NOTE

Mutation analysis of the SDHB and SDHD genes in pheochromocytomas and paragangliomas: identification of a novel nonsense mutation (Q168X) in the SDHB gene

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Abstract. Pheochromocytoma (PCC) and paraganglioma (PGL) are tumors of the autonomic nervous system. The former is a tumor that occurs in only adrenal glands, and the latter can be found in the head and neck or in the thorax and abdomen. In PCC and PGL, genetic mutations account for approximately 30% of functional (secrete catecholamines) and non-functional cases. In addition to *RET*, *VHL* and *NF-1*, genes encoding succinate dehydrogenase complex subunit B (*SDHB*), subunit C (*SDHC*), and subunit D (*SDHD*) are recognized as susceptibility genes for PCC and PGL. Recently, PCC and PGL caused by genetic mutations of *SDHB*, *SDHC* and *SDHD* were established as hereditary pheochromocytoma paraganglioma syndrome (HPPS). Approximately 15% of all PCCs and PGLs are recognized as HPPS. Among these three susceptibility genes, *SDHB* and *SDHD* are known to be strongly related to HPPS. The aim of this study was to analyze *SDHB* and *SDHD* mutations in PCC and PGL patients. Among 18 patients, we identified a novel heterozygous nonsense mutation at codon 168 resulting in a CAG (glutamine) to TAG (stop) substitution (Q168X) in the *SDHB* gene in a patient diagnosed with solitary sporadic PGL. A number of studies have reported that *SDHB* mutation-associated disease demonstrates a higher rate of malignancy. However, all seven patients diagnosed with malignancy in this study did not have genetic mutation of *SDHB* and only one patient with no malignant sign had genetic mutation of *SDHB*. Further accumulation of cases is necessary to confirm the association between *SDHB* mutation and malignant potential.

Key words : Mutation analysis, *SDH*, Pheochromocytoma, Paraganglioma

PHEOCHROMOCYTOMA (PCC) and paraganglioma (PGL) are tumors of the autonomic nervous system and PCC is derived from chromaffin cells. In some cases, PCCs and PGLs can be associated with other endocrine tumors or nervous system diseases. Most of these tumors are benign and secrete catecholamines. Some tumors recur with metastasis within a few years after surgery and, therefore, long-term medical follow-up is needed in all cases.

Hereditary syndromes associated with PCCs are multiple endocrine neoplasia (MEN) type 2, von Hippel-Lindau disease (VHL) and neurofibromatosis

(NF) type 1, which are caused by genetic mutations of *RET*, *VHL* and *NF-1*, respectively. In 2000 and 2001, three genes, *SDHB*, *SDHC*, and *SDHD*, encoding subunits of the succinate dehydrogenase enzyme, a part of the mitochondrial respiratory chain, were identified as susceptibility genes for PCC and PGL [1-3]. Both *SDHB* (35.4kb, 8 exons) and *SDHC* (50.3kb, 6 exons) are located on chromosome 1 at 1p35 and 1q23, respectively. *SDHD* (8.9kb 4 exons) is located on 11q23. PCC and PGL caused by genetic mutation of one of these three genes are named as hereditary pheochromocytoma paraganglioma syndrome (HPPS). Among these, mutations of *SDHB* and *SDHD* play pivotal pathogenic roles in HPPS compared with those of *SDHC* [4, 6, 9]. An Italian study showed that genetic mutations account for approximately 30% of functional and non-functional cases of PCC and PGL, of which approximately 15% were recognized as

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Table 1 Characteristics of the patients.

