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Identification of Somatic Mutations in Parathyroid Tumors Using Whole-Exome Sequencing

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Context: The underlying molecular alterations causing sporadic parathyroid adenomas that drive primary hyperparathyroidism have not been thoroughly defined.

Objective: The aim of the study was to investigate the occurrence of somatic mutations driving tumor formation and progression in sporadic parathyroid adenoma using whole-exome sequencing.

Design: Eight matched tumor-constitutional DNA pairs from patients with sporadic parathyroid adenomas underwent whole-exome capture and high-throughput sequencing. Selected genes were analyzed for mutations in an additional 185 parathyroid adenomas.

Results: Four of eight tumors displayed a frame shift deletion or nonsense mutation in *MEN1*, which was accompanied by loss of heterozygosity of the remaining wild-type allele. No other mutated genes were shared among the eight tumors. One tumor harbored a Y641N mutation of the histone methyltransferase *EZH2* gene, previously linked to myeloid and lymphoid malignancy formation. Targeted sequencing in the additional 185 parathyroid adenomas revealed a high rate of *MEN1* mutations (35%). Furthermore, this targeted sequencing identified an additional parathyroid adenoma that contained the identical, somatic *EZH2* mutation that was found by exome sequencing.

Conclusion: This study confirms the frequent role of the loss of heterozygosity of chromosome 11 and *MEN1* gene alterations in sporadic parathyroid adenomas and implicates a previously unassociated methyltransferase gene, *EZH2*, in endocrine tumorigenesis. **(*J Clin Endocrinol Metab* 97: E1774–E1781, 2012)**

Parathyroid tumors causing primary hyperparathyroidism (pHPT) are common, occurring in 2.1% of postmenopausal women (1). Sporadic (nonfamilial) pHPT occurs predominantly due to a single hyperfunctioning parathyroid adenoma (85%) but can be due to multiglandular (including four gland hyperplasia) disease in 10–15% of cases (2); rarely it is due to carcinoma (<1%) (3).

Allelic loss [loss of heterozygosity (LOH)] of chromosomal loci may identify tumor suppressor genes in neoplasia. LOH at the multiple endocrine neoplasia type 1 (*MEN1*) locus on chromosome band 11q13 has been demonstrated in approximately 25–40% of sporadic parathyroid adenomas. Somatic mutations of the *MEN1* gene are found in 12–21% of adenomas, or about 50% of those tumors with LOH at 11q13 (4). These findings indicate that mutational aberrations in *MEN1* contribute to parathyroid tumorigenesis but also raise the possibility that 11q13 may harbor an additional parathyroid tumor suppressor gene. Apart from the loss of the *MEN1* locus, comprehensive LOH and comparative genomic hybrid-

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Abbreviations: CTNNB1, Complex with Wnt that negatively regulates β-catenin; EZH2, enhancer of zeste homolog 2; LOH, loss of heterozygosity; LRP5, lipoprotein receptor-related protein 5; MEN1, multiple endocrine neoplasia type 1; pHPT, primary hyperparathyroidism; SET, Su(var)3–9, Enhancer of Zeste, Trithorax.

ization studies of parathyroid adenomas have identified locations for several other candidate tumor suppressor genes such as 1p, 1q, 6q, 9p, 11p, and 15q (5). Although inactivating somatic mutations of *HRPT2/CDC73* have been identified in a subset of mainly malignant parathyroid carcinomas, they are not commonly altered in benign parathyroid adenomas (6). Likewise, germline mutations in *CASR* have been associated with familial forms of pHPT, but the gene has not been found to be somatically mutated in sporadic forms of the disease (7). Furthermore, loss of the well-characterized tumor suppressor genes *p53* and *RB* do not appear to contribute to the development of parathyroid adenomas (8, 9). To date, no gene other than *MEN1* has been proven by somatic mutation to be frequently altered in parathyroid adenomas, leaving the majority of sporadic instances of the disease unexplained.

