



ORIGINAL ARTICLE

A novel 8.57-kb deletion of the upstream region of *PRKARIA* in a family with Carney complex

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Abstract

Carney complex (CNC) is a rare hereditary syndrome that involves endocrine dysfunction and the development of various types of tumors. Chromosome 2p16 and *PRKARIA* on chromosome 17 are known susceptibility loci for CNC. Here we report a mother and son with CNC caused by an 8.57-kb deletion involving the transcription start site and non-coding exon 1 of *PRKARIA*. The proband is a 28-year-old male with bilateral large-cell calcified Sertoli cell testicular tumors and pituitary adenoma. Comprehensive genomic profiling for cancer mutations using Foundation One CDx failed to detect any mutations in *PRKARIA* in DNA from the testicular tumor. Single-nucleotide polymorphism array analysis of the proband's genomic DNA revealed a large deletion in the 5' region of *PRKARIA*. Genomic walking further delineated the region an 8.57-kb deletion. A 1.68-kb DNA fragment encompassed by the deleted region showed strong promoter

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activity in a NanoLuc luciferase reporter assay. The patient's mother, who is suffering from recurrent cardiac myxoma, a critical sign for CNC, carried an identical deletion. The 8.57-kb deleted region is a novel lesion for CNC and will facilitate molecular diagnosis of the disease.

KEYWORDS

Carney complex, deletion of a promoter, SNP array, *PRKARIA*

1 | INTRODUCTION

Carney complex (CNC) is a relatively new entity of hereditary tumor syndrome involving myomas, skin pigmentation, and excessive secretion of various hormones with autosomal dominance, and was first described by Carney et al. (1985). A variety of endocrinologic signs and symptoms, including Cushing's syndrome, acromegaly, gynecomastia, precocious puberty, and endocrine organ tumors can occur in CNC patients, with most manifesting as adult-onset (Kamilaris et al., 2019). CNC patients may also develop tumors in a wide variety of other organs, including pituitary, heart, skin, and testes. Clinical diagnosis of CNC requires that two such endocrinologic manifestations be confirmed by histopathology. There are two known susceptibility loci for CNC, chr17q2 (CNC type 1) and chr2p16 (CNC type2), and *PRKARIA* (OMIM accession number: 188830) was identified as the responsible gene for CNC type 1 in 2000 (Kirschner et al., 2000).

The *PRKARIA* gene encodes a regulatory subunit of protein kinase A (PKA) called PKA RI α , which bind to the PKA catalytic subunit to form heterotetramers that suppress PKA activity (Kamilaris et al., 2019). The activity of PKA is known to be induced by the cyclic AMP produced by G-protein-coupled hormone receptors such as those for follicle-stimulating hormone, adrenocorticotrophic hormone, thyroid-stimulating hormone, and melanocyte-stimulating hormone. The endocrinologic symptoms in CNC reflect excessive signaling by these hormones corresponding to PKA RI α as the suppressor of PKA.

Most of the CNC variants that have been identified in *PRKARIA* cause loss-of-function mutations such as frameshifts and stop gains (see Mineo et al. (2016) for example), whereas reports of large structural variations such as deletions of >1 kb are rare (Horvath et al., 2008; Salpea et al., 2014). Recently, comprehensive genomic profiling (CGP) has been widely applied to cancer, and some of the CGP panels, such as FoundationOne CDx (Hellmann et al., 2018), include *PRKARIA* as a target gene. Among carriers of CNC who have submitted samples to identify actionable mutations in their cancers, CGP may show high probability for loss-of-function mutations in the *PRKARIA* gene as a secondary finding. In cases where the onset of

CNC is caused by haploinsufficiency of *PRKARIA* involving a large genomic deletion, however, detection of the variant might be missed under the resolution of CGP testing.

In this report, we describe a CNC patient with malignant testicular tumors whose submission for CGP did not reveal any *PRKARIA* gene mutations. A single-nucleotide polymorphism (SNP) array with patient-parents trio analyses and chromosomal walking revealed that the patient and his mother shared a large structural variant of *PRKARIA*. A functional assay verified that the structural variant is a loss-of-function mutation of *PRKARIA*.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

The study was approved by the in-house ethical committee (registration number 2020-018).

2.2 | Nucleic acid samples

Sources of genomic DNA were 2 ml whole blood from the patient and 2 ml saliva from each of his parents, collected with an Oragene OGR-600 DISCOVER kit (DNA Genotek Inc., Canada). Extraction of genomic DNA was done using a Maxwell DNA purification kit (Promega) for the whole blood sample, and manually following the Oragene protocol for the saliva samples. Total RNA was extracted from 3 ml whole blood from the patient using a NucleoSpin[®] RNA Blood Midi (740210.20, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany, Promega). Written informed consent for the study was obtained from the patient and his parents. Specimens for genetic analyses were anonymized by staff who were not involved in the study.

2.3 | Clinical examinations

Bilateral Sertoli cell tumors were surgically resected, fixed with formalin, and embedded in paraffin using

our hospital's standard protocol (Ito et al., 2021). Magnetic resonance imaging (MRI) was done with a 1.5 T MAGNETOM Avanto Fit (Siemens, Germany), with and without gadolinium enhancement. To confirm the acromegaly, a 75-g oral glucose tolerance test was performed. The FoundationOne CDx CGP for the cancer specimen was done. The paraffin-embedded tumor specimen was sectioned to 5- μ m thickness, and 10 sections were submitted to a Japanese agent of Chugai Pharmaceuticals, who provided a clinical report that was examined by an expert panel of Tohoku University Hospital, Sendai, Japan. The results are given in Supplementary Table S1.