No.	Sex, age at diagnosis (yr)	Primary tumor location, size (cm)	Metastatic location	Family history of PCC/PGL	Additional diagnosis
1	F, 61	Adrenal, 8.0	Negative	Negative	
2	F, 54	Adrenal, 6.0	Negative	Positive	
3	F, 58	Adrenal, 6.5	Liver, Lung	Negative	
4	F, 67	Adrenal, 3.0	Negative	Negative	
5	M, 50	Bilateral adrenal, ND	Bone Lymph node	Positive	VHL disease*, Islet cell tumor Cerebellar tumor, Aortic dissection
6	F, 75	Adrenal, 10.0	Lung	Negative	Ovarian tumor
7	M, 58	Adrenal, 2.8	Negative	Negative	Cephalic lipoma
8	M, 36	Abdominal, 6.5	Negative	Negative	
9	M, 57	Adrenal, 6.0	Lung Lymph node	Negative	Cardiac infarction, Diabetes Cerebral hemorrhage
10	M, 55	Adrenal, 6.0	Negative	Negative	Cardiac infarction, Diabetes
11	F, 65	Adrenal, ND	Liver, Lymph node	Negative	
12	M, 19	Adrenal, 5.4	Negative	Negative	Fundus hemorrhage, Cardiac arrest
13	F, 68	Abdominal, 1.0	Lymph node	Negative	Cerebral hemorrhage
14	M, 24	Abdominal, 2.2	Negative	Positive	VHL disease*
15	M, 30	Abdominal, ND	Bone, Liver, Lymph node	Positive	Diabetes
16	M, 73	Adrenal, 4.0	Negative	Negative	Diabetes
17	M, 78	Bilateral adrenal, 2.3	Negative	Negative	
18	F, 17	Adrenal, 5.4	Negative	Negative	

ND, not determined; M, male; F, female, *No.5 is the father of No.14.

HPPS [4]. *SDHA*, encoding another subunit of the succinate dehydrogenase enzyme, lies on 5p15 and is composed of 15 exons spread over a genomic region of 38.4kb. Genetic mutation of *SDHA* causes Leigh syndrome, a subacute necrotizing encephalomyelopathy during infancy.

SDH mutations are widely distributed along the gene with no hot spots. To date, several studies in Western countries [4-9] have revealed associations between mutated genes (genotype) and clinical characteristics (phenotype) of patients. Patients with mutation of *SDHB* show a high prevalence of abdominal PGL with malignant potential as the initial tumor. In contrast, those with *SDHD* mutation show multiple benign PGL lesions on the head and neck as the main tumor. However, there is no evidence as to whether these associations hold among Japanese cases. Since there are few reports on *SDH* mutations in HPPS in Asian countries [10, 11], we analyzed *SDHB* and *SDHD* mutations in 18 Japanese patients diagnosed with PCC and/or PGL.

Patients and Methods

This study included 18 Japanese patients with PCC

and/or PGL who were surgically treated at Hokkaido University Hospital during 2001-2009. We obtained written informed consent from all patients according to the medical ethics committee of Hokkaido University for the use of human materials for scientific purpose. Patients who presented with a head and neck or thoracic PGL were not included in this study. Four of the 18 patients had a family history of PCC or PGL, and two of them were parent and child suffered from VHL. Clinical and pathological characteristics of the 18 patients are shown in Table 1.

We performed genetic sequencing of the 8 exons of *SDHB* and 4 exons of *SDHD* using tumor specimens. Genomic DNA was isolated by using the DNeasy Blood & Tissue Kit (QIAGEN, Tokyo, Japan). Each exon of the *SDHB* and *SDHD* genes was amplified using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. Briefly, PCR amplifications were performed in 20 μ L reactions and the conditions were as follows: initial denaturation at 95 °C for 9 min, then 35 cycles of denaturation at 95 °C for 15 sec, annealing at 51 °C for 30 sec, and extension at 72 °C for 30 sec, and a final extension at 72 °C for 7 min. The nucleotide sequences of the primers used are list-

Table 2 List of primers in PCR.

