A case of juvenile-onset pheochromocytoma with KIF1B p.V1529M germline mutation

Masahiro Nezu(1,2), Yosuke Hirotsu(2), Kenji Amemiya(2), Miho Katsumata(1), Tomomi Watanabe(1), Soichi Takizawa(1), Masaharu Inoue(1), Hitoshi Mochizuki(2), Kyoko Hosaka(3), Toshio Oyama(4) and Masao Omata(2,5)

1) Department of Endocrinology and Diabetes, Yamanashi Central Hospital, Kofu 400-8506, Japan
2) Genome Analysis Center, Yamanashi Central Hospital, Kofu 400-8506, Japan
3) Department of Urology, Yamanashi Central Hospital, Kofu 400-8506, Japan
4) Department of Pathology, Yamanashi Central Hospital, Kofu 400-8506, Japan
5) The University of Tokyo, Tokyo 113-0033, Japan

Abstract. In 2008, a familial noradrenergic pheochromocytoma (PCC) with a KIF1B germline mutation in exon 41 was reported in a 24-year-old female proband and her family. However, in 2020, the same research group reported that the cause of PCC in this family was a MAX germline mutation and was not due to the KIF1B mutation. In this study, we investigated the pathogenicity of a KIF1B germline mutation detected in a 26-year-old woman with juvenile-onset noradrenergic PCC. She was surgically treated and did not have a family history of PCC. We performed whole-exome sequencing, Sanger sequencing, and immunohistochemical and gene expression analyses of catecholamine-synthesizing enzymes. Three tumors with associated somatic mutations were used as the control group. Whole-exome sequencing revealed a p.V1529M KIF1B germline mutation in exon 41 in our patient, and no other associated germline and somatic mutations, including MAX, were detected. Sanger sequencing confirmed the presence of both mutant and wild-type alleles in the tumor. Among the catecholamine-synthesizing enzymes, the expression of phenylethanolamine-N-methyl transferase was suppressed. An in silico analysis of the p.V1529M mutation showed a score suggestive of pathogenicity. After evaluation with the international guideline for sequence variants, p.V1529M mutation was still classified as a variant with uncertain significance; however, our data, including the in silico analysis data, provided certain evidences that met the criteria supporting its pathogenicity. Therefore, this study can support future studies in proving the pathogenicity of the KIF1B p.V1529M mutation.

Key words: Juvenile-onset pheochromocytoma, Kinesin family member 1B, KIF1B, Whole-exome sequencing, Germline mutation

PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS (PPGLs) are catecholamine-producing neuroendocrine tumors that develop from chromaffin cells. Tumors that develop from the adrenal medulla are termed pheochromocytomas (PCCs) and those that develop from extra-adrenal paraganglia are termed paragangliomas [1]. Approximately 30%–40% of PPGLs were recently reported to harbor various germline mutations [2-4], including SDHB germline mutations that have a strong association with metastatic PPGL [5, 6]. These germline mutations are classified according to their molecular signals into a pseudohypoxia-related cluster (cluster 1), including SDH, VHL, and EPAS1, and a kinase signaling-related cluster (cluster 2), including RET, NF, TMEM127, MAX, and KIF1B [4]. In the somatic mutation of PPGL, a Wnt signaling-related cluster (cluster 3), including CSDE1 and MAML3, has also been classified [4].

KIF1B codes for kinesin family member 1B, a member of the kinesin-3 family, and belongs to the kinesin superfamily. It has two isoforms owing to different mRNA splicing mechanisms [7, 8]. The short form (KIF1BA) lacks a cargo-binding domain, and the long form (KIF1BB) contains a cargo-binding domain, which is coded by KIF1B. KIF1BB transports synaptic vesicle precursors, including synaptic vesicle proteins, such as synaptophysin (SYN), synaptotagmin, and Ras-related protein Rab-3A, to the axon tip [7, 8].

KIF1B germline mutations have been reported in some neurogenic tumors. In particular, the germline missense mutation p.S1481N in exon 41 of KIF1B was first
identified in a female proband with familial PCC in 2008. Consequently, KIF1B was recognized as one of the PPGL-associated mutations [7, 9]. Because the large size of the KIF1B gene makes it difficult for analysis using a genome panel, it has only been the subject of a few genetic studies targeting PPGL [10]. This could have hindered the elucidation of its role and association with clinical features in PPGL. In 2020, the group that reported the first PCC case with a p.S1481N KIF1B germline mutation declared a negative opinion about its role in PCC development [10]. They proposed that the germline mutation of MAX (c.145T>C), and not the KIF1B mutation, was the cause of bilateral PCC [10]. Thus, the clinical significance of KIF1B germline mutation remains controversial.

Herein, we report a case of juvenile-onset PCC with a KIF1B germline mutation in exon 41, which is the exact same exon that was implicated in the first reported case in 2008. In our case, no familial onset was confirmed, but in silico analysis suggested pathogenicity. We also report the endocrinological features based on immunostaining of catecholamine-synthesizing enzymes and gene expression analyses.