Apart from alterations in *MEN1*, genetic abnormalities in other genes appear to occur very rarely. For this reason, the contributions of epigenetic changes to tumorigenesis have been examined previously. Prior studies have shown that epigenetic modifications, like methylation of the *MEG3* locus, often occur in conjunction with LOH across the locus to result in loss of expression (10). Similarly, DNA methylation of a number of other genes has been demonstrated in parathyroid adenomas (11, 12). Although the approach of this study will be unable to detect such epigenetic drivers of tumorigenesis, many microRNA are included in the exome capture array and are analyzed very carefully for mutations. This is important since mutations and expression differences in microRNA have been speculated to play a role in cancers. In fact, differential microRNA expression profiles have been reported to distinguish parathyroid adenomas from parathyroid carcinomas (13). Despite sometimes high frequencies of occurrence, the direct effects of epigenetic modifications on disease are often very difficult to determine without extensive functional studies.

To identify genetic events that may be contributing to parathyroid tumorigenesis, we applied a similar approach to that used previously to identify somatic mutations common in sporadic adrenal aldosterone-producing adenomas via exome sequencing (14). Our study provides, to our knowledge, the first comprehensive evaluation of genetic alterations in parathyroid tumors via whole-exome capture and high-throughput sequencing. The study supports the role of somatic mutations in parathyroid tumorigenesis of the *MEN1* and *EZH2* genes, both critical for proper histone methyltransferase activity.

Materials and Methods

Cases and samples

Eight cases with sporadic pHPT were included in the study using whole-exome sequencing. Inclusion criteria were inappropriate elevation of PTH in relation to serum calcium, normal serum creatinine levels, and no history of familial hyperparathyroidism or exposure to calcimimetic therapy (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). All parathyroid tumors were carefully evaluated and dissected by an experienced endocrine pathologist before use in the study. Preliminary Sanger sequencing determined that all eight cases were free of germline variants in *MEN1*, thereby confirming the sporadic nature of the cases. Tissues from an additional 185 parathyroid adenomas were obtained via the Yale Pathology Tissue Services (Supplemental Table 2). Tissues were snap frozen using liquid nitrogen and stored at −80 C until processing. Written informed consent from patients and approval by the local institutional review board were obtained.

Sample preparation

High-molecular-weight genomic DNA was isolated from matched tumor and constitutional (blood) samples using standard protocols included in the commercially available QIAGEN DNeasy blood and tissue kit (QIAGEN, Venlo, The Netherlands). All specimens were quality control checked for purity using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA).

Whole-exome capture

Constitutional and tumor DNA was captured on a NimbleGen Sequence Capture 2.1M human exome array (NimbleGen, Madison, WI) following the manufacturer's protocols with modifications at the W. M. Keck Facility at Yale University. As previously described, (15), DNA was sheared and adaptors were ligated onto the resulting fragments. Fragments were amplified by ligation-mediated PCR, purified, and hybridized to the array. After washing, fragments were eluted and purified. Resulting fragments were then subjected to high-throughput sequencing.

High-throughput sequencing

Captured libraries were sequenced on the Illumina GA II sequencing system (Illumina, Inc., San Diego, CA) as 74-bp single-end reads and 74- or 99-bp paired-end reads following the manufacturer's protocols. Image analysis and base calling was performed by Illumina pipeline versions 1.3 and 1.4 with default parameters. The summary sequencing statistics for all eight discovery cohort tumor-constitutional pairs are included in Supplemental Table 3.

Analysis

Analysis of raw data from the Illumina sequencing was performed as previously (15). Sequence reads were mapped to the reference genome (hg18) using the Maq program (16). Reads outside the targeted sequences were discarded. Statistics on coverage were collected from the remaining reads using in-house perl scripts. For insertion/deletion detection, the Burrows-Wheeler Aligner was used to allow gapped alignment to the reference genome (17). SAMtools was used to call targeted bases with any base-call deviating from the reference sequence regarded as a potential variant (18). Variants given a SAMtools single-nucleotide polymorphism quality score of less than 100 were assumed to be false positives and thus excluded from further analysis. Identified variants were annotated based on novelty, impact on the encoded protein, and conservation using an

TABLE 1. Somatic mutations identified by exome sequencing included in validation cohort screening

