# A Novel *SDHB* IVS2-2A>C Mutation Is Responsible for Hereditary Pheochromocytoma/Paraganglioma Syndrome

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Pheochromocytomas and paragangliomas are neuroendocrine tumors which arise from adrenal medulla, and sympathetic or parasympathetic nerves, respectively. Hereditary cases afflicted by both or either pheochromocytomas and paragangliomas have been reported: these are called hereditary pheochromocytoma/ paraganglioma syndromes (HPPS). Many cases of HPPS are caused by mutations of one of the succinate dehydrogenase (SDH) genes; mainly SDHB and SDHD that encode subunits for the mitochondrial respiratory chain complex II. In this study, we investigated mutations of SDH genes in six HPPS patients from four Japanese pedigrees using peripheral blood lymphocytes (from one patient with pheochromocytoma and five patients with neck paraganglioma) and tumor tissues (from two patients with paraganglioma). Results showed that all of these pedigrees harbor germline mutations in one of the SDH genes. In two pedigrees, a novel IVS2-2A>C mutation in SDHB, at the acceptor-site in intron 2, was found, and the tumor RNA of the patient clearly showed frameshift caused by exon skipping. Each of the remaining two pedigrees harbors a reported missense mutation, R242H in SDHB or G106D in SDHD. Importantly, all these mutations are heterozygous in constitutional DNAs, and two-hit mutations were evident in tumor DNAs. We thus conclude that the newly identified IVS2-2A>C mutation in SDHB is responsible for HPPS. The novel mutation revealed by our study may contribute to improvement of clinical management for patients with HPPS.

**Keywords:** germline mutation, hereditary pheochromocytoma/paraganglioma syndromes (HPPS), paraganglioma, pheochromocytoma, succinate dehydrogenase

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# Introduction

Paragangliomas are tumors that arise from the neuroendocrine paraganglia that occur along the paravertebral axis from the base of the skull through to the pelvis and are divided into two types; those derived from parasympathetic or from sympathetic paraganglia. Parasympathetic paraganglia associated with paraganglioma syndromes include carotid body paragangliomas at carotid bifurcation; glomus vagal tumors along the vagal nerve; and glomus jugular tumors in the jugular foramen. The carotid body is the most common site for tumorigenesis in familial paraganglioma. The major function of the carotid body is to prevent hypoxia by monitoring partial pressure of oxygen in the arterial blood ( $PaO_2$ ), partial pressure of carbon dioxide in the arterial blood ( $PaCO_2$ ), and pH of arterial blood; once hypoxia is detected, upregulation of blood oxygen is induced through upregulation of ventilation. Clinical symptoms in patients with paraganglioma depend on the site of origin, and tumor formation along with symptoms such as hypertension and headache may occur. About 10% of all head and neck paragangliomas are malignant, and better understanding of their pathogenesis is critical. Invasion or metastases to the lymph nodes or distant organs are the definitive criteria for final diagnoses (Benn et al. 2015).

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Pheochromocytomas are malignant tumors composed of chromaffin cells, which synthesize and release excess amounts of catecholamines and, in some instances, peptide hormones. Pheochromocytomas are characterized by severe hypertension, tachycardia, palpitations, headache, sweating, tremor, and a sense of apprehension. They also cause psychiatric symptom diseases and over all, decrease quality of life. They mainly originate from the adrenal medulla (Benn et al. 2015).

In some pedigrees, patients develop paraganglioma and/or pheochromocytoma: these tumors are categorized as hereditary pheochromocytoma/paraganglioma syndrome (HPPS) (Pritchett 1982). Germline mutations in specific genes are found in 25-30% of HPPS overall (Gimenez-Roqueplo et al. 2012). Several investigators (Baysal et al. 2002; Neumann et al. 2002) have reported that many HPPS cases are caused by germline mutations in genes that encode components of the succinate dehydrogenase (SDH) of mitochondria complex II, namely SDHB, SDHC and SDHD. SDH localizes at the mitochondrial membrane and is one of the important factors in both the mitochondria respiratory chain and the Krebs cycle. SDH works as a tetramer and a defective protein caused by a mutation in one of the SDHB, SDHC or SDHD genes will almost certainly cause the loss-of-function form of complex II, thereby preventing catalytic and electron transport functions. Loss of SDH function is reported to decrease apoptosis and increase the production of reactive oxygen species (Gottlieb and Tomlinson 2005). Succinate, the compound on which the SDH enzyme acts, is an oxygen sensor in the cell and can help turn on specific pathways that stimulate cells to grow in hypoxia. In particular, succinate stabilizes hypoxiainducible factor (HIF) by preventing a reaction that would allow HIF to be broken down (Selak et al. 2005; Cervera et al. 2008; Guzy et al. 2008). HIF controls several important genes involved in cell division and the formation of new blood vessels, including vascular endothelial growth factor (VEGF) in a hypoxic environment, which induces tumorigenesis (Gottlieb and Tomlinson 2005).