**Patient and Methods**

**Patient**

A 26-year-old woman was referred to our department with a 24-mm right adrenal tumor that was detected via abdominal ultrasound and a plain abdominal computed tomography (CT) scan. She was diagnosed with PCC, and we provided information on genetic testing because hereditary PCC was suspected for her age. Peripheral blood samples and tumor specimens were obtained. The genomic profile of the tumor in this patient was compared with those of three other patients who harbored PPGL-associated mutations that were known to be oncogenic or were likely to be oncogenic according to the OncoKB database (https://www.oncokb.org). Of these, two cases had mutations in HRAS (p.Q61K and p.Q61R), and the other case had an EPAS1 (p.P531S) mutation. Written informed consent was obtained from all patients. Genetic analyses, including somatic and germline mutation analysis, were approved by the Institutional Review Board of the Clinical Research and Genome Research Committee at Yamanashi Central Hospital (approval number G-2019-6) and complied with the principles of the Declaration of Helsinki.

**DNA sequencing and targeted sequences**

DNA was extracted from the peripheral blood samples of our patient using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Tumor DNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples using a GeneRead DNA FFPE Kit (Qiagen). The quality of the DNA extracted from the FFPE samples was determined using quantitative polymerase chain reaction (qPCR) with two primers targeting RNase P, as described previously [11, 12].

**Whole-exome sequencing**

Exome sequencing was performed as previously described with minor modification [13]. In brief, multiplex PCR was performed using genomic DNA with buffy coats and tumor FFPE DNA with a premixed primer pool using the Ion AmpliSeq™ Exome RDY (Thermo Fisher Scientific, Waltham, USA). PCR products were pooled and treated with FuPa reagent to partially digest primer sequences. The amplicons were ligated to adapters with the diluted barcodes of the Ion Xpress Barcode Adapters Kit (Thermo Fisher Scientific). Purification was performed using Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA, USA). Library concentration was determined using the Ion Library Quantitation Kit (Thermo Fisher Scientific). Emulsion PCR and chip loading were performed on the Ion Chef using the Ion PI Hi-Q Chef Kit. Sequencing was performed using the Ion PI Hi-Q Sequencing Kit on the Ion Proton Sequencer (Thermo Fisher Scientific).

**Sanger sequencing**

PCR was performed using genomic DNA extracted from the peripheral blood samples of our patient and tumors as templates. The primer pairs were designed to flank the single-nucleotide variant (SNV) sites in exon 41 (accession no. rs756027011; p.V1529M; chr1:10425677) of KIF1B. The PCR products were purified using ExoSAP-IT Express PCR Product Cleanup (Affymetrix, Santa Clara, USA). Sequencing was performed on a 3500 Genetic Analyzer using a BigDye Terminator v3.1 kit (both Applied Biosystems, Waltham, USA) and forward or reverse primers. The PCR products were purified and subsequently analyzed, as previously described [14, 15]. The primer sequences are provided in Table S1.

**Tumor sample preparation**

Tumor tissues were fixed using 10% buffered formalin and embedded in paraffin blocks. Then, 10 μm-thick serial sections were prepared and stained with hematoxylin and eosin. The sections were reviewed by a pathologist to check the tumor location.

**Immunohistochemistry**

Immunohistochemical staining of chromogranin-A (CgA), CD56, neuron-specific enolase, SYN, neurofilaments,
and S-100 were used for routine diagnosis of PPGL. To detect tyrosine hydroxylase (TH), dopa decarboxylase (DDC), dopamine β-hydroxylase (DBH), and phenylethanolamine-N-methyl transferase (PNMT) protein expression, the sections were incubated with mouse anti-TH monoclonal antibody (1:100; cat# T1299; Sigma-Aldrich, St. Louis, USA; RRID:AB_477560), rabbit anti-DDC polyclonal antibody (1:100; cat# AB136; Millipore, Burlington, USA; RRID:AB_90605), rabbit anti-DBH monoclonal antibody (1:100; cat# ab209487; Abcam, Cambridge, United Kingdom; RRID:AB_2892178), and mouse anti-PNMT monoclonal antibody (1:150; cat# TA502822; OriGene, Rockville, USA; RRID:AB_11125428), respectively, at 4°C overnight after blocking with Protein Block Serum-Free (DAKO, Tokyo, Japan), with partial reference to previous articles [16]. Subsequently, a Ventana Benchmark ULTRA fully automated immunostaining system (Roche Diagnostics, Basal, Switzerland) was used with an I-VIEW DAB Universal Kit (Roche Diagnostics) containing secondary antibodies (biotinylated mouse anti-goat immunoglobulin (Ig) G, biotinylated mouse anti-goat IgM, and biotinylated rabbit anti-goat IgG), avidin-horseradish peroxidase, and 3,3’-diaminobenzidine. Images were obtained using an Eclipse Ci upright clinical microscope (Nikon, Tokyo, Japan) and DP2-BSW digital camera software (Olympus, Tokyo, Japan).

**Gene expression analysis**

Total RNA samples prepared using an Ion Torrent Dx FFPE Sample Preparation Kit (Thermo Fisher Scientific) were reverse-transcribed using Superscript IV VILO Master Mix (Thermo Fisher Scientific). Reverse transcription (RT)-qPCR was performed on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) with Power Track SYBR Green Master Mix (Thermo Fisher Scientific) or TaqMan Gene Expression Assays (Thermo Fisher Scientific). Some primer sequences were constructed with reference to previous articles [16] (Table S1). Relative gene expression levels were calculated based on the threshold cycle values (Ct) using each primer set and standardized to Ct values for β-actin (ACTB) expression levels [17].

