



Whole Exome Sequencing Identifies Novel Genetic Alterations in Patients with Pheochromocytoma/Paranglioma

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Background: Pheochromocytoma and paragangliomas (PPGL) are known as tumors with the highest level of heritability, approximately 30% of all cases. Clinical practice guidelines of PPGL recommend genetic testing for germline variants in all patients. In this study, we used whole exome sequencing to identify novel causative variants associated with PPGL to improve the detection of rare genetic variants in our cohort.

Methods: Thirty-six tested negative for pathogenic variants in previous Sanger sequencing or targeted gene panel testing for PPGL underwent whole exome sequencing. Whole exome sequencing was performed using DNA samples enriched using TruSeq Custom Enrichment Kit and sequenced with MiSeq (Illumina Inc.). Sequencing alignment and variant calling were performed using SAM-tools.

Results: Among previously mutation undetected 36 patients, two likely pathogenic variants and 13 variants of uncertain significance (VUS) were detected in 32 pheochromocytoma-related genes. *SDHA* c.778G>A (p.Gly260Arg) was detected in a patient with head and neck paraganglioma, and *KIF1B* c.2787-2A>C in a patient with a bladder paraganglioma. Additionally, a likely pathogenic variant in *BRCA2*, VUS in *TP53*, and VUS in *NF1* were detected.

Conclusion: Exome sequencing further identified genetic alterations by 5.6% in previously mutation undetected patients in PPGL. Implementation of targeted gene sequencing consisted of extended genes of PPGL in routine clinical screening can support the level of comprehensive patient assessment.

Keywords: Pheochromocytoma; Paranglioma; Whole exome sequencing; Germ-line mutation; Molecular diagnostic techniques

Received: 6 July 2020, Revised: 8 October 2020, Accepted: 3 November 2020

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INTRODUCTION

Pheochromocytoma and paragangliomas (PPGL) are the most heritable tumors, with around 30% of cases caused by pathogenic variants. More than 15 germline and 30 somatic variants of causative genes have been associated with the disease, demonstrating a high degree of heterogeneity [1-7].

Molecular PPGL subtypes can be classified into three groups according to the Cancer Genome Atlas [2]. One major cluster is the pseudohypoxic group, which includes *SDHx* (*SDHA*, *SDHB*, *SDHC*, *SDHD*), *SDHAF2*, *FH*, *MDH2*, *IDH1*, *VHL*, *EPAS1*, and *PHD1/2*, with somatic and germline variants. Another cluster is the kinase signaling group, consisting of germline or somatic variants in *RET*, *NF1*, *HRAS*, *MAX*, and *TMEM127*. The third cluster is Wnt signaling group, which includes newly recognized somatic variants in *CSDE1* as well as somatic gene fusions affecting *MAML3*.

Among those genes, *RET*, *NF1*, and *VHL* are involved in three distinct clinical syndromes associated with PPGL: multiple endocrine neoplasia type 2 (MEN2) syndrome caused by *RET*, neurofibromatosis type I caused by *NF1*, and von Hippel-Lindau disease caused by *VHL*. Aside from those three syndromes, germline variants in the succinate dehydrogenase (SDH) genes are the most common cause of PPGL, occurring in up to 25% of all PPGL patients [8]. Clinical practice guidelines of PPGL recommend testing for germline variants in all patients by accredited laboratories [9,10]. Clinical characteristics of PPGL can be classified according to genetic clusters, which can lead to different follow-up tests and treatments guided by the underlying molecular cause. However, some patients may not express causative genes included in the molecular subtypes, thus remaining unclassified.

In this study, whole exome sequencing (WES) was used to screen for novel causative variants associated with PPGL to improve the detection rate of rare genetic variants in our cohort. Additional screening for variants in other genes related to cancerous disease or mitochondrial function was also performed.

