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Analyzing Genetic Differences Between Sporadic Primary and Secondary/Tertiary Hyperparathyroidism by Targeted Next-Generation Panel Sequencing

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Abstract

Secondary hyperparathyroidism (SHPT) is characterized by excessive serum parathyroid hormone levels in response to decreasing kidney function, and tertiary hyperparathyroidism (THPT) is often the result of a long-standing SHPT. To date, several genes have been associated with the pathogenesis of primary hyperparathyroidism (PHPT). However, the molecular genetic mechanisms of uremic hyperparathyroidism (HPT) remain uncharacterized. To elucidate the differences in genetic alterations between PHPT and SHPT/THPT, the targeted next-generation sequencing of genes associated with HPT was performed using DNA extracted from parathyroid tissues. As a result, 26 variants in 19 PHPT or SHPT/THPT appeared as candidate pathogenic mutations, which corresponded to 9 (35%) nonsense, 8 (31%) frameshift, 6 (23%) missense, and 3 (11%) splice site mutations. The *MEN1* (23%, 6/26), *ASXL3* (15%, 4/26), *EZH2* (12%, 3/26), and *MTOR* (8%, 2/26) genes were frequently mutated. Sixteen of 25 patients with PHPT (64%) had one or more mutations, whereas 3 (21%) of 21 patients with SHPT/THPT had only 1 mutation ($p = 0.001$). Sixteen of 28 patients (57%) with parathyroid adenoma (PA) had one or more mutations, whereas 3 of 18 patients (17%) with parathyroid hyperplasia (PH) had just one mutation ($p = 0.003$). Known driver mutations associated with parathyroid tumorigenesis such as *CCND1/PRAD1*, *CDC73/HRPT2*, and *MEN1* were identified only in PA (44%, 7/16 with mutations). Our results suggest that molecular genetic abnormalities in SHPT/THPT are distinct from those in PHPT. These findings may help in analyzing the molecular pathogenesis underlying uremic HPT development.

Keywords Parathyroid adenoma · Parathyroid hyperplasia · Next-generation sequencing · Primary hyperparathyroidism · Secondary hyperparathyroidism · Tertiary hyperparathyroidism

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Introduction

Secondary hyperparathyroidism (SHPT) usually results from the excessive/inadequate secretion of parathyroid hormone (PTH) in response to impaired phosphate excretion, decreasing kidney function, dysregulating calcium homeostasis, and insufficient vitamin D bioactivation in chronic kidney disease patients. PTH synthesis and secretion contribute to the development of a vicious cycle [1]. Prolonged parathyroid stimulation causes initial diffuse polyclonal growth followed by monoclonal hyperplasia, with the degree of parathyroid hyperplasia (PH) determining the severity of SHPT [2]. Tertiary hyperparathyroidism (THPT) is often the result of a long-standing SHPT and is characterized by autonomous proliferation of the parathyroid glands and hypersecretion of PTH [3]. THPT occurs most commonly in renal transplant recipients with SHPT who have continuously elevated PTH and serum calcium levels after receiving kidney transplantation [4]. The development of monoclonal parathyroid expansion is frequent in most patients who receive surgical parathyroidectomy for refractory SHPT and THPT, but the somatic genetic alterations responsible for the selective advantage underlying these clonal expansions have not been investigated yet [5].

On the other hand, primary hyperparathyroidism (PHPT) is caused by autonomous over-secretion by the parathyroid tissue with an unclear etiology, leading to high serum calcium levels [6]. Almost all patients have sporadic, non-familial, and non-syndromic cases. In 85% of the cases, sporadic PHPT is caused by a single parathyroid adenoma (PA), but it may also be caused by PH (10%), double adenomas (2–5%), or parathyroid carcinoma (PC, < 1%) [7]. Several genes are responsible for the pathogenesis of typical sporadic PHPT. Somatic alterations in the *CCND1/PRAD1* and *MEN1* genes have been identified as significant drivers in the development of sporadic PAs [8]. Inactivating somatic mutations of the gene called cell division cycle 73/hyperparathyroidism 2 (*CDC73/HRPT2*) are common in PC [9]. Somatic defects in the genes coding for aryl hydrocarbon receptor-interacting protein (*AIP*) [10], calcium-sensing receptor (*CaSR*) [11], cyclin-dependent kinase inhibitor (*CDKI*) [12], catenin beta 1 (*CTNNB1*) [13], enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*) [14], and protection of telomeres 1 (*POT1*) [15] may contribute to PA formation [16].

Genetic abnormalities are associated with the development of different types of hyperparathyroidism (HPT), but the role of genetic variability in the modulation of PTH function in SHPT/THPT is not well understood. To discover other genetic alterations involved in the etiology of distinct pathologies, we performed next-generation panel-sequencing analysis in Korean patients with HPT. This study aimed to analyze the gene expression differences between PHPT and SHPT/THPT patients and to identify molecular pathways dysregulated in different HPT contexts.