**In silico analyses**

Three in silico tools, namely, Sorting Intolerant from Tolerant (SIFT) [18], Polymorphism Phenotyping v2 (PolyPhen-2) [19], and Combined Annotation Dependent Depletion (CADD) [20, 21], were used to evaluate the impact of mutations on protein function, as previously described [12]. Mutations with a SIFT score of 0–0.05 are predicted as deleterious and those with a SIFT score of 0.05–1.0 are predicted as benign. Mutations with a PolyPhen score of 0–0.15 are predicted as benign, 0.15–1.0 as possibly damaging, and 0.85–1.0 as damaging. The SIFT and PolyPhen scores were obtained using the Ion Reporter software (Thermo Fisher Scientific) analysis. CADD Phred scores were normalized to all potential variants [12]. The bottom 90% (approximately 7.7 billion) of SNVs (approximately 8.6 billion) were compressed into scaled CADD Phred scores of 0–10, whereas the next 9% (top 10%–top 1%) were between 10 and 20, and the top 1% were >20 [20, 22], as previously described [12].

**Results**

**Patient information and clinical course**

Our patient had no relevant medical history and was not on any medication. She had no family history of thyroid, parathyroid, or adrenal tumors. She was 155.8 cm tall and weighed 45.9 kg. Her blood pressure was 112/81 mmHg, pulse rate was 113/min and body temperature was 36.2°C. Laboratory data for blood and 24-h urine samples demonstrated markedly elevated levels of plasma norepinephrine (3,356 pg/mL, reference range: 100–450 pg/mL), urinary norepinephrine (702.7 and 776.4 μg/day, reference range: 48.6–168.4 μg/day), and urinary normetanephrine (1.19 and 1.64 mg/day, reference range: 0.1–0.3 mg/day). The levels of plasma (159 pg/mL; reference range, <100 pg/mL) and urinary epinephrine (59.0 and 61.9 pg/mL; reference range, 3.4–26.9 pg/mL) and urinary metanephrine (0.16 and 0.24 mg/day; reference range, 0.0–0.2 mg/day) were slightly high and normal, respectively, indicating a noradrenergic phenotype (Table 1). Contrast-enhanced CT showed a 25-mm right adrenal tumor and no metastatic lesions, thyroid tumors, or parathyroid tumors. Iodine-123 metaiodobenzylguanidine scintigraphy and single-photon emission CT using iodine-123 were performed, confirming an uptake in the right adrenal tumor (Fig. 1). Based on these results, our patient was diagnosed with right adrenal PCC and underwent laparoscopic adrenalectomy with 32 mg/day doxazosin and 2.5 mg/day bisoprolol. No serious adverse events were observed during the surgery. The norepinephrine and normetanephrine levels were nearly normalized on day 5 after surgery (Table 1), and no recurrence or metastasis has been observed for 3 years postoperatively.

**Other clinical findings in our patient**

The patient had autism-like characteristics, and communication was somewhat difficult. Her younger brother had intellectual disabilities and used the services of a support facility. Micrognathism and funnel chest were observed, and her fingers were elongated and appeared
to have swan neck deformity. Her mother and younger brother also had funnel chest, and her mother had micrognathism. Thus, a genetic disorder, such as Marfan syndrome or related diseases, was suspected because of her elongated fingers and inherited funnel chest, but echocardiography showed an ejection fraction of 60% with no cardiac malformation or valvular disease, and a contrast-enhanced CT scan showed no aortic dissection. An ophthalmic examination did not reveal lens subluxation. She was referred to a gynecologist because of

Table 1  Endocrinology tests

| Blood (Before surgery) | Reference range |
|------------------------|-----------------|
| NE (pg/mL) 3,356      | (100–450)       |
| EPI (pg/mL) 159        | (<100)          |
| DA (pg/mL) 22          | (<20)           |
| PRA (ng/mL/hr) 1.0     | (0.3–2.9)       |
| PAC (pg/mL) 198        | (29.9–158.8)    |
| ACTH (pg/mL) 22.5      | (7.2–63.3)      |
| Cortisol (μg/dL) 12.1  | (6.2–18.0)      |
| DHEAS (μg/dL) 86       | (19–231)        |
| E2 (pg/mL) 111         | (25–550)        |
| Whole PTH (pg/mL) 22.6 | (8.3–38.7)      |

Table 1  Endocrinology tests (24 hr-urine collection)

| Blood (Before surgery) | Reference range |
|------------------------|-----------------|
| NMN (mg/day) 1.19      | (0.1–0.3)       |
| MN (mg/day) 0.16       | (0.0–0.2)       |
| NE (μg/day) 702.7      | (48.6–168.4)    |
| EPI (μg/day) 59.0      | (3.4–26.9)      |
| DA (μg/day) 435.8      | (365.0–961.5)   |
| VMA (mg/day) 7.3       | (1.5–4.3)       |
| HVA (mg/day) 2.7       | (2.1–4.3)       |
| Free cortisol (μg/day) | (11.2–80.3)     |
| ALD (μg/day) 2.7       | (<10.0)         |

Abbreviations: NE, norepinephrine; EPI, epinephrine; DA, dopamine; PRA, plasma renin activity; PAC, plasma aldosterone concentration; ACTH, adrenocorticotropic hormone; DHEAS, dehydroepiandrosterone sulfate; E2, estradiol; PTH, parathyroid hormone; NMN, normetanephrine; MN, metanephrine; VMA, vanillylmandelic acid; HVA, homovanillic acid; ALD, aldosterone.