METHODS

Subjects

Among patients diagnosed with PPGLs at the Seoul National University Hospital, 36 were recruited due to high risk of genetic diseases: metastasis ($n=9$), bilateral diseases ($n=2$), paraganglioma ($n=16$), aged under 35 years ($n=9$). Among them, 20 patients were negative for *SDHB*, *SDHD*, *VHL*, and *RET* genes

using Sanger sequencing and multiplex ligation-dependent probe amplification before March 2014. In March 2014, the targeted next-generation sequencing panel for PPGL was developed and used to test the additional 16 patients. All 16 patients were negative for *MAX*, *NF1*, *RET*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *TMEM127*, and *VHL*. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 2004-012-1115). Informed consent from all patients was obtained.

PPGL was diagnosed by elevated levels of catecholamine and/or histological confirmation after surgery. We conducted serum fractionated metanephrine or 24-hour urine catecholamine/fractionated metanephrine. Hormone type was classified as epinephrine, norepinephrine, or nonfunctioning [11]. Elevated metanephrine with or without high normetanephrine levels was designated as epinephrine type. Elevated normetanephrine levels without high metanephrine levels were considered as norepinephrine type. Nonfunctioning type indicated a normal range of fractionated metanephrine levels.

Thoraco-abdominal computed tomography (CT) or magnetic resonance imaging was performed for anatomical localization. ^{123}I -metaiodobenzylguanidine (MIBG), positron emission tomography/CT with ^{68}Ga -labeled DOTA⁰-Tyr³ octreotide (DOT-ATOC) or ^{18}F -labeled fluorodeoxyglucose (FDG) was conducted to detect multifocal lesions or metastasis. Metastasis was defined as the presence of PPGL tumors in non-chromaffin organs at diagnosis or during follow-up [12].

Molecular genetic testing

DNA was extracted from whole blood samples obtained from 36 patients. WES was performed using DNA samples enriched using TruSeq Custom Enrichment Kit and sequenced with MiSeq (Illumina Inc., San Diego, CA, USA). Sequencing alignment and variant calling were performed using SAMtools. Copy number variation (CNV) analysis for the genes included in the panel was not performed.

Variant filtering and interpretation of clinical significance

Exonic variants with nonsynonymous variants and intronic variants within 10 bp from the exonic region were included. Allele frequencies in normal controls (gnomeAD) and *in silico* prediction results were considered (SIFT, PolyPhen2, and MutationTaster). The highest minor allele frequency (MAF) in the patient population was taken into consideration, and variants that had MAF >0.1% were filtered out. Classification of each retained variant was performed according to the American College of

Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) 2015 guidelines [13]. For previously reported variants, segregation and functional test results were reviewed. Variants were screened for the 32 pheochromocytoma-related genes (*ATRX*, *BRAF*, *CDKN2A*, *DLST*, *DNMT3A*, *EGLN1*, *EGLN2*, *EPAS1*, *FGFR1*, *FH*, *GOT2*, *H3F3A*, *HRAS*, *IDH1*, *IDH2*, *IDH3B*, *KIF1B*, *KMT2D*, *MAX*, *MDH2*, *MERTK*, *MET*, *NF1*, *RET*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *SLC25A11*, *TMEM127*, and *VHL*). Genes related to other types of cancerous disease or encoding for mitochondria-localized proteins were screened for additional variants.

RESULTS

Patients' characteristics

Among the 36 patients included in this study, 19 were female and 17 were male. The mean age at the time of diagnosis was 40.2 (range, 12 to 85) (Table 1). Seventeen patients were diagnosed with pheochromocytoma, four bilateral and two multifocal, while 19 patients were diagnosed with paraganglioma. None of the patients had a family history of pheochromocytoma or paraganglioma. Nine patients presented metastatic lesions, and three patients showed relapse during the follow-up period.