Materials and Methods

Study Samples

A total of 66 patients underwent parathyroidectomy from January 2009 to January 2019 at Daejeon St. Mary's Hospital (Daejeon, Republic of Korea). The diagnosis of PHPT and SHPT/THPT was based on previous medical history, family history, drug history, laboratory tests, clinical features, technetium-99 m-labeled sestamibi scanning or cervical ultrasonography, and pathological diagnosis of surgically removed tumors. Parathyroid tissue samples of PHPT were collected from patients who underwent surgical parathyroidectomy according to appropriate surgical indications for HPT. Parathyroid tissue samples of SHPT/THPT were obtained from dialysis patients who underwent surgical parathyroidectomy due to uncontrolled intact PTH (iPTH) levels refractory to medical treatment consisting of phosphate binders, vitamin D analogs, and calcimimetics. The removed tumors were confirmed to be PA or PH by an experienced pathologist. We applied the following diagnostic criteria to distinguish the histological differences between PA and PH: PA was histologically defined as a well-encapsulated hypercellular lesion usually surrounded by a fibrous capsule, characterized predominantly by a single-cell population, the absence of stromal adipocytes, and a lesion confined to a single gland [17]. PH was defined histologically as presenting an absolute increase in the parenchymal cell mass, occurring as a consequence of the proliferation of chief cells, transitional oncocytes, and oncocytes in multiple parathyroid glands [18]. Parathyroid nodular hyperplasia were included in the category of PH. Patients diagnosed as presenting atypical parathyroid tumors or PC by the histologic pattern of the parathyroid tissues were excluded from the study. Patients with a family history of genetic syndrome including HPT, hyperparathyroidism-jaw tumor (HPT-JT), multiple endocrine neoplasia, hypocalciuric hypercalcemia, and kidney transplant recipients with stable renal function were also excluded. Finally, parathyroid tissue samples were obtained from 46 Korean patients diagnosed with PHPT (n = 25) or SHPT/THPT (n = 21). Of the patients with PHPT, 23 were diagnosed with PA, and 2 patients were diagnosed with PH. Of the patients with SHPT/THPT, pathologic diagnosis revealed 5 PA and 16 PH including parathyroid nodular hyperplasia. Formalin-fixed paraffin-embedded (FFPE) samples of parathyroid tissues were obtained, and histological sections from the same tissues were processed for classification.

Particularly, to confirm the presence of *MEN1* mutations by Sanger sequencing, area of adenoma content > 50% was selected by board-certified pathologist, manually circling, under microscopic examination in six PHPT samples.

Nucleic Acid Preparation

Nucleic acids (DNA and RNA) were extracted from four or five unstained FFPE sections per sample using the Recover-All Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The quantity and quality of genomic DNA was estimated using a Qubit 2.0 Fluorometer with a Qubit dsDNA High-Sensitivity Assay Kit and a TaqMan RNase P Detection Reagent Kit (Thermo Fisher Scientific), respectively. A DNA concentration > 10 ng/μL was considered adequate.

Library Preparation and Next-Generation Panel Sequencing

A custom panel targeting genes associated with parathyroid tumors identified by previous research was designed using the Ion AmpliSeq Designer (www.ampliseq.com). Targeted genes were chosen based on their reported association with parathyroid tumor in previous studies [15, 19–21]. A list of 42 selected genes is presented in Table 1. Libraries were prepared using the Ion AmpliSeq Library Kit Plus 2.0 and the Ion AmpliSeq On-Demand Panel primer pools (Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, two target amplification reactions were merged. The libraries for the custom panel were digested partially, phosphorylated using the FuPa reagent, ligated to Xpress barcode adapters, and purified. The purified libraries were quantified using the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific). The kit's bacterial DNA standard was used for quantification. Pooled purified libraries of eight multiplexed tumor DNAs per chip at a concentration of 50 pM were loaded onto chips and analyzed using the Ion Chef with the Ion 530 chef Kit (ThermoFisher Scientific) and sequenced on S5XL using Ion S530 chips (Thermo Fisher Scientific) for 200-base-read single-strand sequencing as per the manufacturer's instructions. Additionally, Sanger sequencing was performed to confirm some of the detected mutations with a mutant allele burden (MAB) > 20%, and low-level *MEN1* mutations using additional FFPE with adenoma content with > 50%.

Table 1 A list of 42 selected genes associated with hyperparathyroidism for next-generation panel sequencing

ALDH1A3	*EPRS*	*PHKB*
APP	*EZH2*	*PIK3CA*
ASXL3	*GLRA4*	*POT1*
ATAD5	*HGF*	*RAX*
CASR	*INSC*	*RB1*
CCND1	*KIF11*	*RET*
CDC73	*MAP4K5*	*RIZ*
CDKN1B	*MEN1*	*SMC3*
CIT	*MTOR*	*SNRPB*
COL3A1	*NDST1*	*TECPR2*
DENND4B	*NLN*	*TLN2*
DROSHA	*NONO*	*TMEM87A*
EIF1AX	*NR3C2*	*TRIP4*
EIF4G1	*ORC1*	*ZFYVE26*

Bioinformatic Analysis

Automated analysis of raw sequencing data was performed sequentially by Torrent Suite 5.10 and Ion Reporter 5.10 (https://ionreporter.thermofisher.com/ir/) with Oncomine Knowledgebase Reporter (Thermo Fisher Scientific). Human genome build 19 was mapped as the reference sequence for alignment. Mapped sequencing data were regarded as adequate if associated with the following values: mapped read > 2,000,000, read depth > 500×, and Phred quality score > 30. A MAB of 3% was used as a cut-off for variants. Mutations predicted to cause moderate or strong alteration in gene functions were interpreted manually by laboratory geneticists based on ACMG-AMP standards and guidelines [22]. In particular, the pathogenicity of missense variants was assessed using the Catalogue of Somatic Mutations in Cancer (COSMIC, https://cancer.sanger.ac.uk/cosmic). If a candidate missense variant was not listed in COSMIC, its deleterious effect was estimated using in silico computational tools, such as Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), and PhyloP [23] and Grantham [24] conservation scores.

mRNA Expression Analysis for *CDC73*/*HRPT2* Variants

To evaluate the deleterious effect of the two missense *CDC73/HRPT2* variants, complementary DNA was synthesized from RNA extracted from FFPE samples. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) using the 5′ reporter FAM™ dye-labeled and 3′ quencher Minor-groove binder (MGB)-labeled probes (Applied Biosystems) was performed on a CFX96™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA). We used 5′-TGAAGACCAGCTATGT-3′ for p.Asn39Ser and 5′-TACAAAGCACAAGAAA-3′ for p.Gly242Arg of the *CDC73/HRPT2* variants, respectively, and 5′-TGAAGACCAACTATG-3′ and 5′-TACAAAGCACAGGAAA-3′ for the p.Asn39 and p.Gly242 residue of wild-type *CDC73/HRPT2*, respectively. Gene expression levels were normalized to those of

endogenous *ACTB* (Reference Sequence: NM_001101.4) used as a control and determined according to the relative quantification method ($2^{-\Delta\Delta Ct}$). The mRNA expression analysis was performed independently three times.