Case	Chr	Position	Base change	LOH?	Gene	Effect on protein	AA position/length
PTH106	11	64331968	G>A	Y	*MEN1*	Q214X	214/615
	12	67328775	C>T	N	*RAP1B*	R2C	2/184
PTH107	11	64334027	CG	Y	*MEN1*	c.129_130delCG	44/615
	11	67937858	C>T	Y	*LRP5*	R877W	877/1615
PTH108	11	64332147	C	Y	*MEN1*	c.460delG	154/615
PTH113	11	64333905	AGAC	Y	*MEN1*	c.249_252delGTCT	85/615
PTH122	7	148139661	A>T	N	*EZH2*	Y641N	641/707

Chr, Chromosome; Y, yes; N, no; AA, amino acid.

automated pipeline. Somatic mutations were defined as those that were identified in tumor DNA but absent from constitutional DNA.

Mutation validation

Primer3 (http://frodo.wi.mit.edu/primer3/) was used to generate primers for PCR amplification of variants identified via exome sequencing or exons covered in additional screening. Amplification products of appropriate size were identified using agarose gel electrophoresis. Amplicons from constitutional and tumor DNA were sequenced using forward and reverse primers. Variants were confirmed by at least two independent sequences from different primers (Supplemental Tables 4 and 5).

Quantification of contamination using quantitative PCR

Surgically resected tumor tissue often contains constitutional cells surrounding the tumor *in vivo*. Although unavoidable, it is possible to estimate the level of contamination, which can facilitate correction for such during data analysis. Commercially available probes for the TaqMan custom single-nucleotide polymorphism genotyping assay (Applied Biosystems, Inc., Foster City, CA) were designed using the Applied Biosystems web site. Quantitative PCR using probes overlapping a nonsense *MEN1* mutation identified in one of the tumors differentiated between mutant and wild-type alleles. Fluorophores FAM and VIC corresponded to amplification of the somatically-mutated tumor allele and the wild-type allele, respectively. This estimated the presence of tumor DNA in the constitutional sample to be negligible but constitutional DNA in the tumor sample to be 41.3 ± 1.0%. Sanger sequence traces for all heterozygous variants displayed a more pronounced wild-type peak compared with that of the mutant allele, which is consistent with the estimated level of contamination.

Results

Discovery cohort screening

Eight patients with sporadic parathyroid adenomas, in which no germline *MEN1* mutations were present, were included in the discovery cohort for high-throughput analysis. Whole-exome capture and subsequent sequencing was performed on tumor and constitutional DNA samples from each patient as previously described (15). Once raw sequencing data were mapped to the reference human genome (hg18) and variants identified, tumor and constitutional sequences from each patient were cross-referenced to identify somatic mutations unique to each tumor genome.

In the eight tumors, whole-exome sequencing revealed a total of 440 high-quality, somatic, nonsynonymous variants in exons or splice sites (Supplemental Table 6). Variants were cross-referenced with more than 600 control exome sequences. This allowed the list to be narrowed down to 286 variants and then further to 251 variants once amino acid conservation of the mutated residue across 46 orthologs was accounted for. The remaining 251 variants were then examined using computational metrics and manual analysis of read alignments to reveal those mutations with a high likelihood of being false positives. PCR amplification and subsequent Sanger sequencing was performed on the remaining 94 variants, which confirmed 29 somatic mutations. This high false-positive rate can be attributed to our prioritization of sensitivity over specificity, which was done to maximize recovery of somatic, exonic variants.

Four of the 29 mutations were found in *MEN1* in four separate tumors (one nonsense mutation and three frame shift deletions; Table 1 and Supplemental Table 7). All of these mutations encode a truncated menin protein. Additionally, LOH spanning all of chromosome 11 was observed in all tumors with *MEN1* mutations (locus 11q13). Regions were determined to be in LOH if they displayed

TABLE 2. Patterns of LOH in discovery cohort

Case	Region of LOH	Somatic *MEN1* mutation
PTH105	chr1[a]; chr13	—
PTH106	**chr11**; chr21; chr22	Q214X
PTH107	**chr11**	c.129_130delCG
PTH108	chr9; **chr11**	c.460delG
PTH113	**chr11**	c.249_252delGTCT
PTH120	—	—
PTH122	—	—
PTH125	—	—

Common regions of LOH are denoted in *bold*. *Dashes* indicate absence of either LOH or *MEN1* mutation. Chr, Chromosome.