Identification of germline variants in pheochromocytoma-related genes

Average coverage depth in target regions of the whole exome panel was 80.7X; 99.1% of the bases had coverage of $\geq 20X$, which was the minimal level of acceptable coverage considered. Among 36 patients, 14 patients were found to carry at least one variant of interest (VOI) in 32 pheochromocytoma-related genes. A total of 15 VOIs were detected, two were classified as likely pathogenic variants and 13 as variants of uncertain significance (VUS) (Table 2). *SDHA* c.778G>A (p.Gly260Arg) was detected in a patient negative for *SDHB*, *SDHD*, *VHL*, and *RET* genes. This was a previously reported variant in paraganglioma [14-16], known to be a loss-of-function variant according to functional studies [15]. *KIF1B* c.2787-2A>C, a likely pathogenic variant that had not been previously reported, was detected in a patient with a bladder paraganglioma. Other variants detected in pheochromocytoma-related genes lacked strong supporting evidence for pathogenic classification. *SDHC* c.478G>A (p.Val160Met) has not been reported previously, but other missense variants near this amino acid residue such as p. Leu158Pro and p. Leu161Val had been detected in PPGL patients [17-19]. *FH* c.418G>C (p.Val140Leu) had been submitted in ClinVar as a VUS, but

c.419T>G (p. Val140Gly) involving the same amino acid residue has been reported in leiomyomatosis and renal cell cancer patients [20]. In addition, a novel nonsense variant, c.914G>A (p.Trp305*) in *DNMT3A*, showed variant allele frequency of 18% in exome sequencing data, and subsequent validation by Sanger sequencing showed a small alternate peak in the region (Supplemental Fig. S1).

Identification of germline variants in other genes

Also, we screened for germline variants in other cancer-related genes or mitochondria-related genes. One likely pathogenic variant in *BRCA2* and one VUS in *TP53* were detected in cancer-related genes (Table 2). A *BRCA2* splice-site variant, c.8488-1G>A, was detected in a 25-year-old male patient with early-onset paraganglioma. He had no personal cancer history, nor a family history of cancer related to *BRCA2*. A patient with VUS in *TP53*, c.566C>T (p.Ala189Val), had previously been diagnosed with breast cancer, endometrial polyp and also had a brother who had been diagnosed with choriocarcinoma. Additionally, we found a missense VUS c.473G>A in *NFU1*, which is a causative gene of multiple mitochondrial dysfunctions syndrome 1 (MMDS1).

DISCUSSION

Among the 36 patients found to be negative for routine clinical gene testing, only two were found to be positive for likely pathogenic variants (2/36=5.6%). *SDHA* c.778G>A (p.Gly260Arg) was shown to be a loss-of-function variant in functional studies in a yeast strain lacking Sdh1 [15]. Pathogenic germline *SDHA* variants were previously identified in 7.6% of patients with PGL, with diagnosis occurring at a significantly younger age in patients carrying the *SDHA* variants [21]. The patient carrying the likely pathogenic *SDHA* variant in this study was diagnosed with head and neck paraganglioma at the age of 20 and was the second youngest patient of our study cohort. Missense variants in the *KIF1B* gene had been previously detected in samples of pheochromocytoma [22,23], along with a splice site variant [24]. Yet, no previous reports of paraganglioma with a pathogenic *KIF1B* variant have been published. Our patient carrying a *KIF1B* c.2787-2A>C had a bladder paraganglioma, which may be the first paraganglioma to be reported carrying a *KIF1B* variant. The overall positive rate of pathogenic variants in the whole cohort of the apparently sporadic PPGL in our institution was 21.7% (35 among 161 PPGL patients). The most commonly mutated gene was *RET* (31.4%), followed by *VHL* (25.7%),

