Germline deletion and a somatic mutation of the \textit{PRKAR1A} gene in a Carney complex-related pituitary adenoma

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Abstract

\textbf{Objective:} The objective was to assess involvement of loss of the \textit{PRKAR1A} gene encoding a type 1α regulatory subunit of cAMP-dependent protein kinase A located on 17q24 in a Carney complex (CNC)-related pituitary adenoma.

\textbf{Design:} We investigated aberrations of the \textit{PRKAR1A} gene in a CNC patient with a GH-producing pituitary adenoma, whose family has three other members with probable CNC.

\textbf{Methods:} A gene mutation was identified by a standard DNA sequencing method based on PCR. DNA copy number was measured to evaluate allelic loss on 17q24 by quantitative PCR. The breakpoints of deletion were determined by cloning a rearranged region in the deleted allele.

\textbf{Results:} A \textit{PRKAR1A} mutation of c.751_758del8 (p.S251LfsX16) was found in genomic DNA obtained from a pituitary adenoma, but not leukocytes from the patient. Reduced DNA copy number at loci including the \textit{PRKAR1A} gene on 17q24 was detected in both the tumor and leukocytes, suggesting a deletion at the loci at the germline level. The deletion size was determined to be ~0.5 Mb and this large deletion was also found in two other family members.

\textbf{Conclusion:} This is the first case showing a CNC-related pituitary adenoma with the combination of somatic mutation and a large inherited deletion of the \textit{PRKAR1A} gene. Biallelic inactivation of \textit{PRKAR1A} appears to be necessary for the development of CNC-related pituitary adenoma.

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Introduction

The complex of ‘spotty-skin pigmentation, myxomas, endocrine overactivity, and schwannomas’ or Carney complex (CNC) (MIM 160980) is an autosomal dominant multiple neoplasia syndrome (1). CNC is an inherited predisposition to tumors associated with primary pigmented nodular adrenocortical disease (PPNAD), GH- and/or PRL-producing pituitary adenoma, myxomas of heart or skin, psammomatous melanotic schwannoma, and breast ductal adenoma (2).

Previous studies have shown inactivating germline mutations in the \textit{PRKAR1A} gene on 17q24, which may function as a tumor-suppressor gene, in patients with CNC (3, 4). The encoded protein is a type 1α regulatory subunit of cAMP-dependent protein kinase A (PKA). Inactivating germline mutations of this gene are found in ~70% of patients with CNC (2, 5). Salpea et al. (6) have recently reported that 17q24.2−24.3 deletions of varying size including the \textit{PRKAR1A} gene led to haploinsufficiency....
in 11 CNC patients without PRKAR1A mutations. Less commonly, the molecular pathogenesis of CNC is a variety of genetic changes on 2p16 (7).

Previous studies have shown that biallelic inactivation of PRKAR1A is needed for tumor development in the adrenal and other tissues (8, 9). In addition, biallelic, but not monoallelic, inactivation of Prkar1a in mouse pituitary gland leads to the development of a pituitary tumor (10) and several pituitary tumors in CNC patients have been shown to have bilallelic abnormalities of PRKAR1A (4, 11, 12). In this study, we examined the PRKAR1A locus in a Japanese family with CNC and found a large germline deletion of the locus and biallelic inactivation of PRKAR1A in a pituitary adenoma.

**Subjects and methods**

**Case report**

The proband was a young woman who presented with pituitary gigantism and pigmented spots on the face and lips. She had been tall during her childhood and reached a height of 183.5 cm. Incidental pituitary adenoma with a diameter of 15 mm was revealed by magnetic resonance imaging (MRI) upon investigating her head injury (Fig. 1A). She had increased serum levels of GH (11.0 μg/l) and IGF1 (590 μg/l), and the GH levels were not suppressed during oral glucose tolerance test. Serum levels of PRL and cortisol were within the normal range. She had undergone successful endoscopic transnasal adenomectomy at Toranomon Hospital, and the resected pituitary adenoma showed an unusual unique histological feature. The adenoma was composed of extremely enlarged eosinophilic cells harboring large nuclei with prominent nucleoli (Fig. 1B). Immunohistochemical study revealed that tumor cells were positive to an anti-GH antibody (A0570; Dako, Glostrup, Denmark) and negative to an antibody against type 1α regulatory subunit of PKA (#610610; BD Biosciences, San Jose, CA, USA) compared with adjacent normal cells (Fig. 1C and D).

**Family history**

Her family history was remarkable; her mother (II-2 in Fig. 1E) had been operated on for a right adrenal tumor due to Cushing’s syndrome at the age of 19 and her grandmother (I-2) for a cardiac tumor at the age of 48. Both mother and grandmother had been operated on for breast tumors, at ages of 52 and 68 respectively. An elder sister (III-1) underwent surgical operations for a gingival tumor at the age of two and eyelid tumors at ages of 13 and 16. One of the eyelid tumors was pathologically diagnosed as cutaneous myxoma. Spotty facial pigmentation was present.