Statistical Analysis

Continuous variables were compared using the Mann–Whitney U test, because the levels of serum creatinine, vitamin D, alkaline phosphatase (ALP), calcium, phosphorus, and iPTH were not normally distributed. To investigate which laboratory findings were associated with PHPT and SHPT/THPT, logistic regression analysis was carried out. Nonsignificant predictors were removed by the enter method (probability threshold for removal: 0.1). Fisher's exact test was performed to compare genetic differences between PHPT and SHPT/THPT cases according to pathophysiological and/or histological HPT subtypes. The differences in genotype and clinical parameters or histopathology between PHPT and SHPT/THPT according to mutation status were analyzed using the Mann–Whitney U test. In addition, the mean differences in mRNA gene expression when comparing p.Asn39Ser and p.Gly242Arg variants with wild-type *CDC73/HRPT2* were estimated by the Mann–Whitney U test. All the statistical analyses used MedCalc Statistical Software Version 17.6 (MedCalc software, Ostend, Belgium). Differences were considered statistically significant when $p < 0.05$.

Results

Comparisons of Laboratory Findings From Patients With Primary and Secondary/Tertiary Hyperparathyroidism

In SHPT/THPT patients, serum creatinine, phosphorus, iPTH, and ALP levels were significantly higher, whereas serum calcium and 25-hydroxyvitamin D levels were lower than those in PHPT patients (Table 2). However, no laboratory finding was retained by the logistic regression model with the enter method (data not shown).

Quality Control Metrics of Raw Sequencing Data

In quality control metrics for raw sequencing data generated from eight independent experiments, the median mapped reads, on-target read rates, depth of on-target regions, and uniformity of base coverage were 2,425,547 base pairs (range 2,199,789–3,066,694), 92% (86–96), 871× (510–1444), and 90% (85–94), respectively. All the experiments satisfied the manufacturer's specifications (> 95% of amplicons should have a read depth of > 500×).

Mutation Profiles of Primary and Secondary/Tertiary Hyperparathyroidism

Various MABs ranging from 4 to 49% in tumor DNA samples were identified without matched normal tissues as germline controls. Thus, when such a variant is identified in PHPT or SHPT/THPT, its origin could not be defined whether the variant has been somatically acquired or it is constitutional in origin. Missense variants with allele frequency < 0.0001 predicted to be damaging or deleterious which were not registered in COSMIC database as well as null variants (canonical ± one or two splice sites, frameshift, or nonsense mutations) were also included. In addition, missense mutations were examined using computational metrics and in silico analysis (SIFT, PolyPhen2, PhyloP, and Grantham) to reveal variants with a high likelihood of being pathogenic (likely). As a result, 28 single-nucleotide variants or indels were detected initially in the 20 patients. All six low-level *MEN1* mutations were confirmed by Sanger sequencing in manual tissue dissection utilized for adenoma enrichment. However, there was no significant quantitative difference in mRNA gene expression when comparing the two missense *CDC73/HRPT2* variants with wild-type *CDC73/HRPT2* by quantitative RT-PCR. Thus, they were excluded from the candidate driver mutations. As a result, 26 variants in the 19 PHPT or SHPT/THPT were candidate pathogenic mutations: 9 (35%) nonsense, 8 (31%) frameshift, 6 (23%) missense, and 3 (11%) splice site mutations (Table 3). The most frequently mutated gene was *MEN1* (23%, 6/26), which presented three

Table 2 Comparisons of laboratory findings between primary hyperparathyroidism and secondary/tertiary hyperparathyroidism

Laboratory findings	PHPT (n = 25)	SHPT/THPT (n = 21)	*p* value
Creatinine (mg/dL)	0.9 ± 0.46	10.83 ± 3.95	< 0.001
Calcium (mg/dL)	11.88 ± 2.06	9.8 ± 1.15	< 0.001
Phosphorus (mg/dL)	2.60 ± 0.55	6.03 ± 1.76	< 0.001
Intact PTH (pg/mL)	394.77 ± 507.57	1504.8 ± 1147.07	< 0.001
ALP (IU/L)	98.39 ± 47.29	229.6 ± 178.78	< 0.001
25-Hydroxyvitamin D (ng/mL)	6.96 ± 8.58	2.83 ± 6.17	0.037

Results are expressed as mean ± SD. Statistical analysis was performed by Mann–Whitney U test

ALP alkaline phosphatase, *PHPT* primary hyperparathyroidism, *PTH* parathyroid hormone, *SHPT/THPT* secondary hyperparathyroidism/tertiary hyperparathyroidism

Table 3 Possible pathogenic (likely) mutations identified by next-generation panel sequencing in 19 Korean patients with hyperparathyroidism