Table 1. Clinical Characteristics of the Patients Included in This Study

ID	Sex	Age at Dx	uPCC	Bilateral	HNPGL	PGL (others)	Tumor size (max)	Meta	Multifocal	Hormone type	Recurrence	FHx of PPGL	FHx of other cancer	Variant of interest
1	F	46	N	Y	N	N	8.5	Y	N	E/M	Y	N	NA	
2	M	48	Y	N	N	N	NA	Y	N	E/M	N	N	NA	
3	F	39	N	Y	N	Y	6.7	Y	Y	E/M	N	N	NA	
4	M	86	N	N	N	Y	11.8	N	N	E/M	N	N	NA	NM_000143.3(FH);c.260G>A p.(Arg87His)
5	F	54	N	N	N	Y	5.5	N	N	E/M	N	N	NA	NM_002168.3(IDH2);c.424A>C p.(Ile142Leu) NM_001933.4(DLST);c.973C>T p.(Arg325Trp)
6	M	52	Y	N	N	N	NA	Y	N	NE/NM	Y	NA	NA	
7	F	60	Y	N	N	N	NA	Y	N	NE/NM	N	N	NA	NM_003001.3(SDHC);c.478G>A p.(Val160Met)
8	M	60	N	N	N	Y	6.5	N	N	NE/NM	N	N	NA	
9	F	33	N	N	N	Y	9.9	N	N	NE/NM	N	N	NA	
10	M	36	N	N	N	Y	8	N	N	E/M	N	N	NA	NM_022552.4(DNNMT3A);c.914G>A p.(Trp305*)
11	M	38	N	N	Y	N	2.7	Y	N	NA	N	N	NA	
12	M	24	Y	N	N	N	5	N	N	NE/NM	N	N	NA	
13	F	27	N	Y	N	Y	2.5	N	Y	E/M	N	N	NA	NM_001430.4(EPAS1);c.1565A>G p.(Asn522Ser)
14	F	27	N	N	N	Y	3.4	N	N	NE/NM	N	N	NA	NM_000077.4(CDKN2A);c.236C>T p.(Thr79Ile)
15	M	47	N	N	N	Y	5.9	N	N	E/M	N	N	NA	NM_003482.3(KMT2D);c.15707A>G p.(Asn5236Ser)
16	F	20	N	N	Y	N	3.2	N	N	NA	N	N	NA	NM_004168.3(SDHA);c.778G>A p.(Gly260Arg)
17	M	68	N	N	N	Y	5.5	N	N	E/M	N	N	NA	NM_002168.3(IDH2);c.247G>A p.(Asp83Asn)
18	M	67	N	N	N	Y	2	N	N	E/M	N	N	NA	NM_053046.3(EGLN2);c.773C>G p.(Ala258Gly)
19	M	50	Y	N	N	N	9.3	N	N	E/M	N	N	NA	
20	F	45	N	N	N	Y	3.5	N	N	NE/NM	N	N	NA	

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Table 1. Continued

ID	Sex	Age at Dx	uPCC	Bilateral	HNPGL	PGL (others)	Tumor size (max)	Meta	Multifocal	Hormone type	Recurrence	FHx of PPGL	FHx of other cancer	Variant of interest
21	F	55	N	N	N	Y	13	Y	N	E/M	N	N	NA	NM_000143.3(FH):c.418G>C p.(Val140Leu) NM_015700.3(NFUI):c.473G>A p.(Arg158Gln)
22	F	56	Y	N	N	N	3.5	N	N	E/M	N	N	NA	NM_015074.3(KIF1B):c.2787-2A>C p.?
23	F	48	N	N	N	Y	3	N	N	NA	N	N	NA	
24	F	45	N	N	N	Y	4.8	N	N	E/M	N	N	NA	
25	M	36	N	N	N	Y	4.8	N	N	NA	N	N	NA	NM_003482.3(KMT2D):c.4987G>A p.(Glu1663Lys)
26	M	29	Y	N	N	N	3.8	N	N	NE/NM	N	N	NA	
27	M	23	Y	N	N	N	2.4	N	N	E/M	N	N	NA	
28	F	22	Y	N	N	N	6	N	N	E/M	N	N	NA	NM_003482.3(KMT2D):c.4942G>A p.(Asp1648Asn)
29	F	35	N	Y	N	N	5.5	N	N	E/M	N	N	NA	
30	F	23	Y	N	N	N	3.5	N	N	NE/NM	N	N	NA	
31	F	43	N	N	N	Y	4.5	N	N	NE/NM	Y	N	Rt: breast cancer, endometrial polyp Brother with choriocarcinoma, metastasis to lung, kidney, brain	NM_000546.5(TP53):c.566C>T p.(Ala189Val)
32	F	51	Y	N	N	N	8.0	Y	N	NE/NM	N	N	Sister: breast cancer Brother: thyroid cancer	
33	M	27	N	N	N	Y	11.5	Y	N	E/M	N	N	None	
34	F	42	Y	N	N	N	1.5	N	N	E/M	N	N	None	
35	M	12	Y	N	N	N	2.5	N	N	NE/NM	N	N	None	
36	M	25	N	N	N	Y	4.7	N	N	NE/NM	N	N	None	NM_000059.3(BRCA2):c.8488-1G>A p.?