[a] LOH occurred only across 0–103 Mb on the short arm of chromsome 1.

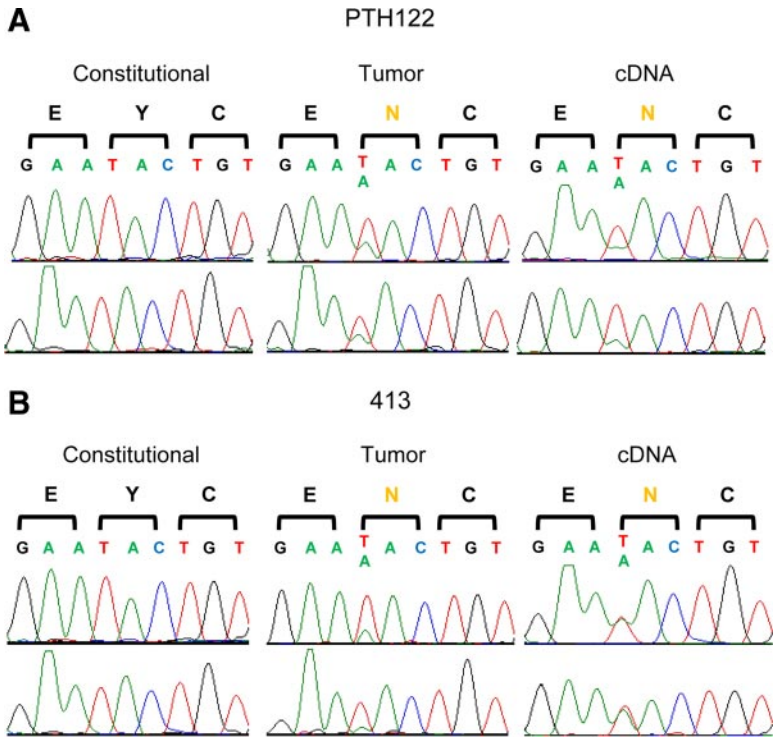


FIG. 1. Somatic mutations in *EZH2*. A, Sequences of constitutional genomic DNA, tumor genomic DNA, and tumor cDNA of *EZH2* codons 640–642 in PTH122 of the discovery cohort. B, Sequences of constitutional genomic DNA, tumor genomic DNA, and tumor cDNA of *EZH2* codons 640–642 in sample 413 from the large-scale *EZH2* SET domain screen. Sequences from the forward direction are shown on the *top row* and the reverse direction are *shown below*.

differential minor allele frequencies between tumor and constitutional DNA (Supplemental Fig. 1). LOH was not limited to chromosome 11, with single instances of chromosomal loss spanning all of chromosomes 9, 13, 21, 22, and a portion of the short arm of chromosome 1 (Table 2 and Supplemental Fig. 1). Based on read coverage, all instances of LOH were determined to be copy neutral.

Although multiple tumors harbored *MEN1* mutations, no recurrent alterations were identified in the discovery cohort. Despite being present in only single instances, several of the confirmed mutations had been previously associated with other types of tumors in the literature. For instance, one of the exome sequenced tumors harbored a heterozygous missense mutation in enhancer of zeste homolog 2 (*EZH2*) gene at a highly conserved residue (Y641N; Fig. 1A). This exact heterozygous mutation in *EZH2* had been identified as common to B cell lymphomas (19).