Fig. 1  Images of the right adrenal tumor in our case. (A) Contrast-enhanced computed tomography (CE-CT) revealing a 24-mm right adrenal tumor (orange arrowhead). (B) Iodine-123 metaiodobenzylguanidine (123I-MIBG) scintigraphy demonstrated intense uptake, which was consistent with the tumor (black arrowhead).
hypertestosteronemia (total testosterone 1.73 ng/mL, reference range: 0.11–0.47) and was diagnosed with polycystic ovary syndrome.

**Gene mutation and in silico analysis**

Whole-exome sequencing (WES) analysis revealed a heterozygous missense germline mutation (c.4585G>A) in exon 41 of \( KIF1B \), leading to the substitution of valine with methionine at position 1,529 (p.V1529M) (Fig. 2A). This mutation has not been previously reported for cases of PPGL (Table 2) \[9, 23-26\]. The online databases, including dbSNP (accession no. rs756027011; https://www.ncbi.nlm.nih.gov/snp/) and ClinVar (accession no. VCV001009684.1; https://www.ncbi.nlm.nih.gov/clinvar/), registered the p.V1529M mutation with the interpretation of uncertain significance. No other germline or somatic mutations of PPGL-associated genes listed in Table S2 were confirmed in SNV and copy number variation obtained from the WES data. Sanger sequencing confirmed the base substitution (c.4585G>A) in both blood and tumor samples (Fig. 2B), suggesting that there was no loss of heterozygosity in the tumor. To predict the pathogenicity, we used in silico analyses. The SIFT and PolyPhen-2 scores were 0.02 (classified as deleterious) and 0.778 (classified as possibly damaging), respectively. Furthermore, the CADD Phred score was 27.0 (i.e., >20) (Table 3). These scores suggested that the \( KIF1B \) missense mutation was pathogenic. The amino acid region where the p.V1529M mutation exists is well conserved across species, from humans to zebrafish, according to the UCSC genome browser (https://genome.ucsc.edu) (Fig. 2C). The in silico analysis of the previously reported \( KIF1B \) germline mutation cases is also shown in Table 3.

**Pathogenicity classification according to an international guideline**

Next, we evaluated the pathogenicity of p.V1529M mutation using an international guideline for sequence variants established by the American College of Medical Genetics and Genomic (ACMG) and Association for Molecular Pathology (AMP) \[27\]. The evaluation criteria of pathogenicity proposed in the guidelines and whether our case met the criteria are shown in Table S3. In the mutation observed in our patient, no evidence that met the criteria of pathogenicity categorized as “Very strong” or “Strong” was found. Next, the criterion categorized as “Moderate” was evaluated. As shown in Fig. 2A and Table 3, our mutations appeared to exist in the region where mutations are observed relatively frequently. Although we could not obtain informed consent for the genetic testing of her parents, no family history of PCC was confirmed. These met two criteria in this category (PM1 and PM6). Regarding PM2, because our patient was Japanese, we referred to a database of whole genome references for a general population consisting of...
8,300 Japanese individuals (Japanese Multi Omics Reference Panel; jMorp, https://jmorp.megabank.tohoku.ac.jp/202109/). According to jMorp, the minor allele frequency (MAF) of p.V1529M mutation in KIF1B was 0.10%, which did not meet the benign criteria (≥5%). However, the global database from the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org) showed that the MAF of this p.V1529M mutation was 0.0004%, suggesting that this mutation was not absent or extremely rare in the Japanese population. As data from a country also located in East Asia, the Korean 1000 Genome Database (Korea1K, http://1000genomes.kr) and the Korean Reference Genome Database (http://coda.nih.go.kr/coda/KRGDB/index.jsp) also showed that the MAF of p.V1529M mutation in KIF1B was 0.05% and 0.13%, respectively; these MAF values were higher than those available in the global data obtained from gnomAD. In

### Table 2  Case series of PPGL with KIF1B germline mutations

| Patient | Age | Gender | Mutation | Coding | Exon | Location | Family onset | Metastasis | NMN | Metanephrine | Norepinephrine | Other tumor | Reported year | Reference |
|---------|-----|--------|----------|--------|------|----------|-------------|------------|-----|-------------|---------------|------------|--------------|-----------|
| 1       | 36  | F      | p.P1217S | c.3649C>T | 33   | Uni-PCC  | No          | Yes        | NA  | Elevate     | Normal        | No         | 2020         | 26        |
| 2       | 48  | F      | p.P1351L | c.4052C>T | 38   | Uni-PCC  | No          | No         | NA  | Elevated    | Normal        | Normal     | 2020         | 25        |
| 3       | 46  | F      | p.Y835C  | c.2504A>G | 24   | Uni-PCC  | No          | No         | NA  | Elevated    | Normal        | Normal     | 2020         | 24        |
| 4       | 54  | F      | p.S1481N | c.4442G>A | 41   | Uni-PCC  | Yes         | No         | NA  | Elevated    | Normal        | Normal     | 2014         | 23        |
| 5       | 22  | F      | p.V1529M | c.4585G>A | 41   | Uni-PCC  | Yes         | No         | NA  | Elevated    | Normal        | Normal     | 2008         | 9         |

**Abbreviations:** PPGL, pheochromocytoma and paraganglioma; PCC, pheochromocytoma; PGL, paraganglioma; F, female; P, proline; S, serine; L, leucine; Y, tyrosine; C, cysteine; N, asparagine; V, valine; M, methionine; Uni, unilateral; Bi, bilateral; NMN, normetanephrine; MN, metanephrine; NE, norepinephrine; EPI, epinephrine; DA, dopamine.