It has been reported that approximately 40% of all paragangliomas and 3% of all pheochromocytomas are associated with SDH deficiency (Gill et al. 2010a, b). Thus, there remains a need for additional investigation of SDH in order to better understand this disorder. In this study, we analyzed six patients from four pedigrees with probable HPPS.

## **Materials and Methods**

#### Patients

A total of six patients in four pedigrees were analyzed, as shown in Fig. 1. Both tumor tissues and peripheral blood lymphocytes were obtained from two patients (pedigree 1 II-1 and pedigree 3 II-1), but only peripheral blood lymphocytes were obtained from the others. Samples from patients II-1 in pedigree 1 and II-1 in pedigree 3 were obtained from Iwate Medical University Hospital (Morioka, Japan), and other samples were obtained from Tohoku University Hospital (Sendai, Japan). Written informed consent was obtained from each patient, and this research was assessed and accepted by our institutional review board under the accession number of 2017-1-1065. One patient (pedigree 2 II-1) was diagnosed with pheochromocytoma, and other five patients with neck paragangliomas. The tumors were resected and, at the same time, the corresponding peripheral blood lymphocytes were collected at the Iwate Medical University Hospital, Iwate, Japan, and Tohoku University Hospital, Miyagi, Japan.

#### DNA extraction

Genomic DNAs were extracted from peripheral blood cells following the standard procedure (Green and Sambrook 2012). Genomic DNAs from the paraganglioma tumor tissues were extracted using DNeasy Blood & Tissue Kit (QIAGEN, Tokyo, Japan), according to supplier's recommendation.

#### Mutation analyses

Based on previous investigations, we analyzed the *SDHB*, *SDHC*, and *SDHD* genes. Target nucleotides were amplified by using the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) (Kimura et al. 1996). Nucleotide sequences were determined by methods described previously (Sakurada et al. 1997) using an ABI PRISM310 Genetic Analyzer (Applied Biosystems).

#### PCR-RFLP analysis

To examine the genetic alterations, PCR-RFLP methods were utilized (Ogawa et al. 2006). The mutated nucleotide sequences of pedigree 1 II-1 and pedigree 2 II-1 created a novel *TaqI* restriction endonuclease recognition site, and pedigree 3 I-1, I-2 and II-1 created a novel *Ava*II restriction endonuclease recognition site. PCR amplified fragments were subjected to *TaqI* and *Ava*II digestion, respectively, followed by electrophoresis in 2% agarose gels. By comparing intensities of bands, we could estimate the ratios of mutant to normal DNA.

## RT-PCR analysis

RNAs were extracted from the tumor tissue specimens using RNeasy Midi kits (QIAGEN, Tokyo, Japan). Then these RNAs were reverse transcribed using Super Script II Reverse Transcriptase (Invitrogen, Carlsbad, CA) (Ogawa et al. 2006) to produce cDNAs. Nucleotide sequences of the primers and the PCR conditions for RT-PCRs are summarized in Table 1.

#### SNV analysis

In order to prove that the mutations we identified are not single nucleotide variants (SNVs) which harbor in the healthy population, we searched the SNV data base (Integrative Japanese Genome Variation Database (IJGVD) which was recently and newly established (Nagasaki et al. 2015) after the Tohoku Earthquake in 2011. The IJGVD data base (https://ijgvd.megabank.tohoku.ac.jp) is originated from 1070 Japanese individuals through whole genome sequencing by NGS. It is a database with the highest definition. To date, most (over 99%) of the SNVs in Japanese ethnic group are thought to be covered in this database.