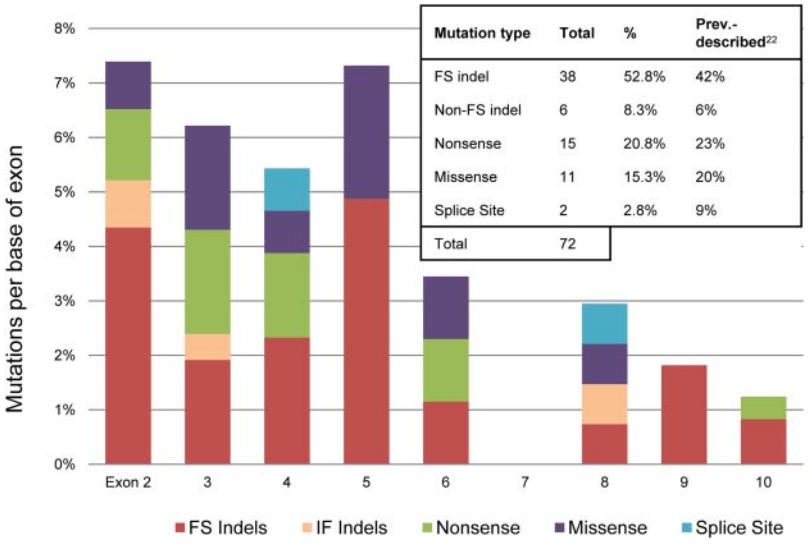
A prior study found expression of an aberrantly spliced, internally deleted low-density lipoprotein receptor-related protein 5 (*LRP5*) mRNA and protein in the majority of sporadic parathyroid adenomas (20). *LRP5* forms a complex with *Wnt* that negatively regulates β-catenin (*CTNNB1*). Although stabilizing mutations in *CTNNB1* have been found in a subset of pHPT patients that possibly account for its reported overexpression, a separate study only identified one such mutation out of 180 tumors from this study's validation cohort (21). Although rare, these stabilizing mutations in *CTNNB1* and loss-of-function mutations in *LRP5*, a negative regulator of *CTNNB1*, may result in similar effects, which could account for the apparent mutual exclusivity of these defects. These prior reports drew attention to a constitutional mutation in *LRP5* (R877W) found in this study that resided at a highly conserved residue within the aforementioned, internally deleted region. This variant was heterozygous in the germline, but the wild-type allele was lost in the tumor due to LOH across chromosome 11. This tumor also displayed a frame shift deletion in *MEN1*, which may alone be sufficient for tumorigenesis. Due to its co-occurrence with a *MEN1* mutation and the fact that LOH across chromosome 11 is frequently observed in these tumors, it is difficult to evaluate the possible role of this *LRP5* variant in tumorigenesis without analyzing additional tumors for mutations in the gene.

Another gene associated with neoplasia that was somatically mutated in one tumor was *RAP1B*. A member of the *Ras* oncogene family, *RAP1B* antagonizes *Ras* mitogenic signals by forming nonproductive complexes with effectors of *Ras*. A mutation in *RAP1B* had previously been associated with myelodysplastic syndromes (22). The identified variant in our cohort was a heterozygous missense mutation (R2C) in a tumor that also harbored a nonsense *MEN1* mutation.

Validation cohort screening

In a cohort of only eight patients, the true frequency of rare mutations is difficult to determine. Therefore, tumor and constitutional DNA from 185 additional sporadic parathyroid adenomas was extracted and screened for the most promising mutations identified via exome sequencing. The best candidates for further sequencing were those that were likely damaging to protein function, at highly conserved residues in highly conserved genes, mutations absent from control exomes, and those previously associated in the literature with tumorigenesis or hormone secretion.

Because *MEN1* mutations are frequently present in parathyroid adenomas and may fully account for tu-



Mutation type	Total	%	Prev.-described[22]
FS indel	38	52.8%	42%
Non-FS indel	6	8.3%	6%
Nonsense	15	20.8%	23%
Missense	11	15.3%	20%
Splice Site	2	2.8%	9%
Total	72		

FIG. 2. Results of the *MEN1* mutation screen. Mutation types per base of exon are displayed across exons 2–10 for all 193 tumors. The table displays the total mutational breakdown across all exons compared with the findings from previous studies. FS indels and IF indels represent frame shift and in-frame insertions or deletions, respectively.

