 1 (MEN1)-related HPT. We sought to perform whole-exome sequencing in HPT patients to identify somatic mutations and associated biological pathways and tumorigenic networks.

Methods—Whole-exome sequencing was performed on blood and tissue from HPT patients (MEN1 and sporadic) and somatic single nucleotide variants (SNVs) were identified. Stop-gain and stop-loss SNVs were analyzed with Ingenuity Pathways Analysis (IPA). Loss of heterozygosity (LOH) was also assessed.

Results—Sequencing was performed on 4 MEN1 and 10 sporadic cases. Eighteen stop-gain/stop-loss SNV mutations were identified in 3 MEN1 patients. One complex network was identified on IPA: Cellular function and maintenance, tumor morphology, and cardiovascular disease (IPA score = 49). A nonsynonymous SNV of TP53 (lysine-to-glutamic acid change at codon 81) identified in a MEN1 patient was suggested to be a driver mutation (Cancer-specific High-throughput Annotation of Somatic Mutations; $P = .002$). All MEN1 and 3/10 sporadic specimens demonstrated LOH of chromosome 11.

Conclusion—Whole-exome sequencing revealed somatic mutations in MEN1 associated with a single tumorigenic network, whereas sporadic pathogenesis seemed to be more diverse. A somatic TP53 mutation was also identified. LOH of chromosome 11 was seen in all MEN1 and 3 of 10 sporadic patients.

Multiple endocrine neoplasia type 1 (MEN1) is a rare, autosomal-dominant disorder caused by alterations in the *MEN1* gene on chromosome 11.¹ Ninety percent of patients with

MEN1 initially present with hyperparathyroidism (HPT), affecting men and women equally (unlike sporadic HPT) and at a younger age of onset (compared with sporadic HPT).² Other commonly affected endocrine glands include the pancreas and pituitary; >20 different types of tumors have been described in MEN1 patients. Of particular concern is the development of pancreatic neuroendocrine tumors (PNET), some of which produce gastrin, insulin, or other secretory hormones; metastatic PNETs are the leading cause of disease-specific death in MEN1 patients.^{3,4} Identifying a cellular mechanism behind the multiple neoplasms occurring in MEN1 patients could eventually enable clinicians to predict and properly screen for the development of PNET at the time of HPT presentation has the important potential to prevent downstream morbidity and mortality.

A number of genes are known to be involved in neuroendocrine tumorigenesis, including *MEN1*, *RET*, *VHL*, *TSC1*, and *TSC2*,⁵ with mutations in *MEN1* remaining the most common form of genetic predisposition to neuroendocrine tumors. More than 1,300 mutations⁶ in the *MEN1* gene have been reported in families with MEN1 syndrome, yet the exact mechanisms by which these mutations cause the MEN1-related pathologies are not known.^{3,7,8} The heterozygous germline-inactivating mutation in the *MEN1* may be followed by loss of the normal copy of this gene or a somatic inactivating mutation (second hit), leading to complete loss of function of the encoded protein menin. However, genotype–phenotype analysis has not revealed a clear pattern of disease penetrance in MEN1 patients.^{9,10} Additionally, it is reported that 5–30% of MEN1 patients do not have an identifiable mutation by standard testing.^{7,11} Loss of heterozygosity (LOH) has been described in parathyroid tissue of sporadic HPT patients, particularly in chromosome 11 in association with *MEN1* gene alterations.^{8,12} No relationship has been found between MEN1-related HPT and tumor suppressor genes, such as TP53,¹³ although mutations of this gene have been associated with other neuroendocrine tumors.

In contrast, whole-exome sequencing of parathyroid tissue from sporadic HPT patients has not revealed clear mutation patterns. Somatic mutations in the *MEN1* gene have been described in 15–35% of non-MEN1 parathyroid adenomas and are implicated in the pathology of sporadic HPT.^{8,12,14} However, few other somatic variants have been found to be harbored with significant frequency in sporadic HPT adenomas.¹² Whole-exome sequencing has previously been used to identify somatic mutations in samples from sporadic HPT adenomas, parathyroid carcinomas, and other sites (ie, PNET), but not MEN1-related HPT.^{8,12,15} Because of the known germline mutation in MEN1 patients, we hypothesized that whole-exome sequencing on blood and tissue samples of HPT patients could aid in the following goals: (1) Identify acquired somatic mutations involved in functional pathways and tumorigenic networks, and (2) elucidate additional germline risk factors associated with outcomes.