2.4 | SNP array analysis

We used hg19 as the human reference genome coordinate because the SNP array used in this study was also based on hg19. The SNP array genotyping was outsourced to LSI Medience (Japan). The CytoScan HD Array (Thermo Fisher) was used for genotyping. One microgram of genomic DNA from the patient-parents trio was submitted for analyses. The regions of copy number alterations were calculated with the Chromosome Analysis Suite version 4.2.0.80, using the default setting (Thermo Fisher). The comparison of copy numbers of the region among the family members was done using the "smooth signal" parameter calculation from the Chromosome Analysis Suite.

2.5 | Histopathology

Surgically resected tumor specimens were fixed with 10% neutral-buffered formalin solution (060-01667; Fujifilm Wako Pure Chemical Co.). The embedding media was a mixture of Histoprep 568 (415-25,791; Fujifilm Wako Pure Chemical Co.) and paraffin (164-13,345; Fujifilm Wako Pure Chemicals Co.) in equal amounts. The melting points of both media were 56-58°C. After embedding, the tumor samples were stored at room temperature. Hematoxylin and eosin-stained specimens were examined by two qualified pathologists (I.S. and K.S.).

Immunohistochemistry was performed using a rabbit polyclonal anti-human PRKARIA antibody (HPA049979, Sigma-Aldrich) at a 1:200 dilution. The following primary antibodies were used to characterize the testicular tumors: steroidogenic factor 1 (SF1), inhibin alpha, and Ki-67 as described in Kasajima et al. (2014), and beta-catenin as described in Konosu-Fukaya et al. (2014).

2.6 | TaqMan probe genotyping and SYBR green copy number analyses

Genomic DNA (10-15 ng) from the patient, his parents, and a healthy control was subjected to TaqMan and SYBR real-time PCR analyses. Probes for genotyping and copy number analyses are summarized in Supplementary Tables S2 and S3, respectively. Real-time quantitative PCR (RT-qPCR) was performed with a COBAS Z 480 (Roche).

TaqMan probes for genotyping of the possible deleted regions (including SNP rs8079916) in the patient and his parents were selected based on the minor allele frequencies (30-55%) among the Japanese general population. The 8.3KJPN data were obtained from the jMorp database (<https://jMorp.megabank.tohoku.ac.jp/202102/>) (Tadaka et al., 2018). After an initial denaturing step (95°C for 10 min), 45 cycles of PCR were performed at 95°C for 15 s and 60°C for 1 min; after the final cycle, 30 s of cooling was done at 40°C. TaqMan Genotyping Master Mix (4,371,353, Thermo Fisher Scientific Inc.) was used for reaction.

To design the SYBR green quantitative PCR probes to detect copy number alterations, we first obtained the nucleotide sequences of deleted regions through the DAS server of the UCSC Genome Browser (<https://genome.ucsc.edu/>); repetitive sequences were masked with RepeatMasker (4.0.7: <http://www.repeatmasker.org>). Next, we employed Primer 3 software to find candidate primer pairs for SYBR green PCR using the default settings except for the product size range, which was set as 150-300 bp (Rozen & Skaletsky, 2000). After an initial denaturing step (95°C for 3 min), 45 cycles of PCR were performed at 95°C for 5 s and 60°C for 10 s; after the final cycle, 30 s of cooling was done at 40°C. All experiments were technically duplicated. Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (600,882, Agilent Technologies, Inc.) was used for reaction.

2.7 | Breakpoint sequencing, cloning, and plasmid construction for promoter characterization

To obtain information on the breakpoint junctions in the upstream region of *PRKARIA* in the patient and his mother, we designed a primer pair for long-range PCR. Based on the TaqMan genotyping results, we assumed that following primers would anneal to the flanking regions of the deletion: upstream primer 66497104F (5'- ATTTTCCCTGTGCCTTTTGA- 3') and downstream primer 66512107R (5'- ACGCTTACTTGCTTTGTTC- 3'). Genomic DNA (50 ng) from the patient and his parents was subjected to PCR amplification with KOD-One thermostable polymerase (Toyobo, Japan) under the following long-range

conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles at 98°C for 10 s (denaturing), 60°C for 5 s (annealing), and 68°C for 1.5 min (elongation); after a final elongation at 68°C for 7 min, reaction products were stored at 4°C and separated by electrophoresis in 0.7% agarose gel with a 1-kb size ladder in the far left lane (Quick-Load 1 kb DNA Ladder, cat no. N0468, NEB, USA).