morigenesis, the first screen covered all coding exons (2–10) of *MEN1* in all 185 tumors. This screen brought the final tally to 72 somatic mutations identified in 68 of the total 193 tumors screened (35.2%) (Supplemental Table 8). This total includes four tumors that each displayed two separate mutations in the *MEN1* gene. Most of these variants were undoubtedly damaging to protein function, with frame shift insertions/deletions and nonsense mutations accounting for 74% of *MEN1* variants. The mutational breakdown is depicted in Fig. 2 (23). The mutation frequency in this cohort of 35.2% was much higher than the previously reported 12–21% (24, 25). It has been suggested that additional tumor suppressors may lie on 11q13 due to the observation that LOH at this locus has been observed more frequently than *MEN1* mutations (25–40% compared with 12–21%, respectively). However, the higher *MEN1* mutation rate observed in this study (and co-occurrence of *MEN1* mutations with every instance of 11q13 LOH in the discovery cohort) suggests that *MEN1* mutations and LOH may reliably co-occur, which indicates against the possibility of an additional tumor suppressor at this locus.

We next evaluated the SET [Su(var)3–9, Enhancer of Zeste, Trithorax] domain of *EZH2* that was frequently mutated in myeloid and lymphoid malignancies (19, 26, 27). Screening of this domain covered four exons (16–19) across all 185 tumors. No mutations were identified in any of the tumors harboring *MEN1* defects. However, one of the tumors with wild-type *MEN1* displayed the exact same heterozygous mutation (Y641N) as previously identified in the discovery cohort (Fig. 1B). Somatic mutation at the same residue in even two separate tumors is very unlikely to occur by chance, which makes our identification of the somatic Y641N mutation in *EZH2* highly significant ($P = 4.13 \times 10^{-10}$) (Supplemental Fig. 2). Furthermore, because identical somatic mutations strongly associated with B cell lymphoma were identified in separate tumors, they are likely driving tumorigenesis in a portion (two of 193) of parathyroid adenomas that cannot be attributed to defective *MEN1*.

We additionally sequenced all coding exons (2–7) of *RAP1B* and exons 9–12 of *LRP5*, which encompassed the entire region containing the previously discussed internal deletion. The initial *RAP1B* and *LRP5* mutations observed in the discovery cohort were in tumors with *MEN1* mutations. Therefore, only the *MEN1*-mutated cohort of 68 tumors was screened for variants in these two genes. However, no additional somatic variants in either gene were uncovered, indicating that the original variants are likely to be passenger mutations or very rare drivers of tumorigenesis.

Discussion

MEN1 mutations are observed at a very high frequency in both familial and sporadic parathyroid adenomas as well as other endocrine tumors (28). This study found a higher mutation frequency of *MEN1* than previously reported, suggesting an even more important role in parathyroid tumors than originally believed. The majority of the identified mutations were very severe, being either frame shift indels or nonsense mutations. There appeared to be an enrichment of these damaging mutations in the earlier exons of *MEN1*, which is consistent with its complete loss of function in endocrine neoplasias.

Despite its alteration in many cancers, the specific role of the ubiquitously expressed, nuclear-localizing (29) menin in the cell is largely unclear. Although this protein is highly conserved from mammals (30) to Drosophila (31), it shares no sequence similarity to any known gene (32). Menin has been demonstrated to play a role as a transcriptional regulator in the nucleus via interactions with histone deacetylases (33), histone methyltransferases (34), and transcription factors (35–37). It has also been shown to be involved in cell proliferation (38), apoptosis regulation (39), and DNA damage repair (40). The knock-

out mouse model has been used to mimic the pattern of *MEN1* loss observed in MEN1 syndrome patients. Homozygous *MEN1* knockout mice are unequivocally lethal during gestation, but heterozygous knockouts are viable and fertile. These $MEN1^{+/-}$ mice typically develop endocrine tumors between 9 and 12 months of age (41). When investigated, these tumors display inactivation of the remaining functional allele of *MEN1* via LOH. However, a conditional, pancreas-specific, homozygous *MEN1* knockout model shows a lag period of about 6 months before the earliest tumors are detectable (42). Such results suggest that additional somatic events may be required for clonal expansion to occur.