Methods

A multidisciplinary, collaborative team was formed between members of the Departments of Surgical Oncology and Epidemiology at our institution. Study phases and mutually agreeable goals of research were established, which included development of a research protocol for this pilot study and subsequent phases to include larger sample size pending

preliminary analysis. The University of Texas MD Anderson Cancer Center Institutional Review Board approved the research protocol.

Patient identification and clinical data collection

We identified patients with HPT who underwent parathyroidectomy at our institution and consented to prospective collection of clinical data, blood, and parathyroid tissue for research purposes. MEN1 status was determined by genetic testing, which was performed on all patients included in this study. Clinical data were reviewed to ensure no sporadic patients met criteria for a clinical diagnosis of MEN1. Patients were matched for age at HPT diagnosis (± 3 years). Age of diagnosis of HPT was determined by onset of renal colic or biochemical documentation of hypercalcemia (whichever occurred first). Clinical data abstracted from the medical records included demographic data (age, gender, race/ethnicity), medical history, family history, disease status, treatment response, survival information, and biochemical studies. Descriptive statistics were calculated using Microsoft Excel (Microsoft Corp, 2010).

Specimen processing and DNA sequencing

Specimens collected for the parathyroid tissue bank consisted of residual fresh-frozen parathyroid tissue and pre-parathyroidectomy blood samples collected at the time of operation. A 5-mg amount of residual parathyroid tumor tissue was used for this protocol and 2 million leukocytes for germline DNA. The samples were de-identified and laboratory staff blinded to the MEN1 status of the patients. DNA was extracted using standard methods and whole-exome sequencing was then performed. The exome capture was performed using the SeqCap EZ Exome Library v3.0. The size of the capture region was 64 MB. Sequencing was performed using the Illumina Hi-seq paired-end reads of 76 nucleotides, resulting in a mean ontarget depth of 100 reads for samples from both parathyroid tissue and blood.

Genomic analyses

Sequenced reads were initially aligned to the human genome reference hg19 using BWA.¹⁶ Aligned reads were subsequently realigned locally using the Genome Analysis Toolkit¹⁷ to improve mapping quality. Somatic mutations were defined as single nucleotide variants (SNVs) seen in the parathyroid tissue which are not seen in the germline DNA extracted from blood, and were identified using the MuTect method by the Broad Institute.¹⁸ Using RefSeq gene transcripts, ANNOVAR¹⁹ was run to annotate mutations to genomic regions. All mutations were then annotated with dbNSFP 2.0²⁰ (an annotation database for nonsynonymous SNVs) and COSMIC²¹ (Catalogue of Somatic Mutations in Cancer) using VariantTools²² to track amino acid substitutions and functional predictions.

Nonsynonymous mutations result in nucleotide changes and may result in functional alteration of the encoded protein, in contrast with synonymous mutations, which do not result in nucleotide change or functional alteration. Therefore, driver statuses of all nonsynonymous mutations were assessed using the Cancer-specific High-throughput Annotation of Somatic Mutations, which prioritizes those mutations most likely to generate functional changes that enhance cell proliferation.²³ Only genes with stop-gain (gain of stop codon and resultant early truncation of encoded protein) or stop-loss (loss of stop codon and

resultant elongation of protein) mutations were assessed via Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com),²⁴ in order to maximize the benefit of the IPA. This software employs an algorithm on the gene list to identify aberrant biological functions and pathways according to its curated Knowledge Base and calculates a score using the right-tailed Fisher's exact test.

LOH

We also conducted an analysis of copy number variation leading to allelic imbalance (eg, LOH, amplification) in the tumor samples using an adaptation of hapLOH²⁵ for sequencing data (FA San Lucas, unpublished; available from www.scheet.org/software). Unlike the whole-exome sequencing somatic mutation analyses, the LOH analyses do not require paired samples.

Results

Paired blood and tissue specimens from 14 patients with HPT (4 MEN1, 10 sporadic) were analyzed. Most patients were female (71%; $n = 10$). The mean age at HPT diagnosis was 26 years (range, 13–45). All patients remained alive at last follow-up (mean, 16 months; range, 1–42). Clinical characteristics of the MEN1 patients are summarized in Table I.