Gene		Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>SDHB</i>	exon 1	CCCTTTCTGAGAAGGTCACG	AGGCAGTCTCTGTGGCTTTC
	exon 2	TTTTTCCTTTTGTGAACTTT	AAGCATGTCCCTAAATCAAA
	exon 3	GAACCTTACATAAATACCACTGGA	CTATCAGCTTTGGCCAGC
	exon 4	ATGGGTGAGGTGTGTTAATG	TGCAAATAAAAACAAAACCA
	exon 5	TGATGATGGAATCTGATCCT	CAGATTGAAACAATAAATAGGGA
	exon 6	CCTCTCTTTTCTCCCCATAC	CAGCAATCTATTGTCCTCTTG
	exon 7	AGCTAATCATCCCTGGTTTT	TTGTGAGCACATGCTACTTC
	exon 8	GTGGGTTTTCCCTTTTCAGTT	CGGCAAGTAAAGGAACAGGT
<i>SDHD</i>	exon 1	ATTGTCGCCTAAGTGGTTCC	AGGCTACGCTAAGCACCTCA
	exon 2	CAGTCCTGTTAAAGG	AGGTAAAGATGGCTAGAGCC
	exon 3	GGGTACTGTGTGGCATATG	CAGCAAACAACTGAGCAGA
	exon 4	GGGCAGCCAAGTTATCTGTA	CAGAGGCAAAGAGGCATACA

ed in Table 2 [1, 12]. Then, the PCR product was purified with QIA quick PCR Purification Kit (QIAGEN), and direct sequence analysis was performed with a Big Dye Terminator sequencing kit version 1.1 (Applied Biosystems), using the same primers on both strand. After direct sequence analysis, we determined the genetic mutation by sequencing of PCR products subcloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA).

Results and Discussion

We sequenced the *SDHB* and *SDHD* genes in 18 Japanese patients with PCC and/or PGL. A novel heterozygous nonsense mutation of CAG > TAG (Q168X) in the *SDHB* gene resulting in a glutamine-to-stop substitution was identified (Fig. 1) in one patient (No.8). Further, the single nucleotide polymorphism (SNP), GCC > GCA (A6A) in *SDHB* was identified in all patients. This SNP in *SDHB* is found in nearly all Japanese people [13]. The patient with the novel heterozygous nonsense mutation was a 36-year-old man who had been referred to Hokkaido University Hospital for further examination of a left peri-adrenal lesion. His height was 176 cm, weight was 80 kg, blood pressure was 117/84 mmHg without medication, and pulse rate was 62/min with regular sinus rhythm. Hematological and biochemical tests showed no abnormalities. Serum and urine catecholamines and their metabolite levels are shown in Table 3. Abdominal computed tomography (CT) scan

revealed a left peri-adrenal mass of 6.5 cm in diameter (Fig. 2) and ¹³¹I-metaiodobenzylguanidine (¹³¹I-MIBG) scintigraphy revealed radioactive iodide uptake only in this lesion. The tumor was laparoscopically resected and pathologically diagnosed as solitary sporadic PGL. He had no family history of PGL. He did not have hypertension, diabetes nor other endocrinological abnormalities.

Patients with a mutation in *SDHB* are known to have the following features. First, they have a high prevalence of abdominal PGL with malignant potential as the initial tumor. Second, patients typically show hypersecretion of noradrenaline and/or dopamine with lower secretion of adrenaline. This probably reflects reduced expression of phenylethanolamine - N - methyl - transferase, an enzyme that exists almost only in medullary chromaffin cells and converts noradrenaline to adrenaline. However, 10% of those tumors are biochemically silent so that diagnosis tends to be delayed by lack of symptoms until the tumor grows. Third, many patients with PGL clinically have no family history of PGL. A study in a Western country revealed that 90% (26 of 29) of patients with *SDHB*-mutated abdominal and thoracic PGL lacked a family history of PGL and all primary tumors but one originated from extra-adrenal locations [8]. As described above, PGL with *SDHB* mutations have been suggested to be primarily identified as an abdominal tumor with high malignant potential. Further, *SDHB* mutations are predictive of rapid metastatic spread and are associated with poor prognosis [14]. Therefore, long-term in-