Neither this study nor others were able to find additional mutations common to tumors with both *MEN1* alleles inactivated. There are several possible explanations for this observation. First, it could be that the inactivation of both copies of *MEN1* is alone sufficient to drive tumorigenesis in the parathyroid gland. If so, additional aberrations present in these tumors could be passenger mutations randomly altered in the *MEN1*-defective cell that clonally expanded that do not contribute to tumor formation or progression. It is also possible that these rare mutations contribute a slight selective advantage to a cell with inactivated *MEN1*, which contribute to, but are not solely sufficient for, tumorigenesis. Another explanation for the absence of common mutations among *MEN1*-mutated tumors is that other factors are necessary for tumorigenesis once *MEN1* is inactivated, but exome sequencing and previous studies failed to detect them. These could include copy number variations, epigenetic modifications, or alterations lying outside the exome, which would be undetectable using the current approach.

In the four discovery cohort tumors with functional copies of *MEN1*, no mutated genes were shared across tumors. However, additional screening in the validation cohort of 185 tumors was able to identify another tumor with an identical *EZH2* mutation. The probability of identifying two tumors with somatic mutations in the same gene merely by chance is exceedingly low. Far less likely is the random occurrence of identifying somatic changes at the exact base in two separate tumors, which makes this finding highly significant. Although this single *EZH2* mutation is likely to be a major driver of tumorigenesis in the tumors in which it is mutated, the fact that it is mutated in such a small percentage of parathyroid adenomas suggests that it plays a minor role in the total pathogenesis of the disease.

The tyrosine residue altered in our tumors is a key component of the *EZH2* active site commonly mutated in B cell lymphomas. The active site resides in the SET domain of *EZH2*, which is the catalytic component of the *PRC2* complex responsible for trimethylating H3K27, a mark of gene repression. Both increased and decreased activity of enzymes regulating H3K27 methylation have been linked to cancer, suggesting a fragile balance for maintenance of normal cell growth (43–45). Consistent with the specific *EZH2* mutation identified in this study as being gain of function, *in vitro* functional studies demonstrated an increased ability of $EZH2^{Y641N}$ to trimethylate H3K27 (46). Given this result, it is interesting to speculate how gain-of-function mutations in *EZH2* could phenocopy loss-of-function mutations in *MEN1*. *MEN1* is a member of the *MLL*-containing histone H3 methyltransferase complex known to methylate the activation mark H3K4 (47), which is associated with activated gene transcription. Based on these opposite functions, it is possible that loss-of-function *MEN1* mutations and gain-of-function *EZH2* mutations could drive tumorigenesis via a common mechanism. For example, the loss of *MEN1* is expected to result in a reduction in H3K4 methylation, which would result in an abnormal loss of expression of a set of genes. Alternatively, these same genes could be silenced through increased methylation of H3K27 by $EZH2^{Y641N}$.

In summary, this study has identified novel mutations possibly contributing to tumorigenesis as well as provided the most comprehensive mutational profile of sporadic parathyroid tumors to date. However, further investigation into this topic is still needed. In some tumor samples, no candidate drivers of tumorigenesis shared among tumors were identified. Although genomic rearrangements have been previously implicated in many tumor types, including parathyroid adenomas (*PTH*-cyclin D1) (48), the approach used in this study would have difficulty detecting such cellular events. Moreover, driver mutations altered at a low frequency in these tumors may have been missed in our discovery cohort of eight tumors, necessitating further exome sequencing using larger cohorts. Alternatively to exome sequencing, tumor samples lacking *MEN1* and *EZH2* defects make excellent candidates for whole-genome sequencing because they may share variants outside the exome, which our approach was not able to identify. Even so, this study demonstrates the power of high-throughput sequencing to identify rare tumorigenic mutations that would not have been identified using traditional approaches. This confirmed the presence of mutations previously found in these tumors and also revealed novel candidates for endocrine tumorigenesis, which suggests mechanisms of sporadic tumorigenesis in the parathyroid gland. Ultimately these results also suggest an increasingly important role for chromatin-modifying methyltransferase genes in tumor formation and progression.

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