## **Results**

In the present study, as summarized in Fig. 1, we analyzed DNAs and RNAs, if available, from six patients with either paraganglioma or pheochromocytoma. Mutations of



Fig. 1. Pedigrees 1 through 4 analyzed with hereditary pheochromocytoma/paraganglioma syndrome in this study. Round and square symbols denote females and males, respectively, and a line with // indicates divorce. Patient symbol filled in right-side has pheochromocytoma (II-1 in pedigree 2), and those filled in left-side have paragangliomas. Two cases from whom both tumor tissue and peripheral blood lymphocytes were available are indicated by double underbars (II-1 in pedigree 1 and II-1 in pedigree 3). Only peripheral blood lymphocytes were available from other patients that are indicated by single underbars (II-1 in pedigree 2, I-1 and I-2 in pedigree 3, and II-1 in pedigree 4).

the entire exonic regions and the exon-intron boundaries of the *SDHB*, *SDHC* and *SDHD* genes were investigated by direct sequencing of genomic DNAs from peripheral blood cells and tumor cells, if available. Results are shown in Figs. 2 and 3.

Patients II-1 of pedigree 1 and II-1 of pedigree 2 (see Fig. 2a) showed the same A to C transversion at the 3' splicing acceptor site of intron 2 of the *SDHB* gene (IVS2-2A>C, A to C alteration at the nucleotide-2 of intron 2); it may cause aberrant splicing. It is notable that the wave intensity of A is much weaker than that of C in tumor DNA from patient II-1 in pedigree 1. This result suggests loss of the wild type A allele in tumor DNA whereas only mutant C allele remains; a two-hit mutation of *SDHB* was suspected. As far as we could determine from a literature survey, this alteration has never been reported. It created a novel *TaqI* recognition site; TCGA. We further performed PCR-RFLP analyses as shown in Fig. 2b. We amplified the 262-bp region containing the exon-intron boundary. If mutated, these 262-bp bands will be split into 166-bp and 96-bp by

*TaqI.* Results clearly indicated that both patients harbored a heterozygous mutation. Furthermore, the 262-bp band is much fainter in the tumor DNA from patient II-1 in pedigree 1; a two-hit mutation is strongly suspected.

To further analyze the effect of this alteration, we extracted mRNA from the tumor tissue from this patient (II-1 in pedigree 1) and performed RT-PCR. Primers were designed in exon 1 (forward) and exon 5 (reverse) to amplify the 517-bp product (see Table 1). Results of RT-PCR are demonstrated in Fig. 2c; the tumor cDNA harbored two distinct sized bands, one normal-sized band and one aberrant-sized band that is 86-bp shorter. The 86-bp correspond to the size of exon 3. On the other hand, only one normal-sized 517-bp band was observed in the wildtype cDNA. These bands were purified for nucleotide sequencing analyses. Results as shown in Fig. 2d, the aberrant band had lost the whole exon 3 sequence. Because exon 3 has 86-bp, the protein product from this aberrant splicing will produce a truncated product resulting from the frameshift.

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Table 1. Nucleotide sequences of the primers and PCR conditions.

	Primer sequ	sence $(5' \rightarrow 3')$	Annealing	DMSO	Size of
	Forward	Reverse	$T(^{\circ}C)$	(%)	product (bp)
SDHB			· /		
genomic PCR					
1F/1R	tattgcgcacgtcgctgt	ccctatgcttcctcagtctc	55	10	360
2F/2R	ccagcgttacatctgttgtg	catcggatgatctcagattttt	55	5	323
3F/3R	gataagctaatacatccaggtg	gtctctatcagctttggccag	58	5	262
4F/4R	tggcacagcaaggaggatcc	ctaatagcgtaacacacatagc	58	10	300
5F/5R	cctggcatagagtggacgag	cttatgttccctgccaagcca	58	5	317
6F/6R	ggcactttgttcatgcactg	cagcaatctattgtcctcttg	55	10	283
7F/7R	7F/7R gctttgagttgagccagggt cacatgctacttctggcgt		58	10	256
8F/8R	8F/8R gtttcacccaagattgtggg tcacattcccctgcggcaagt			5	348
RT-PCR					
F1/R5	cgcgatgttcgacgggacac	gtgcatagaagttgctcaaatc	60	0	517
F4/R8	ggattgacaccaacctcaataag	atgttcagatctgagctggt	60	0	544
F4n/R8n	ataaggteteaaaaatetaceet	gttataaatcatgtttagcatgg	60	0	508
SDHC					
genomic PCR					
1F/1R	gtcacatgacacccccaac	cccaggcacaggataaacag	58	0	251
2F/2R	caaggacactgatactgtcatt	ctccagcctgggtgacag	58	0	266
3F/3R	catgcctggcttggtattg	cetteagaacttteacceact	58	0	264
4F/4R	cagagcgagactctgtctc	ctagggccaatgaaacagcc	65	0	272
5F/5R	gaattgtatgaggtgccagg	cctcccagtctccccact	65	10	315
6F/6R	6F/6R ttttctctagaatcatgctgag tgacaaacagaaagatgggtg		58	0	283
RT-PCR		0 0 0 0000			
F1/R6	tcacttccgtccagaccgga	taccaggtactctactgctc	60	0	527
F1n/R6n	cgtccagaccggaacccaag	ctactgctccaaggagatet	60	0	509
SDHD					
genomic PCR					
1F/1R	ttcotcotcotogotogoaat	aggetacgetaggeacete	60	10	165
2F/2R	gtagettacetatggtcatttag	accattagtgaaggtaaagatgg	59	10	263
3F/3R	tttgggttactgtgtggcatatg	cagacaactatatttggaattgc	60	10	260
4F/4R	cagccaagttatctgtatagtctt	gcatacatcaattcttcaaagtat	60	10	279
RT-PCR	6	6			
F1/R4	attgtcgcctaagtggttcc	ttgtgagcttaatggcatgac	60	0	610
F1n/R4n	ggttggtggatgaccttgag	aggcatacatcaattcttcaaag	60	0	560