### Table 3  In silico analysis of the KIF1B gene mutations

| Patient | Mutation | Coding | Exon | SIFT (score) | PolyPhen (score) | CADD phred score |
|---------|----------|--------|------|--------------|------------------|------------------|
| Our case| p.V1529M | c.4585G>A | 41/47 | Deleterious (0.02) | Possibly_damaging (0.778) | 27.0 |
| 1       | p.P1217S | c.3649C>T | 33/47 | Tolerated (0.41) | Benign (0.050) | 22.1 |
| 2       | Splice site | c.2787-2A>C | — | NA (NA) | NA (NA) | 35.0 |
| 3       | p.P1351L | c.4052C>T | 38/47 | Deleterious (0.03) | Probably_damaging (0.995) | 32.0 |
| 4       | p.Y835C  | c.2504A>G | 24/47 | Deleterious (0.00) | Probably_damaging (0.995) | 29.4 |
| 5       | p.S1481N | c.4442G>A | 41/47 | Benign (0.50) | Torelated (0.094) | 18.6 |

**Abbreviations:** SIFT, sorting intolerant from tolerant; PolyPhen-2, polymorphism phenotyping v2; CADD, combined annotation dependent depletion; P, proline; S, serine; L, leucine; Y, tyrosine; C, cysteine; N, asparagine; V, valine; M, methionine.
contrast, gnomAD showed that MAF of this mutation was 0.005% for 18,394 individuals from the East Asian population, that is less frequent than in the databases from Japan and Korea. Although the rarity of this mutation needs further verification, the MAF is not considered rare enough to meet PM2 at present. No other evidence met the criteria categorized as “Moderate.” We also evaluated the criteria categorized as “Supporting.” Because the p.V1529M mutation was suggested to be pathogenic by in silico analysis (Table S3) and the region including the mutation was conserved between species (Fig. 2C), this met one (PP3) criterion. Because some criteria require experiment or long-term verification such as familial onset, these criteria cannot be verified at present. So far, there appears to be no other evidence of pathogenicity and no other criterion that meets the three evidences of benign impact: “Stand-alone,” “Strong,” and “Supporting” (Table S3 and S4). From the above discussion, among the criteria classified as likely pathogenic in the guidelines, (iv) and (v) lacked one criterion each (described in a footnote in Table S3); therefore, the p.V1529M mutation was classified to be a variant of uncertain significance (VUS).

Histopathologic analysis

The tumor in our patient measured 30 × 25 × 25 mm and was yellow-brown in color. No necrosis, membrane infiltration, or vascular invasion of lymph vessels or veins were observed. Immunohistochemical analysis revealed that the tumor was positive for CgA and SYN, with a Ki-67 labeling index of 1%–3%. The Grading of Adrenal Pheochromocytoma and Paraganglioma score [28] was 3/10, suggesting moderately differentiated PCC.

Analysis of catecholamine-synthesizing enzymes

Because our case showed a high level of norepinephrine alone, although it was not an extra-adrenal tumor, immunostaining and gene expression analysis was conducted to evaluate catecholamine-synthesizing enzymes and were positive for TH, DDC, DBH, and PNMT (Fig. 3). Two tumors with a known HRAS mutation (p.Q61R and p.Q61K) and one tumor with a known EPAS1 mutation (p.P531S) were analyzed as controls, and they were all positive for all catecholamine-synthesizing enzymes (Fig. 3). Interestingly, compared with the control group, our case appeared to have a higher level of mRNA expression of TH, DDC, and DBH. Notably, although an extremely high mRNA expression level of DBH was observed, PNMT expression was strongly suppressed (Fig. 4), which was consistent with this case being a noradrenergic type.

Fig. 3 Histopathologic images. Immunohistochemical analysis of catecholamine-synthesizing enzymes. The tumor tissues were identified using hematoxylin–eosin staining and were positive for TH, DDC, DBH, and PNMT. Our current case (indicated as KIF1B germline) is presented in the upper line, and control tumors harboring known PPGL-associated genes (indicated as HRAS and EPAS1 somatic) are presented in the middle and bottom lines, respectively. Scale bars = 200 μm. Abbreviations: TH, tyrosine hydroxylase; DDC, dopa decarboxylase; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine-N-methyl transferase; and PPGL, pheochromocytoma and paraganglioma.
Discussion