This study was approved by ethics committees of Toranomon Hospital and the University of Tokushima. Individual informed consents for genomic analyses and case presentations were obtained from patients.
Gene mutation analysis

Gene mutation analysis for the PRKAR1A gene using PCR and sequencing was performed as described previously (13). Genomic DNA isolated from leukocytes and a frozen pituitary adenoma was subjected to PCR using TaKaRa Ex Taq Polymerase (TaKaRa, Shiga, Japan) with the PRKAR1A primer sets. PCR products were subjected to direct sequencing in sense and antisense directions.

DNA copy number analysis in a tumor and leukocytes

Relative DNA copy number at each locus on 17q24 was measured by quantitative PCR (qPCR) in a 7300 Real Time PCR System (Applied Biosystems) using THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) with each primer set shown in Supplementary Table, see section on supplementary data given at the end of this article. The specificity of the primer pairs was verified by dissociation curves and the quantitativity was confirmed by slopes in the standard samples for the target loci being between $K_{3.6}$ and $K_{3.2}$ and each R2 value being between 0.99 and 1. The DNA copy number was normalized using that of human TATA-binding protein (TBP) located on 6q27. Furthermore, DNA copy numbers of albumin (ALB) on 4q13.3 or telomerase reverse transcriptase (TERT) on 5p15.33 were confirmed to be almost equal among the samples.

Detection of deleted allele on 17q24 in the subjects

DNA encompassing the deletion junction on 17q24 was amplified from each subject’s leukocyte genomic DNA using TaKaRa Ex Taq Polymerase with the following primers: forward: 5’-GGGACCATCCTGGCTAACACG-3’ and reverse: 5’-GGACATCTGACCTACAAAACTGTGAGC-3’. Normal female DNA (Promega) and pBluescript II SK with a DNA fragment including the deletion junction were used as a negative control and a positive control, respectively. PCR using a primer set for the TERT gene as an internal control was also carried out.

Results

Somatic mutation and one-copy deletion of the PRKAR1A gene in a pituitary adenoma

We examined mutations of the PRKAR1A gene in genomic DNA from a pituitary adenoma and from leukocytes of a proband (III-2 in Fig. 1E) and her parents (II-1 and II-2), using direct sequencing of 11 overlapping PCR products with primer sets that cover the entire coding region and splicing junctions. Sequencing analysis showed no mutations in the PRKAR1A gene in leukocyte genomic DNA from all subjects, but a somatic mutation of c.751_758del8 (p.S251LfsX16) in exon 8 of the PRKAR1A gene in a pituitary adenoma (Fig. 2A). Intensities of WT sequence peaks were weaker than those of mutated allele in the tumor sample, suggesting that these sequence peaks might be derived from the normal allele of slightly contaminated normal tissue and the other allele might be lost at the germline level. To demonstrate the deletion at 17q24, DNA copy number of the PRKAR1A gene was measured by qPCR using five primer sets as shown in Fig. 2B (upper diagram). The DNA copy numbers of the PRKAR1A gene were reduced by almost half in leukocyte

Figure 2

Mutation and deletion analyses of the PRKAR1A gene in a CNC family. (A) Scanned segments of sequencing electropherograms of leukocytes (left) and the tumor (right) DNA from the proband. At the top of each panel, nucleotide sequences of WT (black) and a frameshift mutation (red) are indicated. (B) Validation of PRKAR1A deletion by qPCR. Upper diagram shows the genomic structure of the PRKAR1A gene. The vertical lines indicate positions where DNA copy numbers were measured in introns (Int) 1, 3, 5, and 8 and exon (ex) 11. The graph shows relative DNA copy numbers at each indicated region of the PRKAR1A gene compared with the TBP gene in leukocytes from the proband (III-2, P), father (II-1, F), and mother (II-2, M) and the tumor (T). The albumin (ALB) gene on 4q13.3 was analyzed as a control. A full colour version of this figure is available via http://dx.doi.org/10.1530/EJE-14-0685.
genomic DNA from the proband (III-2) and her mother (II-2), compared with that from the father (II-1) (Fig. 2B), suggesting the inherited deletion of the PRKAR1A gene in the family.

Identification of the breakpoints on 17q24

To identify the range of deletion at 17q24 in the leukocyte genomic DNA, we measured DNA copy number at each locus in the vicinity of the PRKAR1A gene (Fig. 3A) and found that the deletion is in the vicinity of the LOC732538 gene to intron 1 in the FAM20A gene (data not shown). We designed sets of four primers in intron 1 of the FAM20A gene and in the vicinity of the LOC732538 gene (P1–P4 and p1–p4 in Fig. 3B and C) to detect DNA copy number in the loci. The qPCR analyses narrowed the breakpoints to a 1.3 kb region between P2 and P3 in intron 1 of the FAM20A gene (Fig. 3B) and to 14 kb between p2 and p3 surrounding the LOC732538 gene (Fig. 3C).