Dx, diagnosis; uPCC, unilateral pheochromocytoma; HNPGL, head and neck paraganglioma; PGL, paraganglioma; Meta, metastasis; FHx, family history; PPGL, pheochromocytoma and paragangliomas; N, not present; Y, present; E/M, epinephrine/metanephrine; NA, not available; NE/NM, norepinephrine/normetanephrine.

Table 2. Variants of Interest Detected in the Patients

ID	Gene	Transcript	Base change	AA change	<i>In silico</i> prediction (SIFT/Polyphen/Mutation Taster)	gnomAD MAX frequency ^a	ACMG-AMP classification
23	<i>KIF1B</i>	NM_015074	c.2787-2A>C	p.?	-/-/D	-	LP
16	<i>SDHA</i>	NM_004168	c.778G>A	p.Gly260Arg	D/D/D	-	LP [14-16]
14	<i>CDKN2A</i>	NM_000077	c.236C>T	p.Thr79Ile	D/D/D	EAS 0.006%	VUS
5 ^b	<i>DLST</i>	NM_001933	c.973C>T	p.Arg325Trp	D/D/D	AFR 0.012%	VUS
10	<i>DNMT3A</i>	NM_175629	c.914G>A ^c	p.Trp305 ^a	-/-/D	AFR 0.0062%	VUS
18	<i>EGLN2</i>	NM_053046	c.773C>G	p.Ala258Gly	T/B/D	EAS 0.011%	VUS
13	<i>EPAS1</i>	NM_001430	c.1565A>G	p.Asn522Ser	T/B/N	EAS 0.033%	VUS
4	<i>FH</i>	NM_000143	c.260G>A	p.Arg87His	D/D/D	EAS 0.033%	VUS
21 ^b	<i>FH</i>	NM_000143	c.418G>C	p.Val140Leu	D/B/D	EAS 0.0054%	VUS
17	<i>IDH2</i>	NM_002168	c.247G>A	p.Asp83Asn	D/D/D	-	VUS
5 ^b	<i>IDH2</i>	NM_002168	c.424A>C	p.Ile142Leu	D/P/D	-	VUS
28	<i>KMT2D</i>	NM_003482	c.4942G>A	p.Asp1648Asn	D/P/D	EAS 0.015%	VUS
25	<i>KMT2D</i>	NM_003482	c.4987G>A	p.Glu1663Lys	D/D/D	NFE 0.001%	VUS
15	<i>KMT2D</i>	NM_003482	c.15707A>G	p.Asn5236Ser	T/P/D	EAS 0.006%	VUS
7	<i>SDHC</i>	NM_003001	c.478G>A	p.Val160Met	D/P/D	-	VUS
36	<i>BRCA2</i>	NM_000059	c.8488-1G>A		-/-/D	-	Pathogenic for breast/ovarian cancer
21 ^b	<i>NFUI</i>	NM_015700	c.473G>A	p.Arg158Gln	D/D/D	EAS 0.0054%	VUS
31	<i>TP53</i>	NM_000546	c.566C>T	p.Ala189Val	D/D/D	EAS 0.027%	VUS

AA, amino acid; ACMG-AMP, American College of Medical Genetics and Genomics and the Association for Molecular Pathology; D, deleterious/damaging/disease causing; LP, likely pathogenic; EAS, East Asian; VUS, variant of uncertain significance; AFR, African; T, tolerated; B, benign; N, polymorphism; P, possibly damaging; NFE, non-Finnish European.

^aHighest minor allele frequency among different populations (gnomAD); ^bTwo variants detected in the same patient (ID5, ID 21); ^cA likely hematopoietic somatic mosaic variant.