Pathogenicity of p.V1529M and p.S1481N mutations in KIF1B

The KIF1B germline mutation (p.V1529M) observed in our case was located in the same exon (exon 41) as the first KIF1B germline mutation (p.S1481N) in early-onset bilateral asynchronous PCC with other types of tumors, which was reported in 2008 [7, 9]. Our case also showed no clear family history of PCC; however, onset in the 20s suggests that the PCC in the patient was hereditary. No contralateral PCC or other tumors were observed, but because only a few years have elapsed after the surgery, careful follow-up might help in the detection of those tumors. Unlike the case’s family with the p.S1481N mutation in KIF1B, our case did not have any germline or somatic mutations, including MAX, except for the KIF1B germline mutation. Therefore, we considered that the germline mutation of p.V1529M in KIF1B was responsible for the development of PPGL. However, the evaluation of the pathogenicity of sequence variant according to the ACMG/AMP guideline still classified this p.V1529M mutation as a VUS at this time, we could not clearly prove its pathogenicity. However, because this present report, including in silico analysis, has accumulated certain supportive evidence, the evidence will be adequately stronger to prove the pathogenicity if familial onset and functional analysis of gene mutations are proven in the future. Reports on the p.S1481N mutation in KIF1B suggested that the pathogenic mechanism of PCC was haploinsufficiency of KIF1B or epigenetic silencing of the wild-type allele in the tumor [7, 9]. In our case, the mutant allele (c.4585G>A) and the wild-type allele were present in the same levels in both blood and tumor samples. There is also a report of a KIF1B germline mutation (c.4052C>T, p.P1351L) in exon 38, where the loss of heterozygosity (LOH) of KIF1B in the tumor was proposed as the mechanism of PCC development [24]. Assuming that the KIF1B mutation in our case is pathogenic, the mechanism of PCC pathogenesis might be due to haploinsufficiency of the KIF1B gene, as proposed in the p.S1481N mutation case, rather than LOH.
Unfortunately, we could not conduct cell-based experiments to prove this aspect. However, we believe that a neural crest-derived cell line with the p.V1529M heterozygous mutation of KIF1B would be a useful tool to prove its pathological mechanism. Because KIF1B suppresses apoptosis when its function is suppressed [7], if the mutant cell line shows reduced expression level of KIF1B protein and mRNA, suppression of apoptosis via apoptosis assay, and cancellation of apoptosis suppression by KIF1B overexpression assay using transfection, haploinsufficiency might be regarded as the main pathological mechanism of this mutation.

**Catecholamine profile**

Interestingly, although our case was a primary adrenal tumor, only norepinephrine was overproduced. The p.S1481N mutant case also showed bilateral adrenal PCC with only norepinephrine overproduction and shared the same clinical features [9] as our case.

In our case, immunostaining revealed that the samples were positive for all catecholamine-synthesizing enzymes. However, the intratumoral gene expression analysis of the control group revealed that the expression of PNMT, which converts norepinephrine to epinephrine, was suppressed, and the expression of DBH, which converts dopamine to norepinephrine, was remarkably enhanced. These findings suggest that the gene expression analysis results were consistent with the catecholamine secretion profiles in our case.

KIF1B germline mutations in PCC are considered transcriptionally similar to RET and NF1 germline mutations and are classified into the kinase signaling-related cluster (cluster 2) [29]. In contrast, noradrenergic PPGL is mainly observed in genes in the pseudohypoxia-related cluster (cluster 1) [30]; thus, our case appears to have a discrepancy between the intratumoral transcriptional and hormonal characteristics.

High levels of both norepinephrine and epinephrine were observed in recent cases of PCC with a KIF1B germline mutation in exon 33 (p.P1217S) [26] and exon 38 (p.P1351L) [24], for which hormonal data were available. The latter case was diagnosed at the age of 46 years, and lymph node metastasis was observed during the course of the disease [24]. These cases had clinical features that were slightly different from those in our case and the case with the S1481N mutation [7, 9]. Although the less number of reports is a hindrance, reassessment of the relationship between intratumoral transcription signals and catecholamine profiles in KIF1B mutations may be necessary for future studies.

**Other clinical symptoms**

Other than juvenile-onset PCC, the autism-like characteristics of our proband, the intellectual disability in her younger brother, and familial history of micrognathism and funnel chest prompted the suspicion of a hereditary syndrome. Although the p.Q87L loss-of-function mutation, which is located in the ATP-binding domain of KIF1B, is known as the causative mutation of Charcot–Marie–Tooth disease type 2A (CMT2A), the hereditary peripheral neuropathy [31] symptoms of CMT2A or the clinical features observed in our case were not mentioned in the other reports of KIF1B mutant cases that caused PCC [9, 24]. We also consulted with a pediatrician and searched for germline mutations in our WES data, but no candidate gene mutations or syndromes were detected. Because we could not conclude whether the various familial features in our patient are related to the KIF1B mutation, more cases are necessary to provide sufficient evidence to resolve this issue.

In conclusion, we presented a case of juvenile-onset PCC with a KIF1B germline mutation in exon 41 and a primary adrenal tumor of the noradrenergic type, similar to the first reported case in 2008. Although we could not prove its pathogenicity, our clinical and genetic data provided some evidence that might lead to the clarification of pathogenicity in the future.

**Acknowledgments**

We thank the corporate member of the Department of Pathology, Yamanashi Central Hospital, for expert technical assistance and clinical work. We also thank Enago (https://www.enago.jp) for the English language review. The authors declare that they have no conflicts of interest.

**Disclosure**

The authors have nothing to disclose.
### Table S1 Primer information

For sanger sequencing

| Gene name | Sequence |
|-----------|----------|
| KIF1B forward | 5’-TCAGCACCTCCACCAAGTATC-3’ |
| KIF1B reverse | 5’-AACCAGAGGGCTAGTTTGTG-3’ |

For RT-PCR analyses

| Gene name | Sequence |
|-----------|----------|
| TH forward | 5’-CGGATGAGGAAATTGAGAAGCT-3’ |
| TH reverse | 5’-TTGTGTTGCACACAGGCTGCA-3’ |
| DDC forward | 5’-GGCCGGAGATGAGGATCGT-3’ |
| DDC reverse | 5’-TGTTGGCCAGCTCCCCGCTT-3’ |
| DBH forward | 5’-GCAGCCACTTTGAGGACATCA-3’ |
| DBH reverse | 5’-GGCTGTACATGCTCCAGTTGAA-3’ |
| PNMT forward | 5’-CCAACCGCGAGAAGATGA-3’ |
| PNMT reverse | 5’-CCAGAGGCGTACAGGGATAG-3’ |
| ACTB forward | 5’-CCAACCGCGAGAAGATGA-3’ |
| ACTB reverse | 5’-CCAGAGGCGTACAGGGATAG-3’ |