Sample	S/A	Dx	Histology	Genes	Transcript	Base change	AA change	Effect	MAB	SIFT	PP2	PhylP	GT
PTH03*	F/54	PHPT	Adenoma	*MEN1*	NM_000244.3	c.556C > T	p.His186Tyr	Missense	6	D	D	8.69	83
				SMC3	NM_005445.3	c.551delG	p.Gly184Alafs*10	Frameshift	31				
PTH04*	F/43	PHPT	Adenoma	*MEN1*	NM_000244.3	c.833delT	Leu278Argfs*8	Frameshift	4				
				ASXL3	NM_030632.2	c.962_963delAG	p.Gln321Profs*20	Frameshift	41				
PTH06	M/46	PHPT	Adenoma	*INSC*	NM_001031853.4	c.1672C > T	p.Gln558*	Nonsense	16				
				MTOR	NM_004958.3	c.817C > T	p.Arg273*	Nonsense	18				
PTH07	F/57	PHPT	Adenoma	*EZH2*	NM_004456.4	c.1936 T > A	p.Tyr646Asn	Missense	30	D	D	9.05	143
PTH13*	F/48	PHPT	Adenoma	*MEN1*	NM_000244.3	c.493G > C	p.Ala165Pro	Missense	4				
PTH16	F/57	PHPT	Adenoma	*ASXL3*	NM_030632.2	c.4827G > A	p.Trp1609*	Nonsense	16				
				MTOR	NM_004958.3	c.4469 + 1G > A	p.?	Splice site	17				
				TRIP4	NM_016213.4	c.1377G > A	p.Trp459*	Nonsense	10				
PTH29*	M/47	PHPT	Adenoma	*MEN1*	NM_000244.3	c.252dupT	p.Ile85Tyrfs*32	Frameshift	4				
PTH30	F/54	PHPT	Adenoma	*DENND4B*	NM_014856.2	c.1841G > A	p.Arg614His	Missense	22	D	D	7.56	29
PTH32	M/52	PHPT	Adenoma	*EZH2*	NM_004456.4	c.2111-1G > A	p.?	Splice site	17				
PTH33	F/69	PHPT	Adenoma	*ASXL3*	NM_030632.2	c.451C > T	p.Gln151*	Nonsense	26				
PTH34	M/72	PHPT	Adenoma	*CDC73*	NM_024529.4	c.165C > A	p.Tyr55*	Nonsense	7				
PTH36	F/55	PHPT	Adenoma	*EZH2*	NM_004456.4	c.1747_1748insT	p.Arg583Leufs*4	Frameshift	13				
PTH40*	F/39	PHPT	Adenoma	*MEN1*	NM_000244.3	c.1339C > T	p.Gln447*	Nonsense	4				
PTH44*	F/59	PHPT	Adenoma	*CCND1*	NM_053056.2	c.875A > G	p.Asp292Gl	Missense	6	D	D	8.49	94
				MEN1	NM_000244.3	c.752_753delCCinsTT	p.Ser251Phe	Frameshift	6				
				NONO	NM_001145408.2	c.943 + 1G > A	p.?	Splice site	19				
PTH41	F/37	PHPT	Hyperplasia	*POT1*	NM_015450.3	c.955G > T	p.Gly319*	Nonsense	19				
PTH45	F/53	PHPT	Hyperplasia	*DROSHA*	NM_013235.4	c.1431_1432insC	p.Glu478Argfs*5	Frameshift	11				
PTH24	M/53	SHPT/THPT	Adenoma	*CIT*	NM_001206999.1	c.5683C > T	p.Gln1895*	Nonsense	16				
PTH31	M/26	SHPT/THPT	Adenoma	*ASXL3*	NM_030632.2	c.5992G > C	p.Glu1998Gln	Missense	49	D	D	4.73	29
PTH21	M/47	SHPT/THPT	Nodular hyperplasia	*HGF*	NM_000601.5	c.687_689delCAA	p.Lys230del	Frameshift	15				

Computation of p values for conservation or acceleration either lineage-specific or across all branches; Grantham, a prediction of the effect of substitutions between amino acids based on chemical properties

S/A sex/age, *Dx* diagnosis, *Ex* exon, *AA change* amino acid change, *MAB* mutant allele burden, *SIFT* Sorting Intolerant From Tolerant, *PP2* Polymorphism Phenotyping v2, *D* damaging, *PhylP* PhyloP conservation score (deleterious > 1.6), *GT* Grantham conservation score (conservative ≦100, radical > 101), *PHPT* primary hyperparathyroidism, *SHPT/THPT* secondary hyperparathyroidism/tertiary hyperparathyroidism

*All six low-level *MEN1* mutations were confirmed by Sanger sequencing in manual tissue dissection utilized for adenoma enrichment