To identify the breakpoints, cloning of a region encompassing the deletion junction was attempted (Supplementary Fig. 1A, B, and C, see section on supplementary data given at the end of this article). The cloned DNA contained sequences of intron 1 of the FAM20A gene and the LOC732538 gene, and a 24 bp region in which they overlapped (Supplementary Fig. 1D and E), suggesting that each breakpoint should be located in the overlapping region and the deletion size in genomic DNA was ~0.5 Mb. The deletion size and the location were consistent with results obtained from comparative genomic hybridization (CGH) array analysis (Supplementary Fig. 2). Finally, we designed a primer set to detect the deletion and confirmed that the family members (II-2, III-1, and III-2), except for the proband’s father (II-1), had the germline deletion by the presence of 325 bp PCR products (Fig. 3D). Her grandmother’s leukocytes were unavailable (Supplementary Materials and methods).

Discussion

The most common type of endocrine tumor in CNC is PPNAD, which was detected in 25–30% of patients (2). In this family, her mother developed bilateral adrenal tumors, while pathological findings of right resected tumors were unavailable. Although almost all CNC patients (75%) exhibit asymptomatic elevations in serum GH, IGF1, or PRL level, acromegaly with pituitary adenomas occurs in a smaller population of patients (~ 10%) (14). Gigantism is frequent in CNC; in addition to two adolescents with gigantism published in individual

Figure 3

Deletion analyses at several loci on 17q24. (A) Schematic diagram of the 17q24 region. Arrows indicate the orientation of genes in the 5’ to 3’ direction. (B) Relative DNA copy number at positions in intron 1 of FAM20A downstream of the PRKAR1A gene compared with the TBP gene. Using the set of four primers (P1–P4) indicated in the upper diagram, relative DNA copy number at four positions in intron 1 of FAM20A was measured by qPCR in leukocytes from the proband (III-2, P), father (II-1, F), and mother (II-2, M) and in the tumor (T). (C) Relative DNA copy number at loci in the vicinity of the LOC732538 gene compared with the TBP gene. Using the set of four primers (p1–p4) in each position, relative DNA copy number was measured in the same way. (D) Identification of the large deletion on 17q24 in family members. The estimated size of PCR products amplified from a WT allele and an allele with the deletion using a primer set (arrow) to detect the deletion (upper diagram). Representative electropherogram of PCR products for leukocyte DNA from the proband (III-2, P), her father (II-1, F), mother (II-2, M), and sister (III-1, S). PCR using a primer set for the TERT gene on 5p15.33 as an internal control was also carried out. NC, no template control; C, control genomic DNA; PC, a plasmid including DNA encompassing a deletion junction. A full colour version of this figure is available via http://dx.doi.org/10.1530/EJE-14-0685.
case reports (15, 16), several large series of patients with CNC and GH-secreting pituitary tumors have been published, which have included cases with gigantism (5).

To our knowledge, this is the first proven CNC-related pituitary adenoma with the combination of a large inherited deletion on 17q24 and a somatic frameshift PRKAR1A mutation. This study suggests that the standard sequencing method alone is insufficient to detect the genetic abnormalities in leukocytes and tumors. In this study, we estimated the existence of a deletion at the germline level in the family because of the weaker sequencing peaks of the normal allele in addition to somatic frameshift mutation in the tumor (Fig. 2A).

However, if the tumor had a large biallelic deletion, we might misdiagnose it as normal due to the sequencing peak derived from contaminated normal tissue. Therefore, even if no mutations were detected in both leukocytes and tumors by sequencing analysis, measurement of DNA copy number at the locus of tumor suppressor gene may be necessary. Owing to the expense of CGH array and the commercial unavailability of multiplex ligation-dependent probe amplification for the PRKAR1A gene, qPCR of genomic DNA may be the first-line method for detecting a large deletion.

In ~75% of CNC patients, germline mutations in the PRKAR1A gene have been reported (17). Large deletions of the gene loci at 17q24.2–24.3 have recently been detected in 7.7% (18) and 21.6% (6) of PRKAR1A mutation-negative patients with CNC. The mutations and large deletions leading to PRKAR1A haploinsufficiency have been considered to cause CNC. However, whether haploinsufficiency of PRKAR1A is sufficient for the development of pituitary adenomas in CNC remains unknown. Owing to the low frequency of pituitary adenomas in CNC, reports on biallelic inactivation of PRKAR1A in pituitary tumors have been scarce. In the first report about inactivating mutations of the PRKAR1A gene in CNC, one of the two GH-producing pituitary adenomas showed germline mutation and somatic loss of heterozygosity (LOH), while another adenoma was uninformative (4). Bossis et al. (11) and Takano et al. (12) reported LOH in pituitary adenomas with PRKAR1A-inactivating germline mutation. On the other hand, CGH revealed that loss of 17q was not observed in four pituitary adenomas from patients with CNC (19); however, this method may be inadequate to identify whether these tumors have inactivating mutations or a small deletion. In this study, we demonstrated the biallelic inactivation of the PRKAR1A gene in a CNC-related pituitary adenoma and loss of the protein expression in the tumor cells (Fig. 1D).

In conclusion, we found somatic inactivating mutation in GH-producing adenoma in a family with a large inherited deletion of the PRKAR1A locus. This suggests that the complete loss of PRKAR1A might be necessary for the development of at least some pituitary adenomas in CNC.

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