Reporter plasmids were constructed to characterize *PRKARIA* promoter activity. We employed the seamless ligation cloning extract (SLiCE) technology to clone PCR products made using primers with ~15-nucleotide flanking tags added to the 5' ends (Motohashi, 2017). Genomic DNA from a pancreatic cancer cell line, PANC-1, was used as PCR template for the plasmids expressing the normal putative *PRKARIA* promoters. The primers used to amplify the two promoter regions were as follows: promoter1, 17_66506033_HindIII5cloning (5'- GGCCTCGGCGGCCAAGCTGCAGCTGAATGGGGACAC -3') to 17_66508391_HindIII3cloning (5'- GTACCGGATTGCCAAGCTCTTACTCGTCCCTTATCCA -3'); and promoter2, 17_66507404_HindIII5cloning (5'- GGCCTCGGCGGCCAAGCTCTA AAGCAGCCCCTAGCCAGACAC-3') to 17_66509086_HindIII3cloning (5'- GTACCGGATTGCCAAGCTGACGATGGCGGACCCAGTAAAAT -3'). Plasmids containing the mutant alleles were constructed using the same strategy, with genomic DNA from the patient and his mother as PCR template and the following primers: 17_66497104F_HindIII (5'- GGCCTCGGCGGCCAAGCTATTTTCCCTGTGCCTTTTGA -3') and hg19chr17_66512107R (5'- GTACCGGATTGCCAAGCTACGCTTACTTGCTTTGTTCA -3'). The promoter regions of *PRKARIA* in genomic DNA (50 ng) from the patient and his parents was subjected to PCR amplification using KOD Fx thermostable polymerase (Toyobo, Japan) under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles at 98°C for 10 s (denaturing), 60°C for 30 s (annealing), and 68°C for 2 min (elongation); after a final elongation at 68°C for 7 min, The PCR primer pair was 17_66507404_HindIII5coning (5'- GGCCTCGGCGGCCAAGCTCTAAA GCAGCCCCTAGCCAGACAC -3') and hg19chr17_66512107R (5'- GTACCGGATTGCCAAGCTACGCTTACTTGCTTTGTTCA -3'). PCR products were stored at 4°C. The PCR products were cloned into *HindIII*-digested pNL1.1-neo, a reporter plasmid modified using the pNL1.1 vector (Promega). The reporter pNL1.1-neo was constructed by inserting a kanamycin/neomycin resistance gene fragment (1.51 kbp) from pZS-Green-DR (Clontech) into the *BamHI/SalI* sites of pNL1.1. One-shot MAX DH10B (12331013, Thermo Fisher) competent cells were used for transformation.

To visualize the CNC pathogenic deleted regions, the custom track file was generated for UCSC Genome Browser following the instruction provided in the web site (Hinrichs et al., 2006). The genome positions of previously reported large pathogenic deletion (>1 kbp) of CNC around the *PRKARIA* region were downloaded from ClinVar database. In addition, we identified a manuscript describing two pathogenic deletions of *PRKARIA* for CNC although exact genomic positions were not available (Horvath et al., 2008). To obtain the correct genomic positions of the deletion described in Horvath et al., we obtained the nucleotide sequences of the flanking region from Figure 1 of the paper, mapped them onto JG1, a reference genome sequence for the Japanese population (Takayama et al., 2021) using the NCBI search tool BLAST, and transferred the coordinates to hg19 using LiftOver software (Hinrichs et al., 2006). In search of the deletions in the general population, we searched the database of genomic variants (MacDonald et al., 2014).

The cloned mutated DNA fragments from the patient and his mother were subjected to the DNA sequencing with primer-walking method. The mutant allele sequence will be submitted to Genbank accession number of mutant allele identified in this study is OL629246

2.8 | RT-qPCR analyses of the patient's *PRKARIA* cDNA

Total RNA from the patient's whole blood and three cancer cell lines (ST-1 from an adult T-cell leukemia, HLE from a human hepatoma, and HPCM2 from a head and neck cancer) was obtained using standard protocols (Mochizuki et al., 2016). One microgram of total RNA was subjected to reverse transcription with random primers using a Takara PrimeScript2 cDNA synthesis kit (#6210, Takara, Shiga, Japan) following the manufacturer's instructions. Hydrolysis probes for TaqMan reverse transcriptase assays of the *PRKARIA* 5' untranslated region (a FAM dye label on the on the 5' end and minor groove binder [MGB] on the 3' end) and an *RPLP2* gene reference as an internal control (VIC dye label with MGB) were obtained from Thermo Fisher (Hs00978277_m1, cat. no. 4351372 and Hs01115128_gH, cat. no. 4448489, respectively). RT-qPCR was performed with a COBAS Z 480 (Roche) in 2× TaqMan Fast master mix (#4444556, Thermo Fisher). After an initial polymerase activation step at 95°C for 20 s, 45 cycles of PCR were performed at 95°C for 15 s and 60°C for 1 min; after the final cycle, 30 s of cooling was done at 40°C. All experiments were technically duplicated.