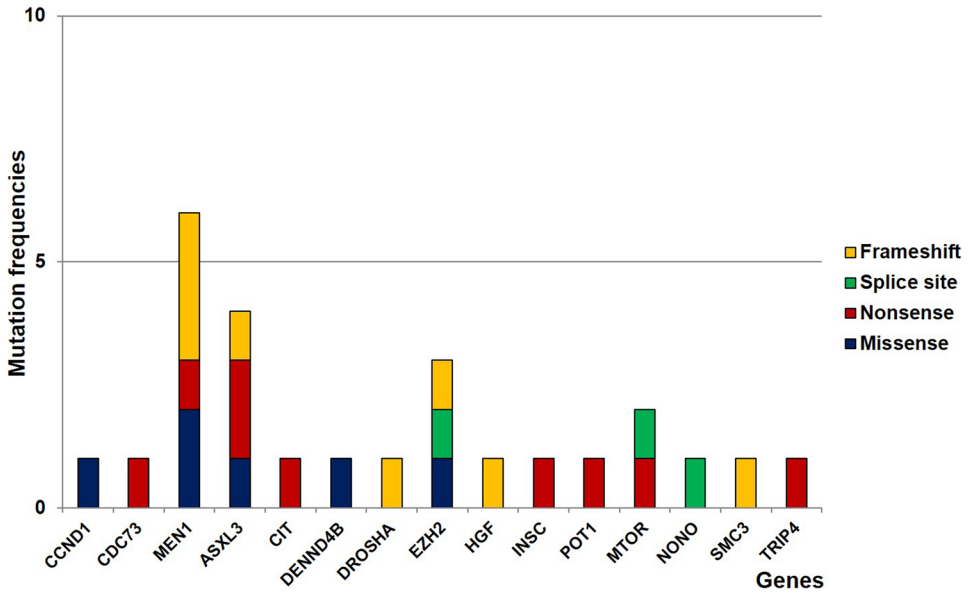

Fig. 1 Frequencies of somatic mutations identified by next-generation panel sequencing in the various genes in 19 primary or secondary/tertiary hyperparathyroidism. Gene identities are depicted on the x-axis, and frequency of mutations on the y-axis

frameshift, two missense, and one nonsense mutations. The second, third, and fourth most frequently mutated genes were ASXL Transcriptional Regulator 3 (*ASXL3*, 15%, 4/26), *EZH2* (12%, 3/26), and Mammalian Target of Rapamycin (*MTOR*, 8%, 2/26), respectively. One mutation (4%, 1/27) was detected in *CCND1/PRAD1*, *CDC73/HRPT2*, Citron Rho-Interacting Serine/Threonine Kinase (*CIT*), DENN Domain-Containing 4B (*DENND4B*), Drosha Ribonuclease III (*DROSHA*), Hepatocyte Growth Factor (*HGF*), INSC spindle orientation adaptor protein (*INSC*), Protection of Telomeres 1 (*POT1*), Non-POU Domain-Containing Octamer Binding (*NONO*), Structural Maintenance of Chromosomes protein 3 (*SMC3*), and Thyroid Hormone Receptor Interactor 4 (*TRIP4*) (Fig. 1). Five PHPT revealed more than one mutation in different genes: *MEN1* and *SMC3* mutations in PTH03; *MEN1* and *ASXL3* mutations in PTH04; *INSC* and *MTOR* mutations in PTH06; *ASXL3*, *MTOR*, and *TRIP4* mutations in PTH16; and *CCND1/PRAD1*, *MEN1*, and *NONO* mutations in PTH44 (Fig. 2).

Genetic Differences Between Primary and Secondary/Tertiary Hyperparathyroidism According to Pathophysiology and Histology

Figure 2 illustrates the distribution of somatic mutations identified in each parathyroid tissue sample. Sixteen of 25 patients with PHPT (64%) had one or more mutations, whereas 3 (21%) of 21 patients with SHPT/THPT had only one mutation (*ASXL3*, *CIT*, and *HGF*; $p=0.001$). Known driver mutations associated with parathyroid tumorigenesis—*CCND1/PRAD1*, *CDC73/HRPT2*, and *MEN1* mutations—were identified in 44% (7/16) of PHPT patients but not in SHPT/THPT patients. Sixteen of 28 PAs (57%) had one or more mutations, whereas 3 (PTH41, PTH45, and PTH21) of 18 PHs (17%) had just one mutation (*POT1*, *DROSHA*, and *HGF*; $p=0.003$). Likewise, for PHPT, known driver mutations associated with parathyroid tumorigenesis such as *CCND1/PRAD1*, *CDC73/HRPT2*, and *MEN1* were identified only in PA (44%, 7/16 with mutations).

Comparison of Age at Diagnosis Between Patients With Primary and Secondary/Tertiary Hyperparathyroidism According to Their Mutation Status

Next, we compared the differences in genotype and clinical parameters or genotype and histopathology between PHPT and SHPT/THPT patients according to their mutation status. Due to the relatively small sample size, genotype–histopathologic correlations were not found in the study. In patients with PHPT, any mutations in PHPT appeared at an earlier age (mean age, 53 years; 95% confidence interval [CI], 48 to 58) compared to patients with no PHPT mutation (mean age, 68 years; 95% CI, 58 to 78) ($p=0.003$). In patients with mutated PHPT, PHPT with *MEN1* mutation occurred at an earlier age (mean age, 51 years) compared to patients having PHPT with other mutations than *MEN1* (mean age, 53 years). However, these differences were not statistically significant ($p=0.265$). In addition, there was no significant difference in the age of onset between SHPT/THPT patients with any mutations and SHPT/THPT patients with no mutation (mean age, 42 years vs. 48 years; $p=0.391$) or between PHPT patients with any mutations and SHPT/THPT patients with any mutations (mean age, 53 years vs. 42 years; $p=0.115$).