Whole-exome sequencing performed on 4 MEN1 and 10 sporadic cases demonstrated an average of 83 and 78 SNV mutations, respectively. Table II summarizes the number of SNV somatic mutations we observed in 4 MEN1 and 10 sporadic cases. The majority of mutations seen were nonfunctional SNVs; only 5% of SNVs in each group were determined to result in either stop-gain or stop-loss mutations. In the MEN1 cases, 18 stop-gain SNVs were identified in 3 patients; 1 MEN1 patient had neither stop-gain nor stop-loss SNVs (Table III). Based on IPA analysis, genes with stop-gain SNVs are associated with the network of “Cellular function and maintenance, tumor morphology, and cardiovascular disease” (IPA score 49), and centered around ubiquitin C (Fig 1).

Additionally, a somatic driver mutation on the *TP53* gene, causing a lysine (K) to glutamic acid (E) change at codon 81 ($P = .002$), was identified in a MEN1 patient. This patient did not have any additional germline or stop-gain or stop-loss somatic mutations detected via whole-exome sequencing. A summary of germline SNVs identified in the blood DNAs is listed in Supplementary Table I and demonstrates that the distribution of mutations between groups was similar.

In the sporadic group, on average, we observed 78 SNVs in 10 cases (Table II). We found 38 stop-gain and 3 stop-loss somatic SNVs among sporadic cases. Several functional pathways were suggested to have significance in the IPA analysis performed on the 41 genes with somatic stop-gain or stop-loss SNVs. These include: (1) lipid metabolism, small molecule biochemistry, and cell cycle (IPA score 36); (2) cell death and survival, cancer, cellular function and maintenance (IPA score 22); (3) DNA replication, recombination, and repair, energy production, and nucleic acid metabolism (IPA score 22); and (4) cellular assembly and organization, DNA replication, recombination, and repair, gene expression (IPA score 3). Germline variants in *MEN1* were seen in 9 of 10 samples including 1 stop-gain, 5

nonsynonymous, and 1 frameshift deletion. One sporadic sample had a somatic frameshift deletion in the gene. These were confirmed by visual inspection of the sequencing reads, because MuTect does not detect deletions.

For the LOH analysis, we included 2 additional MEN1 parathyroid specimens for which there were no paired germline samples (blood). Thirteen tissue samples (6 of 6 MEN1 and 7 of 10 sporadic) had 1 chromosomal allelic imbalance event. The MEN1 specimens each exhibited multiple chromosomal events. Figure 2 illustrates a greater degree of chromosomal allelic imbalance across the MEN1 tumor genomes compared with the sporadic tumors. Of note, there was consistent LOH of chromosome 11 (where the MEN1 gene resides) across all 6 of the MEN1 patient tumor samples. Three of 10 sporadic tumors also exhibited LOH of chromosome 11, including the patient with a somatic SNV of the *MEN1* gene. Additionally, 3 of the 10 sporadic patients exhibited no chromosomal LOH events.

Discussion

We performed whole-exome sequencing in samples of tissue and blood of patients with sporadic and MEN1-related HPT. This represents the first reported whole-genome or whole-exome sequencing performed on MEN1-related HPT specimens. In the MEN1 patients, we identified 18 somatic stop-gain mutations, which may result in early truncation of the encoded proteins. Of these, a mutation in the *RBBP6* tumor suppressor gene has previously been reported in a WES study of sporadic HPT adenomas.¹²

Using IPA, the mutations were determined to associate in a single biological network of “Cellular function and maintenance, tumor morphology, and cardiovascular disease.” The IPA score of 49 suggests a prominent association between the involved cellular pathways, a lesser probability that they would be interrelated by chance alone. These findings could be consistent with clinical observation that PNETs and cardiovascular disease commonly occur in MEN1 patients; it has recently been reported that the leading non-MEN1 causes of death is cardiovascular disease, whereas disease-specific death is owing to PNET.³ These cellular networks, as well as the contributions of epigenetic modifications, may be important for cancer susceptibility, drug metabolism, and immune response. Further understanding the microenvironment is critical for targeted care.