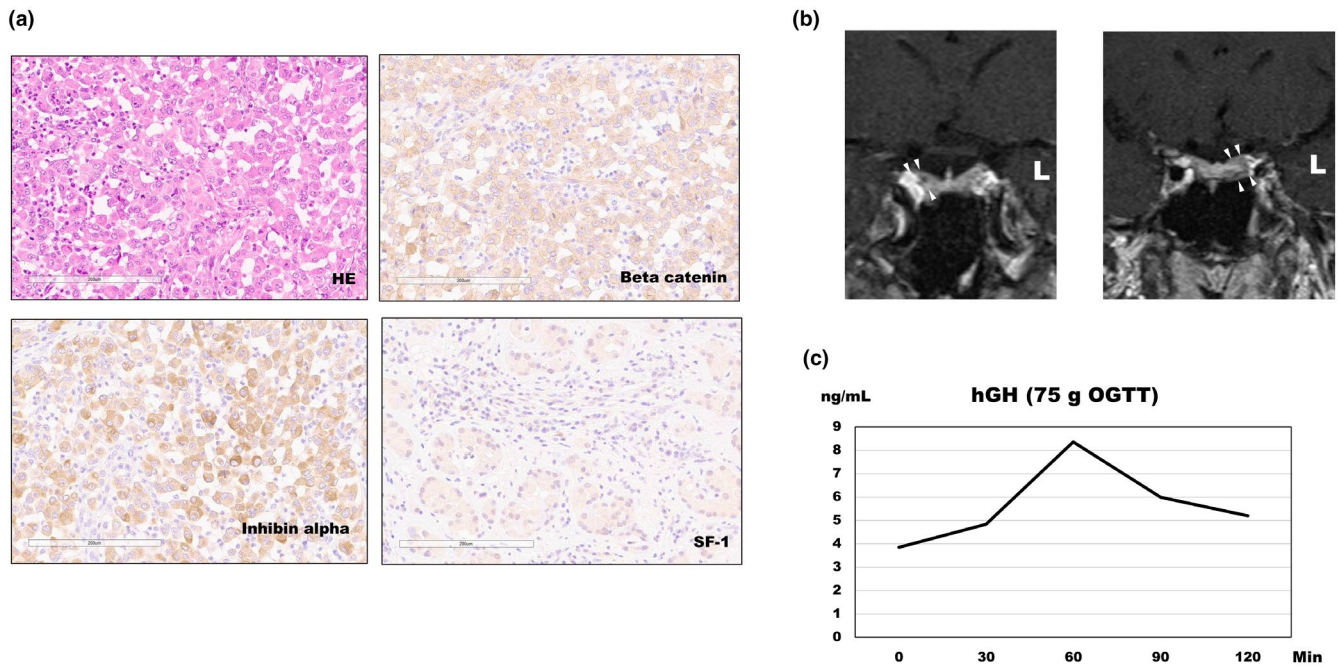


FIGURE 1 Clinical manifestations of Carney complex in a patient based on histopathology and magnetic resonance imaging (MRI). (a) Histopathology of the testicular tumor: Hematoxylin and eosin (HE) staining and immunohistochemical positivity for beta-catenin, inhibin alpha, and SF-1. (b) MRI of the pituitary adenoma, L indicates the left side of the patient body. Arrowheads indicate the pituitary adenoma (c) time course of changes in the level of human growth hormone (hGH; ng/ml) in the blood following administration of 75 g glucose (oral glucose tolerance test; OGTT)

To calculate mRNA expression data for *PRKARIA* relative to *RPLP2* in normal human peripheral blood cells, we accessed the Genotype-Tissue Expression (GTEx) project portal (<https://www.gtexportal.org/home/>).

2.9 | Luciferase reporter assay

HEK-293 cells were cultured in RPMI1640 medium with 10% fetal bovine serum supplemented with L-glutamate and 1% ampicillin-streptomycin at 37°C in 5% CO₂. Collagen-coated 96-well white plates were inoculated with the cells and incubated for 24 h before being transfected with 0.3 μL FuGENE HD Transfection reagent (E2312, Toyobo, Japan) containing a total of 25 to 50 ng reporter plasmid DNA (pNL1.1 or 3.1 vectors) and pGL4.5 as an internal control (Promega). pNL3.1 is similar to pNL1.1 but has minimal promoter activity and used as a positive control for promoter activity. The transfectants were assayed for NanoLuc luciferase (NLuc) activity at 48 h of transfection. The luminometer was the SYNERGY H1 plate reader (BIOTECH, USA) and the reagents were from the Nano-Glo® Dual Luciferase Reporter Assay System (N1610, Promega). Results from five wells were combined for one data point, and all experiments were technically duplicated.

3 | RESULTS

3.1 | Clinical manifestation of CNC in the patient

A 26-year-old Japanese man noticed the presence of tumors in the bilateral testes and visited our hospital in 20XX. Based on a needle biopsy of the testes and subsequent histologic examination, the lesions were diagnosed as bilateral calcified Sertoli tumors, for which the patient underwent resection surgery. Histopathologic examination of the resected tumors revealed tubular patterns and immunohistochemical positivity for beta-catenin, inhibin alpha, and SF-1 (Figure 1a), which were all consistent with Sertoli cell tumors, albeit there was only partial nuclear staining of beta-catenin. Abundant Ki-67 immunoreactivity indicated the actively growing nature of the tumors, while negative CK immunoreactivity ruled out a mesothelial origin (Supplementary Figure S1a). The patient's countenance also showed some features of acromegaly, namely high eyebrows and a large mandible. Suspecting a pituitary adenoma, we examined contrast-enhanced MRI of the sella turcica and estimated the level of growth hormone in the blood (Figure 1b,c). Contrast-enhanced T1-weighted coronal images of the sella turcica showed lesions with low enhancement at the left and right sides of the pituitary gland,

indicating pituitary adenoma (Figure 1b). The pituitary tumor showed invasion along the septum of the sphenoidal sinus (Supplementary Figure S1b). A 75-g oral glucose tolerance test indicated quite a high basal level of growth hormone of ~4 ng/ml (average normal adult, 0.4 ng/ml) and a slight increase 60 min after the glucose. These results indicated that the patient was suffering from a pituitary adenoma and unregulated secretion of growth hormone, and supported a diagnosis of CNC despite the lack of *PRKARIA* mutations in the FoundationOne CDx CGP panel results (Supplementary Table S1). The patient's mother suffered from recurrent cardiac myxoma (Figure 2a), which is one of

the signs associated with CNC (Kamilaris et al., 2019). This family history suggested that the patient's phenotype was the result of genetic inheritance.