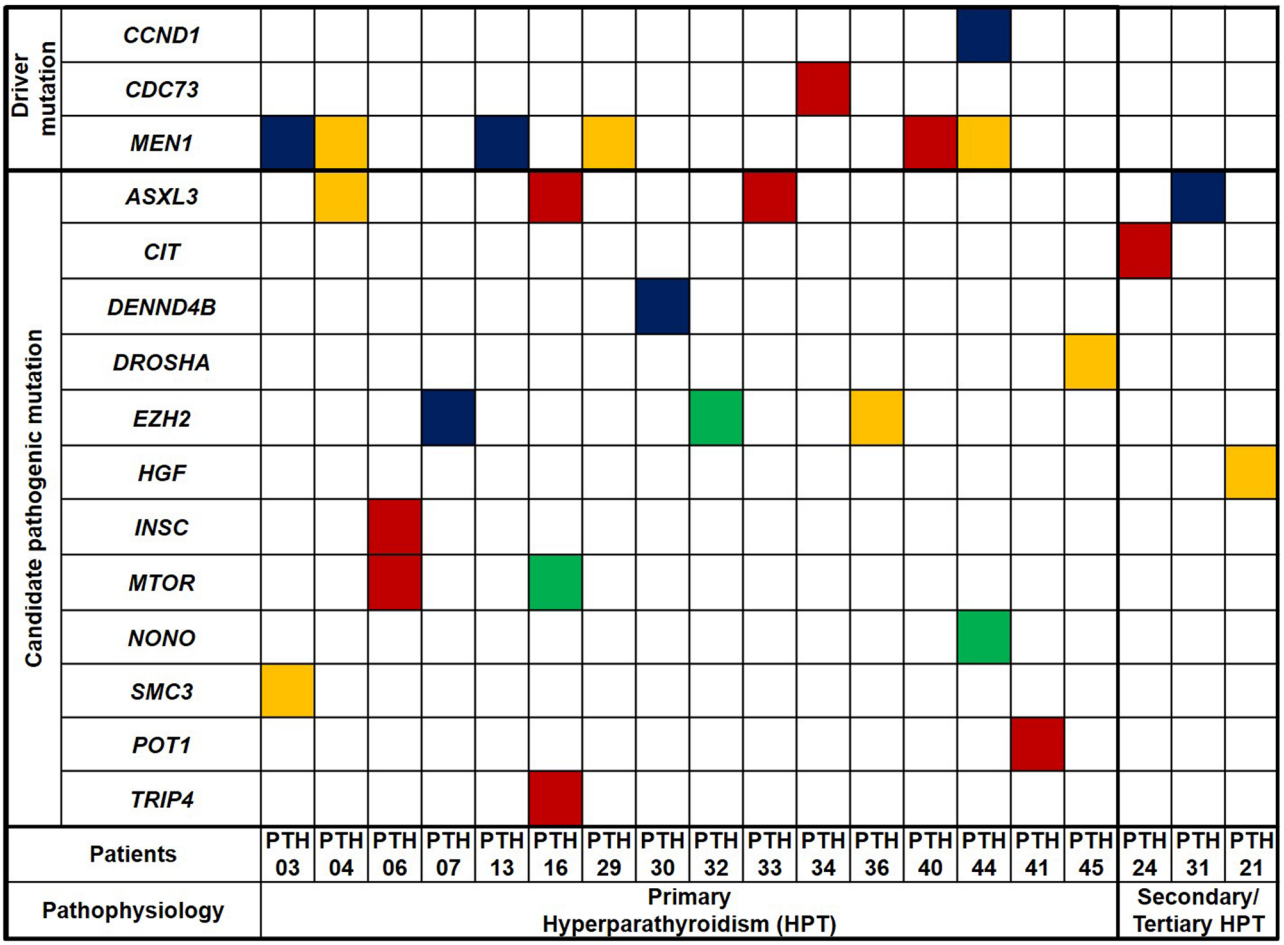

Fig. 2 Distribution of somatic mutations identified by next-generation panel sequencing in each primary or secondary/tertiary hyperparathyroidism. Individual patients are depicted on the x-axis, and identity of genes on the y-axis. Green box, splice site mutation identified; navy box, missense mutation identified; red box, nonsense mutation identified; yellow box, frameshift mutation identified; white box, no mutation identified

Discussion

This study is the first identifying genetic abnormalities and comparing genetic alterations in parathyroid tissues between PHPT and SHPT/THPT Korean patients using next-generation panel sequencing. We confirmed a recurrent mutation phenomenon that implicates *CCND1/PRAD1*, *CDC73/HRPT2*, and *MEN1* genes in parathyroid tumorigenesis in patients with PHPT [20]. However, somatic mutations in these three genes were not detected in SHPT/THPT. This indicates that a substantial number of parathyroid tumors may harbor somatic mutations in undetected genes that contribute to parathyroid tumorigenesis. Nineteen possible (likely) pathogenic somatic mutations were identified in different genes. In our study, the incidence of genetic mutations in SHPT/THPT was lower than that in PHPT.

Consistent with our results, previous studies reported the detection rates of genetic mutations in SPHT. Falchetti et al. reported allelic loss of heterozygosity on chromosome 11 in only 2 of 12 enlarged parathyroid glands [25]. Inagaki et al. also reported the loss of heterozygosity of the *Ha-ras* locus in only 1 of 11 SPHT patients (9%) and no rearrangement or allelic loss of *CCND1/PRAD1*, Insulin-like growth factor 2 (*IGF-2*), *PTH*, or REarranged during Transfection (*RET*) genes [26]. The differences in the frequency of genetic mutations between PHPT and SHPT/THPT were explained by Imanishi et al. [27], who described significant activation of proliferative pathways in parathyroid tissue before monoclonal outgrowth in SHPT/THPT. In contrast, different somatic genetic alterations may be required to drive clonal growth in PHPT, with PAs developing on a substrate lacking underlying proliferative activity [27]. Although certain crucial functional factors may be common to both SHPT/THPT and PHPT, significant differences in genetic alterations between SHPT/THPT and PHPT indicate that these tumors occur and develop through distinct pathogenetic mechanisms.