SDHB (17.1%), and *SDHD* (14.3%) (unpublished data). Positive rate for *SDHA* was 2.9%, though the result seems underestimated since *SDHA* has been included in the panel recently. Our data showed targeted gene panel with extended genes related to PPGL would benefit by increasing the positive rate of pathogenic variants.

Among the VUSs, a novel nonsense variant, c.914G>A (p.Trp305*) was detected in *DNMT3A*. This variant may be a likely hematopoietic somatic mosaic variant unrelated to the paraganglioma. Germline variants of *DNMT3A* previously reported in paraganglioma had been gain-of-function missense variants [25], while most of the likely hematopoietic somatic mosaic variants detected in multiple cancers were loss-of-function variants [26].

In other cancer-related genes, *BRCA2* c.8488-1G>A, detected in a 25-year-old male patient with early-onset paraganglioma. The patient had no personal history or family history of

BRCA2 related cancer. Germline *BRCA1/2* variants, most commonly associated genes in familial breast and ovarian cancer, are also known to be associated with other cancers such as prostate, colon, gastric, pancreatic cancer. *BRCA1/2* variants are not regarded as genetic causes for adrenal tumors, but there had been a previously reported case of pheochromocytoma who carried *BRCA2* variants [27]. A 40-year-old Ashkenazi woman was diagnosed with pheochromocytoma, and later diagnosed with infiltrating ductal carcinoma at 61 years of age. The patient carrying a *BRCA2* splicing variant in our study had been diagnosed with paraganglioma at the age of 25, and yet he had not been diagnosed with additional cancer until now. However, at 15 months of age, the patient underwent a Fontan operation [28]. Thus, hypoxic condition may be the second hit for development for paraganglioma. Although the causative role of this variant for the diagnosis of pheochromocytoma cannot be proven, the *BRCA2* germline variants may be associated with an increased

risk for adrenal tumors. Another missense VUS, c.473G>A (p.Arg158Gln) in *NFUI*, was found in an individual who also carried a missense variant c.418G>C (p.Val140Leu) in *FH*. *NFUI* is an essential iron–sulfur (Fe/S) protein implicated in multiple metabolic pathways and energy production, and acts as a maturation factor of respiratory complex II (SDH) [29]. Though this gene is known to be associated with MMDS1, which is inherited in an autosomal recessive pattern, a variant that affects the function of the protein may be involved with compromised SDH function [30,31]. Association of the disease and the *BRC42* variant, as well as VUS detected in other genes, should be assessed in further studies.

This study has several limitations. Though VUS reclassification is considered important as the genetic testings are becoming more available [32], familial screening was not performed in any of the patients. Segregation data would have provided more evidence that can lead to the reclassification of numerous VUS detected. Also, gross defects such as CNV were excluded, only analyzing single nucleotide variants or small insertion/deletions. Moreover, somatic variants in the tissue were not analyzed though it would have explained the additional driver alteration of the disease other than the germline portion. Further evaluation regarding family testing, CNV analysis, and sequencing of the tissue samples would improve the overall detection rate of the causative genetic variants.

In conclusion, we analyzed the WES data of PPGL patients with no causative genetic variant detected in routine clinical gene testing. Likely pathogenic variants were detected in two patients, which led to a 5.6% increase in molecularly confirmed PPGL patients. While implementation of WES for detection of germline variants in PPGL patients has not yet been widely adopted in clinical laboratories, implementation of targeted gene sequencing consisted of extended genes of PPGL in routine clinical screening can support the level of comprehensive patient assessment.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea by the grant NRF-2020R1A2C1014419.

AUTHOR CONTRIBUTIONS

Conception or design: S.H.S., J.H.K., M.W.S. Acquisition, analysis, or interpretation of data: S.H.S., J.H.K., M.J.K., S.I.C., S.J.K., H.K., C.S.S., S.S.P., K.E.L., M.W.S. Drafting the work or revising: S.H.S., J.H.K., K.E.L., M.W.S. Final approval of the manuscript: K.E.L., M.W.S.

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