3.2 | Genomic copy number loss upstream of *PRKARIA* in the patient and his mother

To address the possible large deletions in *PRKARIA*, we employed SNP array analyses of the patient and his parents. A 9.7-kb deletion (chr17:66496283–66,505,948)

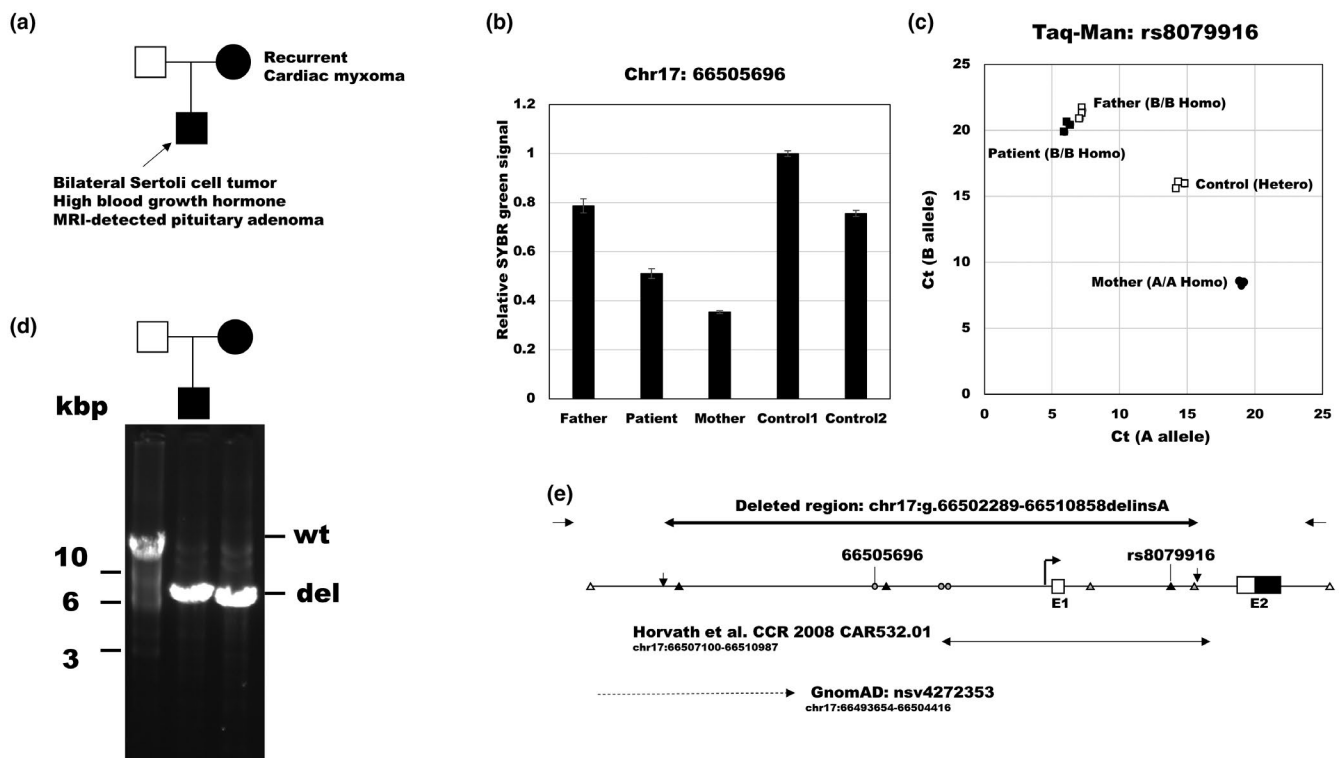


FIGURE 2 Genomic alteration identified in the patient and his mother. (a) family tree of the patient. (b) sybr green pcr copy number analysis of the upstream region of the *PRKARIA* gene in the patient and his parents at CHR17: 66505696, indicating the 5' position of the amplicon; the healthy control 1 DNA was set as one. The signal for the DNA from the patient and his mother was less than half that of the healthy control. (c) Scatter plot of taqman genotyping of a and b alleles at the single-nucleotide polymorphism RS8079916 in the patient family trio (all homozygous) and a healthy control (heterozygous). CT = CYCLE THRESHOLD. (d) Fluorogram of agarose gel electrophoresis of products of long-range PCR of the region flanking the putative deletion mutation of *PRKARIA*. The PCR products from the father, patient, and mother loaded from left to right. The father, a wild-type homozygote, showed only longer PCR products of around 15 KB in size. The patient and mother, heterozygotes of wild-type and deletion mutant alleles, showed strong signals for the shorter product from the mutant allele (6.4 KB) and weaker signals for the longer 15-kb product, whose PCR amplification is theoretically less competitive in vitro. (e) Schematic of the deletion detected in the patient and his mother. The longest horizontal line represents genomic DNA (CHR17:66501103–66,512,084) upstream of the *PRKARIA* locus. Open, hatched and closed triangles indicate the position of TAQMAN genotyping probes and heterozygote, concordant homozygote (either parent is homozygous), and discordant homozygote genotypes, respectively. hatched circles indicate the position of SYBR green probes with decrease in genomic copy number in the patient and his mother. the positions of the probes used in the panel B and C are indicated with short vertical lines. Open and closed rectangles indicate the noncoding and coding exons (e), respectively. The bent arrow indicates the position of the major promoter of the *PRKARIA* gene. Horizontal arrows indicate the positions of PCR primers used for the amplification shown in panel D. Vertical arrows indicate the breakpoints detected in the PCR products of the patient and his mother. double-headed arrows indicate the deleted promoter region of *PRKARIA* responsible for carney complex in the patient. The horizontal arrow with a broken line indicates the region of the deletion in the GNOMAD database. the genbank accession number of mutant allele identified in this study is OL629246

was identified in the upstream region of *PRKARIA* in the genome of the patient (Supplementary Figure S2a), but not his parents. Except for rs4968898 (chr17: 66505856), the genotypes of the parent–offspring trio showed no discordant SNPs. At the locus, the father's and mother's genotypes were A/A and B/B, respectively. The patient's genotype was B/B, indicating that the patient's genome had lost one copy at the locus. Given the length of the deletion, it should have been possible to bridge the flanking sequences using long-range PCR, but we failed to obtain the shorter PCR products, indicating that further detailed characterization of the deleted region was needed. Additionally, no large deletions were identified in the 2p15 region, where another locus responsible for CNC (CNC2) is localized, suggesting that copy number loss at chr17 upstream of *PRKARIA* may be responsible for the patient's CNC.