Somatic *MEN1* mutations in both alleles were found in 25–40% of sporadic PAs in PHPT patients, but *MEN1* mutations in PH in SHPT/THPT patients and in PCs are rare [28]. Patients with *MEN1*-mutated tumors usually show a germline origin followed by the appearance of a somatic mutation such as an inactivating mutation or loss of heterozygosity. These somatic alterations, similarly to what occurs with germline mutations, are dispersed throughout the gene coding region [29]. Known molecular alterations of sporadic benign parathyroid tumors include cyclin D1 protein overexpression and genetic abnormalities in the *MEN1* gene [30]. Our study confirmed that somatic *MEN1* mutations are common in patients with nonfamilial sporadic PHPT [31], even though the MAB of *MEN1* in six PHPT were very low, ranging from 4 to 6%. However, PHPT with *MEN1* mutations occurred at an earlier age than PHPT with other than *MEN1* mutations, though without statistical significance. Although young age is the only common criterion for suspicious *MEN1* mutations, Skandarajah and colleagues suggest that patients of any age diagnosed with PHPT should be treated with an identical preoperative and surgical approach [32]. Interestingly, PHPT carrying any mutations tends to be relatively early in onset compared to PHPT without any mutations. Thus, mutation profiling using targeted next-generation panel sequencing may contribute to the early diagnosis and proper treatment of Korean PHPT patients, providing insights into the genome landscape of PHPT and further evidence of gene mutations' role in PHPT tumorigenesis.

As the regulatory subunit of a holoenzyme, *CCND1/PRAD1* promotes progression through the G1–S phases of the cell cycle, as well as inactivates and phosphorylates the retinoblastoma protein in a manner-dependent on cyclin-dependent kinases. Overexpression or amplification of *CCND1/PRAD1* plays critical roles in the development of melanoma, prostate cancer, breast and colon cancer, and lymphoma [33]. *CCND1/PRAD1* may also play a role in PAs and PCs, with cyclin D1 overexpression found in 20–40% of PAs in patients with PHPT, 31% of PH in patients with SHPT/THPT, and up to 90% of PCs [28, 34]. *CCND1/PRAD1* is a target gene for the Wnt/β-catenin signaling pathway, and cyclin D1 overexpression may be explained in part by the activation of this pathway through aberrant accumulation of non-phosphorylated β-catenin [35]. Aberrant activation of the Wnt/β-catenin signaling pathway may elevate *CCND1* expression in PHPT and SHPT/THPT [13]. In our study, one missense p.Asp292Gly mutation (COSM6918225) of *CCND1/PRAD1* was identified in PHPT with PA, but no somatic mutation of *CCND1/PRAD1* was shown in SHPT/THPT.

On the other hand, two missense p.Asn39Ser and p.Gly242Arg variants of the *CDC73/HRPT2* gene were excluded because of the lack of evidence of a pathogenic effect. Even though pathogenic missense mutations cause functional defects in *CDC73/HRPT2* proteins, lack of parafibromin expression is required for a pathogenic effect. If immunohistochemistry (IHC) analysis is insufficient to detect variations in parafibromin expression, it would be helpful to gather morphologic evidence of the presence of *CDC73/HRPT2* mutations [36]. *CDC73/HRPT2* may be a tumor-suppressor gene, whose inactivation is associated with predisposition to HPT-JT and occurrence of some sporadic parathyroid tumors [37]. As parathyroid tumors have a higher incidence of malignancy in patients with HPT-JT syndrome than in MEN type 1 or MEN type 2 patients, mutations in *CDC73/HRPT2* are likely an important factor in increasing the risk of PCs [38]. *CDC73/HRPT2* mutations occur in up to 77% of PCs but in less than 1% of unselected, apparently benign sporadic PAs [36]. A somatic p.Tyr55* mutation (COSM26053) in the *CDC73/HRPT2* gene in PAs was also reported previously in PCs [39]. Because complete analysis such as IHC was not available to rule out any carcinomatous changes in these cases, the possibility of selection bias or histological intra-tumor heterogeneity remained. By contrast, no somatic mutation of *CDC73/HRPT2* was found in SHPT/THPT.

ASXL3 belongs to a group of vertebrate Asx-like proteins considered to be transcriptionally functional regulators [40]. Somatic *ASXL* gene mutations were identified in all myeloid leukemias and in solid tumors including melanoma, pancreatic cancer, prostate cancer, and breast cancer [41]. As found in a previous study of a Chinese population [21], somatic *ASXL3* mutations were detected at the same frequency as *CDC73/HRPT2* mutations in three PHPTs and one SHPT/THPTs with PAs. In particular, we found one p.Gln151*, one p.Gln321Profs*20, one p.Trp1609* (COSM337508) [42], and one p.Glu1998Gln mutations in this study. Only deleterious mutations such as nonsense or frameshift mutations of *ASXL3* were identified in PHPT with PA, whereas missense mutations were predicted to be pathogenic in SHPT/THPT. A better understanding of the pathological and physiological functions of *ASXL3* in parathyroid tumorigenesis in both PHPT and SHPT/THPT may provide biomarkers and therapeutic targets for parathyroid tumors. Functional analysis is required to confirm this finding and elucidate the underlying pathogenesis.