Despite identifying *MEN1* gene variants in the sporadic HPT group, only 1 patient was found to have a somatic frameshift deletion in this gene. It has been widely reported that 35% of sporadic HPT cases may be associated with acquired mutations in the *MEN1* gene. Given the sample size, seeing mutations in only 1 of our 10 patients could reasonably be expected. It is also possible that multiclonality within adenomas was missed owing to sampling bias, because only 1 sample per gland per patient is collected under our prospective tissue bank protocol.

The stop-gain and stop-loss mutations identified in the patients with sporadic HPT patients differed from those seen in MEN1-related HPT. Three genes in our sporadic HPT cohort (*EIF4G1*, *PCNT*, and *TP53BP1*) had SNVs that were not annotated as stop-gain or stop-loss; different mutations in these genes were reported in the previous whole-exome sequencing

studies.^{8,12} Additionally, the IPA revealed 4 functional pathways associated with these mutations, suggesting that pathogenesis includes more diverse biological pathways in sporadic HPT. These findings are consistent with recent reports that few somatic variants are seen in sporadic parathyroid adenomas.¹²

We also identified a somatic *TP53* mutation that could be a driver mutation. Although the *TP53* mutation is a finding in a single patient, it did not have other truncating mutations. In addition, *TP53* is an important tumor suppressor gene that has been implicated in other malignancies. Evaluating additional specimens of parathyroid or other MEN1-related neoplasms will be essential to determine the significance of this finding in the pathogenesis of MEN1. Being able to predict PNET development at the earliest manifestation of disease---or even preclinically using genetic testing as a diagnostic tool---has the potential of earlier diagnosis and treatment. Clinical application of whole-exome sequencing information could personalize the frequency of screening modalities for patients based on risk.

The consistent LOH of chromosome 11 seen across all MEN1 patients is another important finding, because we know they already had a germline mutation in 1 copy of their *MEN1* gene. The MEN1 patients also demonstrated a higher degree of chromosomal allelic imbalance across the genome compared with the sporadic patients. Consistent with the literature, 30% of our sporadic adenomas also demonstrated LOH of chromosome 11.^{8,12}

We acknowledge several limitations of our study, including the small sample size. This preliminary investigation was carried out to determine the feasibility of using whole-exome sequencing to identify somatic mutations and associated potentially relevant biological pathways. Given the limited patient samples available, it was important to establish preliminary data before pursuing a larger, multiinstitutional investigation. Nonetheless, our sample size of 10 sporadic and 4 MEN1 patients is comparable with the prior 2 whole-exome sequencing studies performed in sporadic HPT of 8 and 16 (with respective validation sets of 185 and 24).^{8,12}

Another potential limitation is the lack of a control group consisting of normal parathyroid specimens. There is no previous report in the literature of sequencing of normal parathyroid tissue. As in prior whole-exome sequencing studies,^{8,12} we focused on identifying acquired mutations in tumor samples through comparison to germline DNA from blood samples, and then contrasting acquired mutations in samples from sporadic and MEN1-related HPT patients to elucidate unique tumorigenic networks. Additionally, our protocol for the prospective collection of tissue samples or blood for research does not include the routine collection of tissues (including normal parathyroid) from patients without disease.

Last, our prospective parathyroid tissue bank protocol calls for a sample from a single gland from each patient to be submitted for future research. In the setting of multiglandular disease, such as in MEN1 patients, a more extended approach to collect tissue from multiple glands (as well as from other affected sites, importantly including pancreas) could be helpful to expand the availability of tissue. More important, this would enable us to analyze whether hyperplastic glands from the same patient exhibit different somatic mutations. Evaluation for multiclonal cells would also be made possible if multiple samples were taken from each

gland, although potentially difficult to obtain when the parathyroid is only slightly enlarged. This would make the bank a more valuable resource in the study of patients with multiglandular disease and is under consideration.

This pilot study illustrates the importance of interpreting genomic data and the susceptibility of germline and somatic data as a framework for comprehensive cancer patient genomics. Centralized analytic extraction of these variables will be an important addition to the traditional prognostic factors needed to design individual treatment choices. Although beyond the scope of the current investigation, the ability to predict risk, pattern of disease development, response to treatment, treatment-related toxicity, and quality of life are important goals of personalization of care through comprehensive biologic specimen analysis. Genetic heterogeneity is likely an important determinant of variation in outcomes. Although germline sequence variants can influence tumorigenic pathways, epigenetic mechanisms may also be important in patients with both MEN1 and sporadic HPT. This is suggested by the absence of functional SNVs in 1 of 4 MEN1 patients investigated in this series, and the relatively low frequency of mutations identified in sporadic HPT patients in this and other investigations. We hope that this study represents a first step toward building an engine that allows a systemic approach to leveraging the interpretation of genomic variation in patients with MEN1-associated and sporadic HPT.