To characterize the deleted region more precisely, TaqMan genotyping and SYBR green RT-qPCR assays were done, which showed a different deletion pattern from that in the SNP array. Figure 2b shows the loss in copy number in the genomes of the patient and his mother detected by SYBR green quantitative PCR, which was localized downstream of the deleted region indicated by the SNP array. Genomic walking with SYBR green quantitative PCR and TaqMan genotyping (see Figure 2c for loss of typical heterozygosity pattern in the patient) revealed that the deleted region in the patient and mother may lie farther downstream, and involve the promoter region of *PRKARIA*.

Long-range PCR revealed that the patient and his mother shared the shorter sequence between 66,497,104 and 66,512,107 at chr17 (Figure 2d). Retrospectively, the smooth signals of the SNP array probes corresponding to a region of the deletion (chr17:66501103–66,512,084) were examined further, revealing decreased signals in the patient and his mother (both 1.75) compared with his father (2.0) (Supplementary Figure S2b), indicating that the deleted chromosome in the patient was indeed inherited from his mother. The discrepancy between the SNP array data and the actual deletion might be explained by the uneven distribution of copy number probes, i.e. there is no probe for the disputed region in this particular SNP array (Supplementary Figure S2c).

After comparing the nucleotide sequence of the flanking region of the deletion generated by long-range PCR of the genome in the patient and his mother with a human reference genome, the breakpoints in the region were identified (chr17:g.66502289–66510858delinsA: Figure 2e, Supplementary Figure S3a,b). We then compared the deleted region identified in the patient and his mother with many previously reported deletion mutations, which mainly cover the all coding exons (Supplementary

Figure S3a). There was one exception: CAR 532.01 (Horvath et al., 2008), a case of sporadic CNC involving a deletion of the 5' region of *PRKARIA* with a downstream breakpoint very near to that of our deletion (Figure 2e and Supplementary Figure S3a). There is one deletion, nsv4272353, has been registered in the gnomAD database (Collins et al., 2020) and this deletion does not cover the promoter and exon one (Figure 2e). Because our patient and his mother share the same deletion allele that could cause loss of expression of *PRKARIA*, we suspected that this allele was likely to be responsible for the patient's CNC.

3.3 | Relatively low expression of *PRKARIA* in the patient's blood cells

If the deletion identified in this study causes the loss of promoter activity of *PRKARIA*, the expression of *PRKARIA* in patients who carry the deletion in one of their chromosomes would be expected to be lower compared with healthy individuals. The RT-qPCR assay revealed that the level of RNA expression of *PRKARIA* relative to *RPLP2* in the patient's peripheral blood cells was similar to that in three cancer cell lines (Figure 3a). Interestingly, the mRNA expression of *PRKARIA* relative to *RPLP2* calculated from GTEx data on normal human peripheral blood (0.167) was about twofold higher than that in our patient (0.0780). Additionally, *PRKARIA* protein expression was lower in the Sertoli cells than the invaginated non-cancer cells on immunohistochemistry of the patient's testicular tumor (Figure 3b).

3.4 | Molecular validation of the non-functional deletion of the *PRKARIA* upstream region

Our patient's deletion covers the H3K27 acetylated region, many transcription factor binding sites, and the first exon of *PRKARIA* based on the image obtained from the UCSC Genome Browser. To examine the promoter activity in the deleted region, we first identified two possible promoters based on the position of exon 1 of *PRKARIA*. There are two different groups of transcripts for *PRKARIA*. Two transcripts, NM_001369389 and NM_002734, were used to represent the upstream and downstream first exons, respectively (Figure 4a). We constructed two NLuc reporter plasmids to differentiate between the activities of the two promoters. In HEK293 cells, one of the promoters, downstream promoter 2, showed strong transactivation of the NLuc reporter gene, indicating that the deleted region has