The *EZH2* gene encodes a histone methyltransferase constituting the catalytic component of the polycomb repressive complex 2 (*PRC2*), which initiates epigenetic silencing of genes involved in cell fate decisions [43]. *EZH2* specifically methylates nucleosomal histone H3 at lysine-27 (H3K27) [44]. The function of *EZH2* as a histone methyltransferase suggests that *EZH2* can act as a tumor-suppressor gene and likely influences epigenetic modifications that lead to cancer [45]. In this study, somatic *EZH2* mutations were detected frequently in three PHPTs with PAs: one p.Arg583Leufs*4,

one p.Tyr646Asn (COSM37031), and one c.2111-1G > A mutation. In contrast, no somatic mutation of *EZH2* was found in SHPT/THPT. The identification of the p.Tyr646Asn mutation revealed the role of the *EZH2* gene in parathyroid tumorigenesis. Consistent with the same missense *EZH2* mutation identified in our study and in a previous report being a gain-of-function mutation [14, 21], in vitro functional studies demonstrated an increased ability of *EZH2* with p.Tyr646Asn to trimethylate H3K27 [46]. Svedlund et al. suggested that amplification of the *EZH2* gene in 29% of PHPT tumors, 50% of SHPT tumors, and 60% of PCs and *EZH2* depletion interfered with Wnt/β-catenin signaling by elevating the expression of growth-suppressive Axis Inhibition protein *2* (*AXIN2*), a negative regulator of β-catenin stability [47]. Further investigations are needed to elucidate the different pathogenetic roles of this *EZH2* mutation in PHPT and SHPT/THPT.

MTOR is a highly conserved protein kinase present in two functionally and structurally distinct protein complexes: TOR complex-1 (*TORC1*) and *TORC2*. *TORC1* is a critical regulator of mRNA translation and cell growth and proliferation, whereas *TORC2* promotes cell cycle progression, cell survival, and actin cytoskeletal rearrangement [48]. In our study, an *MTOR* mutation associated with parathyroid tumorigenesis was detected in two cases of PHPT with PAs: one p.Arg273* and one c.4469 + 1G > A. The phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway plays a key role in regulating cell cycle progression, whereas dysregulation of the PI3K/AKT/mTOR pathway disturbs the balance between cell apoptosis and proliferation, resulting in cancer metastatic competence, therapy resistance angiogenesis, and competitive growth advantage [49]. To date, *MTOR* mutations have been reported in one sporadic PC and one case of PHPT with PA (c.5664C > G; COSM462604) [21].

Our study suffers from several limitations. Our main concern about low MAB was that FFPE samples of parathyroid tissues were obtained, without laser capture microdissection for enrichment of adenoma or hyperplasia content. This problem may lead to underestimation of the MAB while many mutations in this study had MAB's around 5–20%. Maximizing the burden of target tissue before molecular testing is crucial for limiting the need for additional biopsies to obtain a sample for molecular testing as well as for eliminating potential false negative results. When needle biopsies or cell block preparations with small populations of target samples are used, selective sample preparation such as digitally guided microdissection is a highly effective method for enriching the content of target tissue while preserving DNA yield and quality [50]. Second, this was a single-center, cross-sectional study, and the total number of enrolled patients was relatively small. This could cause selection bias. Third, although disease-related genes were chosen carefully, novel drivers or critical genes could not be discovered due to the inherent underrepresentation of genes in next-generation panel sequencing. However, this technique offered improved uniformity of coverage and sufficient coverage of exon regions at typical sequencing depths compared with whole-exome sequencing or whole-genome sequencing [51]. Fourth, whether these were true somatic mutations in the affected tissue or germline mutations was not determined using matched germline DNA from peripheral blood in parallel to the tumor DNA from parathyroid tissue. In some cases, germline mutations lead to an increased inheritable risk for cancer occurrence, even though most actionable genomic alterations are acquired somatically. The identification of inherited cancer-predisposing mutations can promote the early detection and prevention of future tumors in affected families as well as in the probands. Finally, it is possible that the absence of mutations in 27 studied patients was due to a low tumor burden carrying "actual" mutations, even with a cut-off of mutant allele burden of 3%. Both adequate analysis by pathologists to correctly detect tumor cellularity and regular quality assessment processes by laboratory doctors in molecular diagnostics laboratories can contribute to reducing the errors of a false negative result.

Despite these limitations, this report supplies the first mutational profile of PHPT and SHPT/THPT with PA or PH in Korean patients. Our study also reveals that PHPT with PA may harbor known driver mutations of *CCND1/PRAD1*, *CDC73/HRPT2*, and *MEN1*, along with candidate pathogenic somatic variants of PT. Some parathyroid tissues may also present mutations at a low frequency (i.e., < 20% mutant alleles as the limit of detection by Sanger sequencing) in other driver genes, such as genes involved in DNA repair and genome stability. Functional studies and further investigations are required to comprehensively describe the pathological functions of mutations in driving SHPT/THPT. Studies on larger study populations are necessary to discover valid recurrent mutations that may drive SHPT/THPT and verify our findings.

In conclusion, we confirmed that known driver mutations in *CCND1/PRAD1*, *CDC73/HRPT2*, and *MEN1* in PHPT and recurrent mutations in *ASXL3*, *EZH2*, and *MTOR* may be associated with the pathogenesis of PHPT. However, no somatic mutations of *CCND1/PRAD1*, *CDC73/HRPT2*, and *MEN1* were found in SHPT/THPT, and one mutation in each of the genes *ASXL3*, *CIT*, and *HGF* may be associated with the pathogenesis of SHPT/THPT. These observations improve our understanding of the differences in tumorigenesis between PHPT and SPHT, provide insights into the molecular pathogenesis of SHPT/THPT, and suggest guidelines for cloning genes that may serve as novel therapeutic targets in SHPT/THPT treatment.

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Author Contribution YAH designed the study and wrote the first draft of the manuscript. BKK, JL, and WYS participated in data collection. KCP contributed to sample preparation. WJC, YKC, and SYK interpreted the data. SS and JP performed molecular experiments and interpreted the sequencing data. KYH contributed to mRNA gene expression analysis. YAH and JP reviewed/edited the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Ethics Approval The study protocol was approved by the Institutional Review Board of The Catholic University of Korea (DC19SESI0016). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Conflict of Interest The authors declare no competing interests.

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