### Table S2 PPGL-associated genes analyzed according to whole exome sequencing

| | Germline mutation analysis | Somatic mutation analysis |
|-----------------|---------------------------|---------------------------|
| | BAP1 | KIF1B | SDHC | ARNT | FH | KMT2D | SETD2 |
| DLST | MAX | SDHD | ATRX | GNAS | MAML3 | TERT |
| DNM3A | MDH2 | SLC25A11 | BAP1 | GDNF | MAX | TP53 |
| ECLN2 | MERTK | TP53 | CDKN2A | HRAS | MYCN | VHL |
| EPS1 | N1 | VHL | CSDE1 | IDH1 | MYOSB |
| GH2 | RET | DNMT3A | IDH2 | NF1 |
| G0T2 | SDHA | EPASI | IRP1 | RET |
| HRAS | SDHA | EZH2 | JMD1C | SDHB |
| ID1H8 | SDHB | FGR1 | KIF1B | SDHD |

Abbreviation: PPGL, pheochromocytoma and paraganglioma.

### Table S3 Summary of the international guideline about evidences of pathogenicity [26] for sequence variants and our case

| Evidence of pathogenicity | Category | Our case |
|---------------------------|----------|----------|
| Very Strong | PVS1 | Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multixenon deletion) in a gene where loss of function is a known mechanism of disease | N |
| Strong | PS1 | Same amino acid change as a previously established pathogenic variant regardless of nucleotide change | N |
| | PS2 | De novo (both maternity and paternity confirmed) in a patient with the disease and no family history | NV |
| | PS3 | Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product | NV |
| | PS4 | The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls | N |
| Moderate | PM1 | Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation | Y |
| | PM2 | Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium | N |
| | PM3 | For recessive disorders, detected in trans with a pathogenic variant | N |
| | PM4 | Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants | N |
| | PM5 | Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before | N |
| | PM6 | Assumed de novo, but without confirmation of paternity and maternity | Y |
| Supporting | PP1 | Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease | N |
| | PP2 | Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease | NV |
| | PP3 | Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) | Y |
| | PP4 | Patient’s phenotype or family history is highly specific for a disease with a single genetic etiology | N |
| | PP5 | Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation | N |

To be classified as “Likely pathogenic” in the international guideline, the following criteria must be met. (i) 1 Very strong (PVS1) and 1 moderate (PM1–PM6), (ii) 1 Strong (PS1–PS4) and 1–2 moderate (PM1–PM6), (iii) 1 Strong (PS1–PS4) and ≥2 supporting (PP1–PP5), (iv) ≥3 Moderate (PM1–PM6), (v) 2 Moderate (PM1–PM6) and ≥2 supporting (PP1–PP5), (vi) 1 Moderate (PM1–PM6) and ≥4 supporting (PP1–PP5).

Abbreviation: N, no; Y, yes; NV, not verified.
Table S4 Summary of the international guideline about the evidences of benign impact [26] for sequence variants and our case

| Evidence of benign impact | Category | Our case |
|--------------------------|----------|----------|
| Stand-alone | BA1 | Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium | N |
| Strong | BS1 | Allele frequency is greater than expected for disorder | N |
| | BS2 | Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age | N |
| | BS3 | Well-established *in vitro or in vivo* functional studies show no damaging effect on protein function or splicing | NV |
| | BS4 | Lack of segregation in affected members of a family | NV |
| Supporting | BP1 | Missense variant in a gene for which primarily truncating variants are known to cause disease | N |
| | BP2 | Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern | NV |
| | BP3 | In-frame deletions/insertions in a repetitive region without a known function | N |
| | BP4 | Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) | N |
| | BP5 | Variant found in a case with an alternate molecular basis for disease | N |
| | BP6 | Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation | N |
| | BP7 | A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved | N |

Abbreviation: N, no; Y, yes; NV, not verified.