Numbers for the name of primers correspond to exon numbers, and primers with n are used for nested RT-PCR.

In pedigree 3, three individuals suffered from paraganglioma; I-1, I-2 and II-1. All the patients showed the same G to A transition in exon 4 of the *SDHD* gene (see Fig. 3a), which causes a G106D mutation, and this mutation co-segregated with disease. This alteration was heterozygous in DNA from peripheral blood cells, but it was homozygous in tumor DNA. This alteration created a novel *Ava*II recognition site, GG(T/A)CC, and results by the PCR-RFLP analyses using blood and tumor DNA supported the detected mutation by the sequencing analyses (data not shown).

In pedigree 4, patient II-1 showed a G to A transition in exon 7 of the *SDHB* gene (see Fig. 3b) that causes an R242H mutation. This alteration was heterozygous in the DNA from peripheral blood cells. An additional G to A transition in intron 2 of the *SDHB* gene (IVS2+33 G>A) and an insertion of additional TG dinucleotide in intron 1 of the *SDHC* gene (IVS1+12insTG) were also observed and were reported as SNV (data not shown). Results of our present study are summarized in Table 2.

## Discussion

In this study, we analyzed six patients' peripheral blood samples, five paraganglioma and one pheochromocytoma, from four pedigrees, and found that all of them harbored mutations in either *SDHB* or *SDHD*. Two pedigrees harbored a novel splice-site mutation in *SDHB* that causes exon skipping, one pedigree harbored a reported missense mutation in *SDHB*, and one pedigree harbored a reported missense mutation in *SDHD*; mutation in this family showed co-segregation with disease.



Fig. 2. Mutation analyses in pedigree 1 and 2 with hereditary pheochromocytoma/paraganglioma syndrome.
(a) Nucleotide sequencing results of DNAs from both tumor tissue and peripheral blood cells in patient II-1 in pedigree 1 and DNA from peripheral blood cells in patient II-1 in pedigree 2 are shown. A normal DNA sample from a healthy

1 and DNA from peripheral blood cells in patient II-1 in pedigree 2 are shown. A normal DNA sample from a healthy volunteer is also shown. An A to C transversion at the acceptor site of intron 2 of the *SDHB* gene (IVS2-2A>C, boxed) was exclusively observed in patient samples.

(b) Results of PCR-RFLP analyses are shown. Genetic alteration found in (a) creates a novel *TaqI* recognition sequence (TCGA). A PCR amplicon harboring this mutation site spanning 262-bp is subjected to *TaqI* digestion followed by a 2% agarose electrophoresis. Two *TaqI*-digested fragments from DNA samples with altered alleles, 166-bp and 96-bp, along with undigested 262-bp wild-type allele were clearly observed in patients' samples whereas only the wild-type 262-bp band was observed in DNA sample from a healthy volunteer.

(c) Results of RT-PCR clearly demonstrate two bands corresponding to full length (517-bp) and exon 3-deleted sized band (431-bp) in cDNA from the tumor of patient II-1 in pedigree 1, whereas only full length band was visible in wild-type cDNA.