Fig. 1 a) Genetic analysis of the SDHB gene in Patient 8. Nucleotide sequence around codon 168 in exon 5 of *SDHB*. Left, direct sequencing; Right, sequencing of PCR product subcloned into pGEM®-T Easy Vector. b) A summary of germline mutations of the SDHB gene. The notation of mutations is based on the TCA Cycle Gene Mutation Database (http://chromium.liacs.nl/lovd_sdh/home.php?select_db=SDHB). A novel mutation (Q168X), which is identified in the present study, is described in bold. Abbreviations: fs, frameshift; Ex, exon

tensive follow-up is needed for patients who are positive for *SDHB* mutation. According to Knudson's two-hit theory, patients with a germline mutation should have a somatic mutation or loss of the other allele. In general, somatic point mutations of the SDH gene seem to be very rare. To date, no somatic point mutations have been reported as part of a possible two-hit theory. In contrast, a

number of studies reported a somatic deletion mutation at a site proximal to the SDH gene on the wild-type *SDH* allele in both familial and sporadic tumors [9, 15-17]. These findings raise the possibility that unknown tumor suppressor genes may be involved in tumorigenesis. In the present case, loss of heterozygosity (LOH) was not examined. A more precise characterization of the somatic alterations associated

Table 3 Results of endocrinological tests in the patient with solitary sporadic PGL with a novel heterozygous mutation in *SDHB*.

Endocrinological tests	Result	Normal range
Serum catecholamines		
Adrenaline (pg/mL)	19	<100
Noradrenaline (pg/mL)	531	100 - 450
Dopamine (pg/mL)	19	<20
Urine catecholamines		
Adrenaline (μg/day)	13.2	<28
Noradrenaline (μg/day)	208	19 - 151
Dopamine (μg/day)	987	130 - 1200
Metanephrine (mg/day)	0.17	0.04 - 0.19
Normetanephrine (mg/day)	0.41	0.09 - 0.33

with *SDH* mutation will increase our knowledge of the mechanisms underlining tumorigenesis.

Although seven patients including five PCC and two abdominal PGL in the present study were strongly suspected of being mutation carriers based on their malignant signs, only one patient with no malignant sign had genetic mutation of *SDHB*. This result was fewer than expected. A number of studies have reported that patients with abdominal PGL have a higher incidence of mutation of *SDHB* than those with PCC. For example, an American study [5] reported that 16 of 44 (36.4%) patients with abdominal PGL had *SDHB* mutation compared to 9 of 313 (2.9%) patients with PCC, and similarly, another French study [6] reported that 17 of 58 (29.3%) patients with thoracoabdominal PGL had *SDHB* mutation compared to 7 of 314 (2.7%) patients with PCC. Furthermore, an American study [14] which analyzed *SDHB* mutation in a series of patients with malignant PCC and PGL reported that 12 of 29 (41.4%) patients with abdominal PGL had *SDHB* mutation compared to 1 of 15 (6.7%) patients with PCC. In the present study, 5 of 7 patients with malignancy had PCC and the other only 2 patients had abdominal PGL, lower rate of *SDHB* mutation might be due to the difference of tumor origin. Another reason for the lower rate of *SDHB* mutation may be due to racial differences. As a result, it would be difficult to indicate an association between *SDHB* mutation and malignancy from this study. On the other hand, a lack of *SDHD* mutation in the pres-

Fig. 2 Abdominal CT-scan in Patient 8. The arrow indicates the tumoral lesion in the left peri-adrenal gland.

ent study may have been caused by the source of tumor specimens. Because the prevalence of *SDHD* mutation is known to be higher at head and neck PGL than PCC or abdominal PGL [5, 7, 9].

We did not examine other susceptibility genes, *RET*, *VHL* and *NF-1*, for mutations. Indeed, a *VHL*-mutated parent and child were included in the present study. However, testing of all susceptibility genes in patients is not recommended from a cost-effectiveness perspective [4]. It is desirable to test for mutations after estimating the pathogenic gene from the patient's clinical characteristics. Several studies in Western countries have revealed associations between genotype and phenotype among patients and indicated the strategy as to which genes should be screened first [4]. However, there is no evidence to support that such correlations are suitable for patients in Asian countries including Japan. We analyzed *SDH* mutations in 18 patients of which number was too small to conclude the association. Finally, we hope that genotype-phenotype analysis of *SDH* mutations will lead to the development of an appropriate genetic screening test that would be performed on all patients with PCC and PGL.

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