References

1. Lenders JW, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SK, et al. (2014) Pheochromocytoma and paraganglioma: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 99: 1915–1942.
2. Buffet A, Venisse A, Nau V, Roncellin I, Boccio V, et al. (2012) A decade (2001–2010) of genetic testing for pheochromocytoma and paraganglioma. *Horm Metab Res* 44: 359–366.
3. Mannelli M, Castellano M, Schiavi F, Filetti S, Giacche M, et al. (2009) Clinically guided genetic screening in a large cohort of Italian patients with pheochromocytomas and/or functional or nonfunctional paragangliomas. *J Clin Endocrinol Metab* 94: 1541–1547.
4. Fishbein L, Leshchiner I, Walter V, Danilova L, Robertson AG, et al. (2017) Comprehensive molecular characterization of pheochromocytoma and paraganglioma. *Cancer Cell* 31: 181–193.
5. Benn DE, Gimenez-Roqueplo AP, Reilly JR, Bertherat J, Burgess J, et al. (2006) Clinical presentation and penetrance of pheochromocytoma/paraganglioma syndromes. *J Clin Endocrinol Metab* 91: 827–836.
6. King KS, Prodanov T, Kantorovich V, Fojo T, Hewitt JK, et al. (2011) Metastatic pheochromocytoma/paraganglioma related to primary tumor development in childhood or adolescence: significant link to SDHB mutations. *J Clin Oncol* 29: 4137–4142.
7. Schlissio S, Kenchappan RS, Vredeveld LC, George RE, Stewart R, et al. (2008) The kinesin KIF1Bbeta acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor. *Genes Dev* 22: 884–893.
8. Munirajan AK, Ando K, Mukai A, Takahashi M, Suenaga Y, et al. (2008) KIF1Bbeta functions as a haploinsufficient tumor suppressor gene mapped to chromosome 1p36.2 by inducing apoptotic cell death. *J Biol Chem* 283: 24426–24434.
9. Yeh IT, Lenci RE, Qin Y, Buddavarapu K, Ligon AH, et al. (2008) A germline mutation of the KIF1B beta gene on 1p36 in a family with neural and nonneural tumors. *Hum Genet* 124: 279–285.
10. Cardot Bauters C, Leteurtre E, Carnaille B, Do Cao C, Espiard S, et al. (2020) Genetic predisposition to neural crest-derived tumors: revisiting the role of KIF1B. *Endocr Connect* 9: 1042–1050.
11. Amemiya K, Hirotsu Y, Goto T, Nakagomi H, Mochizuki H, et al. (2016) Touch imprint cytology with massively parallel sequencing (TIC-seq): a simple and rapid method to snapshot genetic alterations in tumors. *Cancer Med* 5: 3426–3436.
12. Hirotsu Y, Yokoyama H, Amemiya K, Hagimoto T, Hosaka K, et al. (2020) Genomic profiling identified ERCC2 E606Q mutation in helicase domain respond to platinum-based neoadjuvant therapy in urothelial bladder cancer. *J Clin Oncol* 29: 4137–4142.
13. Hirotsu Y, Zheng TH, Amemiya K, Mochizuki H, Guleng B, et al. (2016) Targeted and exome sequencing identified somatic mutations in hepatocellular carcinoma. *Hepatol Res* 46: 1145–1151.

14. Hirotsu Y, Nakagomi H, Amemiya K, Oyama T, Inoue M, et al. (2017) Intrinsic HER2 V777L mutation mediates resistance to trastuzumab in a breast cancer patient. *Med Oncol* 34: 3.

15. Kojima Y, Hirotsu Y, Omata W, Sugimori M, Takaoka S, et al. (2018) Influence of NUDT15 variants on hematological pictures of patients with inflammatory bowel disease treated with thiopurines. *World J Gastroenterol* 24: 511–518.

16. Grouzmann E, Matter M, Bilz S, Herren A, Triponez F, et al. (2012) Monoamine oxidase A down-regulation contributes to high metanephrine concentration in pheochromocytoma. *J Clin Endocrinol Metab* 97: 2773–2781.

17. Hirotsu Y, Nakagomi H, Sakamoto I, Amemiya K, Oyama T, et al. (2015) Multigene panel analysis identified germ-line mutations of DNA repair genes in breast and ovarian cancer. *Mol Genet Genomic Med* 3: 459–466.

18. Ng PC, Henikoff S (2001) Predicting deleterious amino acid substitutions. *Genome Res* 11: 863–874.

19. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, et al. (2010) A method and server for predicting damaging missense mutations. *Nat Methods* 7: 248–249.

20. Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M (2019) CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res* 47: D886–D894.

21. Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, et al. (2014) A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 46: 310–315.

22. Nakagomi H, Mochizuki H, Inoue M, Hirotsu Y, Amemiya K, et al. (2018) Combined annotation-dependent depletion score for BRCA1/2 variants in patients with breast and/or ovarian cancer. *Cancer Sci* 109: 453–461.

23. Welander J, Andreasson A, Juhlin CC, Wiseman RW, Backdahl M, et al. (2014) Rare germline mutations identified by targeted next-generation sequencing of susceptibility genes in pheochromocytoma and paraganglioma. *J Clin Endocrinol Metab* 99: E1352–E1360.

24. De Filpo G, Contini E, Serio V, Valeri A, Chetta M, et al. (2020) Germline mutation in KIF1Bbeta gene associated with loss of heterozygosity: usefulness of next-generation sequencing in the genetic screening of patients with pheochromocytoma. *Int J Endocrinol* 2020: 3671396.

25. Seo SH, Kim JH, Kim MJ, Cho SI, Kim SJ, et al. (2020) Whole exome sequencing identifies novel genetic alterations in patients with pheochromocytoma/paraganglioma. *Endocr Metab (Seoul)* 35: 909–917.

26. Ma X, Li M, Tong A, Wang F, Cui Y, et al. (2020) Genetic and clinical profiles of pheochromocytoma and paraganglioma: a single center study. *Front Endocrinol (Lausanne)* 11: 574662.

27. Richards S, Aziz N, Bale S, Bick D, Das S, et al. (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 17: 405–424.

28. Kimura N, Takayanagi R, Itagaki E, Katabami T, et al. (2014) Pathological grading for predicting metastasis in phaeochromocytoma and paraganglioma. *Endocr Relat Cancer* 21: 405–414.

29. Zhikrivetskaya SO, Snezhkina AV, Zaretsky AR, Alekseev BY, Pokrovsky AV, et al. (2017) Molecular markers of paragangliomas/pheochromocytomas. *Oncotarget* 8: 25756–25782.

30. Nolting S, Bechmann N, Taieb D, Beuschlein F, Fassnacht M, et al. (2021) Personalized management of pheochromocytoma and paraganglioma. *Endocr Rev*. Online ahead of print.

31. Zhao C, Takita J, Tanaka Y, Setou M, Nakagawa T, et al. (2001) Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. *Cell* 105: 587–597.