In conclusion, we performed the first whole-exome sequencing of parathyroid tissue and blood from patients with MEN1-related HPT. Our work suggests that mutation patterns among MEN1 patients may be distinct from patients with sporadic HPT. We identified somatic SNV mutations that may result in early protein truncation and associated them with a single tumorigenicity network centered around ubiquitin C. In addition, the *TP53* somatic mutation may be important in development of MEN1-related HPT. The role of LOH of chromosome 11, which harbors the *MEN1* gene, was seen in both MEN1-related and sporadic disease. Further research using additional technologies, such as cDNA array, a larger cohort, and other tissues---such as neuroendocrine pancreas tumor specimens---may aid better understand the impact of acquired mutations and the role of aberrant pathways in development of MEN1-related neoplasms. Larger scale molecular epidemiologic studies that integrate susceptibility, exposure profiles, and genetic and epigenetic markers are needed. In the future, we additionally wish to employ cDNA array to evaluate the significance of gene expression alterations in the manifestation of HPT and other endocrine neoplasias seen in patients with MEN1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Network identified on Ingenuity Pathways Analysis (IPA) analysis for stop-gain single nucleotide variants (SNVs) detected in 3 patients with multiple endocrine neoplasia type 1 (MEN1)-related hyperparathyroidism (HPT). The network centers on the protein ubiquitin C.

Fig 2.

Allelic imbalance events across the genome of multiple endocrine neoplasia type 1 (MEN1) and sporadic hyperparathyroidism (HPT) patients (*black bars*). MEN1 samples are labeled *A* and sporadic samples are labeled *C*. Loss of heterozygosity (LOH) of chromosome 11 is seen in all 6 MEN1 samples and 3 of 10 sporadic adenomas. Samples C4, C8, and C10 (not shown) exhibited no chromosome LOH events.

Table I
Characteristics of 4 patients with multiple endocrine neoplasia type 1 (MEN1) evaluated for hyperparathyroidism

Characteristic	Value
Total MEN1 patients	4
MEN1-related neoplasms	
Hyperparathyroidism	4
Pancreatic neuroendocrine tumors	2
Pituitary adenoma	1
Family history	
Hyperparathyroidism	3
Pancreatic neuroendocrine tumors	3
Pituitary adenoma	2
Zollinger-Ellison syndrome	1
MEN1	3

Table II
Number of somatic single nucleotide variant (SNV) mutations observed in 4 multiple endocrine neoplasia type 1 (MEN1) and 10 sporadic patients

Variants in samples	MEN1										Sporadic									
	1	2	3	4	1	2	3	4	5	6	7	8	9	10						
	1	2	3	4	1	2	3	4	5	6	7	8	9	10						
Nonsynonymous SNV	105	23	90	20	116	43	62	34	31	52	37	42	34	33						
Stop-gain SNV	8	3	7	0	14	2	1	7	1	4	2	4	2	1						
Stop-loss SNV	0	0	0	0	2	0	0	0	0	0	0	0	0	1						
Synonymous SNV	16	19	29	19	32	37	58	19	33	40	17	20	9	13						
Total	129	45	126	39	164	82	121	60	65	96	56	66	45	48						

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Table III
Somatic nonsynonymous mutations resulting in stop-gain or stop-loss of function in DNA extracted from parathyroid tissue of patients with multiple endocrine neoplasia type 1 (MEN1)-related hyperparathyroidism

Patient	Specific mutations									
1	RALGPS2	TROVE2	TRAT1	ELOVL7	ASCC3	CNBD1	RCBTB1	PRKAA2		
2	MYO10	ARHGAP20	ANKFN1							
3	APOB	DLEC1	IL5	RIOK5	RBBP6	POLD1	MX2			
4*	—									

* Patient had no stop-gain or stop-loss mutations, but did have a *TP53* somatic mutation.