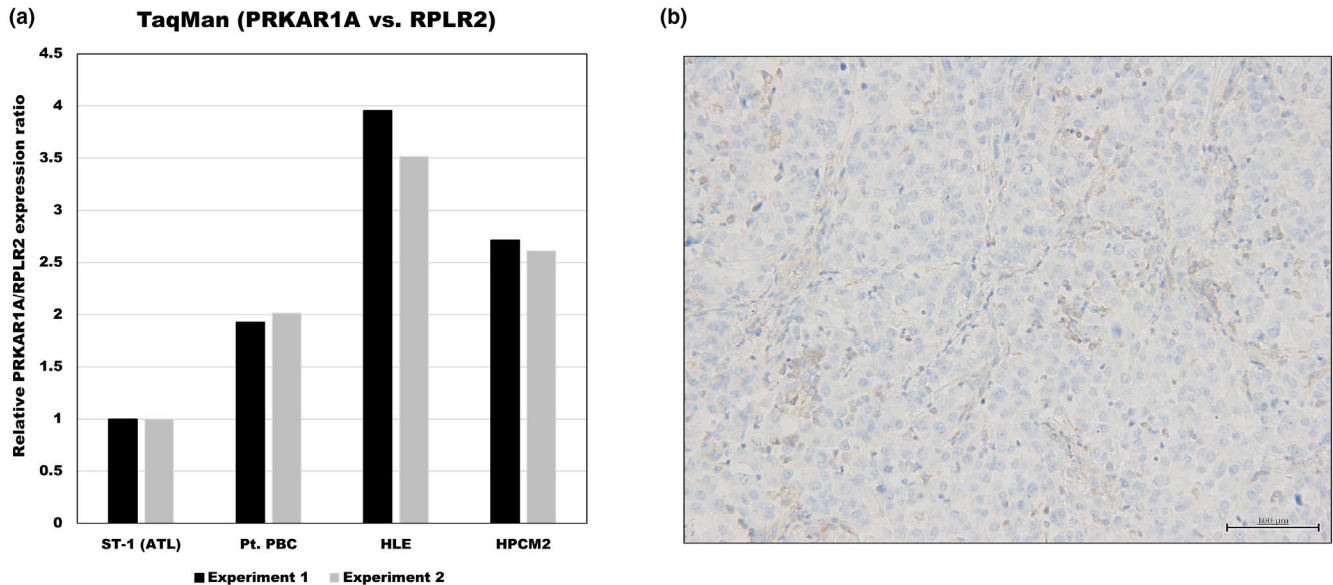


FIGURE 3 relative expression of *PRKAR1A* mRNA in the patient's blood and testicular tumor. (a) mRNA expression of *PRKAR1A* relative to *RPLP2* in the patient's peripheral blood cells (Pt PBC) and three cancer cell lines (ST-1, HLE and HPCM2). The ratio of *PRKAR1A* to *RPLP2* expression in ST-1 was set as 1. Black and hatched boxes indicate two independent experiments. (b) Immunohistochemistry showing PKAR1A protein in the patient's testicular tumor

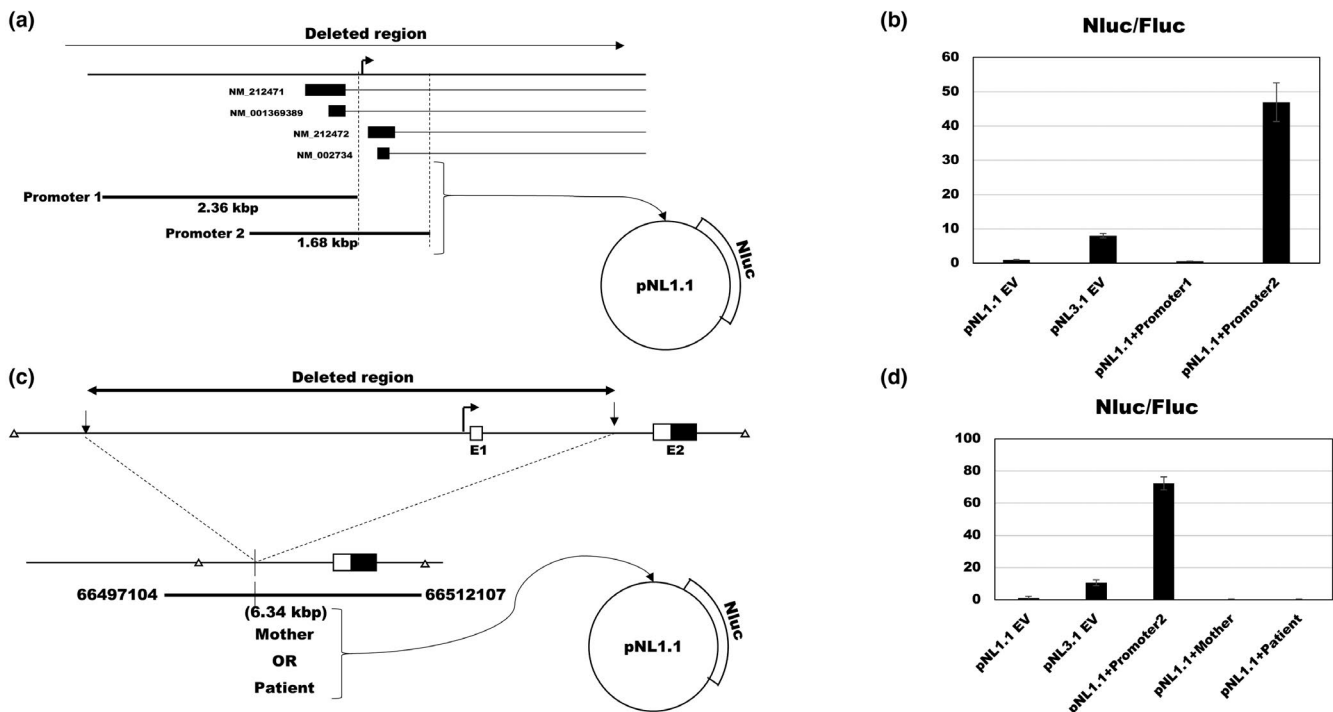


FIGURE 4 Promoter activity of the *PRKAR1A* deleted region in HEK-293 cells. (a) Schematic diagram of reporter plasmid construction for testing promoter activity. Closed boxes indicate the first exons of four representative isoforms of *PRKAR1A* transcripts and their genbank accession numbers. The regions of promoters 1 and 2 cloned into PNL1.1-NEO are indicated by thick horizontal lines. (b) Relative reporter gene expression of the promoters. relative nanoluciferase (NLUC) activity of pNL1.1-neo transfectants comparing with the control firefly luciferase (FLUC) activity of PGL2.2 in HEK-293 cells was set as 1. EV = empty vector (c) schematic of reporter plasmid construction for testing promoter activity of the mutant alleles. symbols are the same as described in Figure 2e. (d) Relative reporter gene expression of the promoters. NLUC activity of PNL1.1-Neo transfectants was set as 1. PRO, promoter; MO, mother; PT, Patient

promoter activity that could manifest as CNC in the patient (Figure 4b).