(d) Results of nucleotide sequences of both bands from patient II-1 in pedigree 1 (in Fig. 2c) are shown. The 431-bp band seemed to derive from an mRNA with exon 3-skipping, and the 517-bp band derived from that without skipping. Because size of exon 3 is 86-bp, this short mRNA would produce a truncated protein due to frameshift. A minor fraction of contaminated the 431-bp band was also observed.

The novel *SDHB* IVS2-2A>C mutation in DNA from peripheral blood cells was heterozygous, but it was homozygous in the corresponding tumor DNA in patient II-1 in pedigree 1. Patient II-1 in pedigree 2 also harbored the same mutation in the constitutional DNA. The individuals in these pedigrees are not related. This A to C transversion mutation causes exon skipping of the 86-nt exon 3. Predicted protein product only harbors 67 of 180 full-length (a)



(a) Nucleotide sequencing results of DNAs from both tumor tissue and peripheral blood cells in patient II-1 in pedigree 3 and DNA from peripheral blood cells in patient II-1 in pedigree 3 are shown. A normal DNA sample from a healthy volunteer is also shown. A G to A transition at codon 106 (boxed) in exon 4 of the *SDHD* gene that would cause a substitution of aspartic acid for glycine (G106D) was observed in patient samples. A two-hit mutation in tumor DNA is suspected; no wild-type peak was observed.
(b) Nucleotide sequencing result of DNA from peripheral blood cells in patient II-1 in pedigree 4 is shown. A normal DNA sample from a healthy volunteer is also shown. A G to A transition at codon 242 (boxed) in exon 7 of the *SDHB* gene that would cause a substitution of histidine for arginine (R242H) was observed in the patient's sample.

Table 2. Summary of mutations found in six patients.

Pedigree 1	Patient	Age	Gender	tumor type	initial site of tumor	gene	mutation	treatment	predicted result
1	II-1	31	Female	paraganglioma	right carotid body	SDHB	IVS2-2A>C	resection	skipping of exon 3 that causes frameshift
2	II-1	18	Female	pheochromocytoma	left adrenal gland	SDHB	IVS2-2A>C	resection	skipping of exon 3 that causes frameshift
3	I-1	56	Female	paraganglioma	right carotid body	SDHD	G106D	resection	missense mutation
3	I-2	51	Male	paraganglioma	right vagal paraganglia, neck	SDHD	G106D	resection	missense mutation
3	II-1	30	Male	paraganglioma	right carotid body	SDHD	G106D	resection	missense mutation
4	II-1	50	Male	paraganglioma	left carotid body	SDHB	R242H	radiation	missense mutation

amino acids with unrelated frameshift-mediated 21 additional amino acids followed by a termination codon. Benn et al. (2015) reported that exons 3-4 and 6-7 of *SDHB* code for iron-sulfur cluster binding domain that is very important for SDHB function; a serious damage to SDHB function is expected. Therefore, based upon this study, we propose that this novel A to C transversion is likely responsible for carcinogenesis. Databases IJGVD (Nagasaki et al. 2015) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) show that this IVS2-2A>C mutation in *SDHB* does not match any SNV or variation. Furthermore, exon-skipping resulting in truncation of the protein product was observed.

SDHD codes for 159 amino acid-protein and the G106D mutation was previously reported by Ogawa et al. (2006). At that time, the G to A mutation was not found in

100 healthy control volunteers. Co-segregation of these mutation and onset of disease also supports the argument that this germline mutation is causative. Based on this previous report and our results, we suggest that this G to A mutation leads to the expression of cancer rather than a polymorphism. Furthermore, according to the InterPro database (https://www.ebi.ac.uk/interpro), this mutation appears to occur within the methyltransferase domain.

The R242H mutation found in pedigree 4 II-1 has been reported as a germline mutation of hereditary pheochromocytoma/paraganglioma syndromes (Young et al. 2017). This R242H mutation may be due to a common mutational mechanism that involves deamination of CpG to TpG.

In conclusion, mutations in *SDH* genes play important roles in the pathogeneses of HPPS. Elucidation of patho-

geneses of this syndrome may lead to better understanding of tumorigeneses of other cancers. However, our knowledge of the underlying mechanisms behind the carcinogeneses in *SDH* mutations is still limited. Further studies are needed to improve early detection and prevention, which may help to achieve effective clinical management for patients with HPPS.

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## **Conflict of Interest**

The authors declare no conflict of interest.

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