Next, we tested the promoter activity of the new mutant allele. Using PCR, we generated mutated allele fragments (7.92 kb) from the patient and his mother's genomic DNA and cloned them into pNL1.1-neo (Figure 4c). Neither of the mutated alleles from either the patient or his mother showed any promoter activity, indicating that the loss of *PRKARIA* promoter activity caused CNC in the patient (Figure 4d).

4 | DISCUSSION

The present study identified a new pathogenic deletion mutation for CNC. Our patient, the proband, suffered from bilateral calcified Sertoli tumor and a pituitary adenoma detected by MRI imaging, as well as acromegaly. CGP of the patient did not detect small deleterious mutations in the *PRKARIA* locus, but SNP array analyses revealed possible copy number loss in the genomes of the patient and his mother. Genome walking and long-range PCR clarified that the mutated allele comprises a deleted region spanning 8.57 kb, which includes an active promoter and non-coding exon 1 of *PRKARIA*. This deletion is shared with the patient's mother, who suffers from recurrent cardiac myxomas, indicating the deletion is responsible for the patient's CNC. The level of mRNA expression of *PRKARIA* was decreased both in the testicular tumor and the peripheral blood of the patient. Finally, the presence and absence of promoter activity were confirmed in the deleted region and the mutated allele, respectively.

The first report of a large deletion in *PRKARIA* being responsible for CNC was by Horvath et al., in 2008 (Horvath et al., 2008). The same group reported other large deletions associated with CNC, but there was no familial cases in their cohort (Salpea et al., 2014). Iwata et al. described a single 0.5-Mb deletion involving the entire gene sequence of *PRKARIA* shared by a mother and two of her offspring (Iwata et al., 2015). They clinically diagnosed their patient with CNC and identified the loss of copy number of the *PRKARIA* gene using SYBR green quantitative PCR, but the details regarding identification of the breakpoints of this large 0.5-Mb deletion were not made available. Stelmachowska-Banaś et al. also reported a large deletion of 41 to 107 kb involving *PRKARIA* that was shared by a pair of siblings, but not their parents, and was detected using a comparative genomic hybridization array (Stelmachowska-Banas et al., 2017). In both of those studies, the deletions involved the entire *PRKARIA* gene. To the best of our knowledge, this is the first report of a family with CNC caused by a deletion in *PRKARIA* not involving the coding exons.

The method used to detect large structural variants at the *PRKARIA* locus was SNP array analysis, specifically the Cytoscan HD array. The potential resolution of this SNP array varies at different loci and between populations. Zarrei et al. reported that they used Cytoscan HD to detect a 16-kb deletion in people with a hemiplegic form of cerebral palsy (Zarrei et al., 2018). Because the mean spacing of the probes in CytoScan HD is one probe/1.1 kb, a minimum stretch of 25 probes would be needed to detect our patient's 8.57-kb deletion, i.e. it might not be routinely detected on this array. As shown in Supplementary Figure S2c, the density of the copy number probes was quite high in one part of the deleted region, and this part would be critical for detection of the deletion in the patient's genomic DNA. Fluctuations in CytoScan HD probe signals in the region just upstream from the deletion, which was present as two copies in our patient's DNA, were initially missed, but ultimately indicated a copy number alteration. Therefore, we suggest that careful examination of SNP array data is necessary to detect structural variations at the *PRKARIA* locus that may be responsible for CNC. Alternatively, long-range PCR or multiplex ligation-dependent probe amplification (Schouten et al., 2002) may be helpful to detect middle-size deletions that might not be detectable by the short-read sequencers (from 500 bp to several kilo-bases) in the *PRKARIA* gene.

The functional importance of the deleted region in our case was shown by the NLuc reporter assay, in which only one of two possible promoter-containing fragments showed activity. RNA-Seq mapping signals were similar between the two possible first exons of *PRKARIA* (data not shown). However, it is possible that these two promoter sequences function differently in vivo, and the use of alternating promoters may be important for fine tuning of the PKA signaling pathways.

The prevalence of CNC in the Japanese population is unclear, in part because a comprehensive collection of data on patients with CNC has not been yet undertaken. A Japanese whole-genome reference panel comprising sequences from more than 8300 Japanese individuals, the 8.3KJPN (an earlier version of this dataset was described by Tadaka et al. (2019)), does not include variants reported in the ClinVar-pathogenic archive, nor potential loss-of-function variants in *PRKARIA*, indicating that CNC would be quite rare among Japanese. The relatively recent establishment of CNC as a disease entity, and its varied manifestations among patients, may explain the underrepresentation of the studies of this disease in Japan.

Recently, CGP of cancer tissues has become popular in Japan, with susceptibility genes for some hereditary cancer syndromes being analyzed at the bedside. Our

patient's CGP showed a negative result for *PRKARIA* mutations. The known causative variants of *PRKARIA* in CNC mainly consist of short, nonfunctional variants such as stop gains (Kamilaris et al., 2019). Thus, most CNC type 1 cases caused by inactivation of the germline *PRKARIA* should be detectable by CGP panels that include the gene (for example, see Mineo et al. (2016)). However, in suspected cases of CNC with no common pathogenic variants in *PRKARIA*, it is recommended that the potential for large deletions in the locus be investigated (Salpea et al., 2014; Stelmachowska-Banas et al., 2017). Our results indicated that the precise clinical diagnosis of CNC is critically dependent on histopathologic results, clinical symptoms and signs, and selection of an appropriate modality to detect structural variants in *PRKARIA* in the absence of small nonfunctional variants.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The Genbank accession number of mutated allele sequence is OL629246. This item provisionally will open on 22nd December 2022. We will ask Genbank to open the item just after the publication of the manuscript.

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