

Title: Germline deletion and a somatic mutation of the *PRKAR1A* gene in a Carney complex-related pituitary adenoma

Authors: T Iwata¹, * T Tamanaha², * R Koezuka², M Tochiya², H Makino², I Kishimoto², N Mizusawa¹, S Ono¹, N Inoshita³, S Yamada⁴, A Shimatsu⁵ and K Yoshimoto¹

*These authors contributed equally to this work.

Affiliations:

¹Department of Medical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

²Department of Endocrinology and Metabolism, National Cerebral and Cardiovascular Center, Osaka, Japan

³Department of Pathology, Toranomon Hospital, Tokyo, Japan

⁴Department of Hypothalamic and Pituitary Surgery, Toranomon Hospital, Tokyo, Japan

⁵Clinical Research Institute, National Hospital Organization Kyoto Medical Center, Kyoto, Japan

Correspondence:

Katsuhiko Yoshimoto, M.D., Ph.D.

Department of Medical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto-cho 3-18-15, Tokushima City 770-8504, Japan

E-mail: yoshimoto@tokushima-u.ac.jp

Short title: *PRKAR1A* mutations in a pituitary tumor

Key words: Carney complex, PRKAR1A, gigantism, large deletion, DNA copy number

Word count: 3,480 words for the manuscript including abstract, references, and figure legends.

Abstract

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

Design: We investigated aberrations of the *PRKARIA* gene in a CNC patient with a GH-producing pituitary adenoma, whose family has 3 other members with probable CNC.

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.

Results: A *PRKARIA* 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 *PRKARIA* 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 approximately 0.5 Mb and this large deletion was also found in other 2 family members.

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 *PRKARIA* gene. Biallelic inactivation of *PRKARIA* may be necessary for the development of CNC-related pituitary adenoma.

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 *PRKARIA* gene on 17q24, which may function as a tumor-suppressor gene, in patients with CNC (3, 4). The encoded protein is a type 1 α regulatory subunit of cAMP-dependent protein kinase A (PKA). Inactivating germline mutations of this gene are found in about 70% of patients with CNC (2, 5). Less commonly, the molecular pathogenesis of CNC is a variety of genetic changes on 2p16 (6).

Salpea *et al.* have recently reported that 17q24.2-24.3 deletions of varying size including the *PRKARIA* gene led to haploinsufficiency in 11 CNC patients without *PRKARIA* mutations (7); however, biallelic inactivation in CNC-related tumors was not

shown. In the present study, we examined the *PRKARIA* locus in a Japanese family with CNC and found a large germline deletion of the locus and biallelic inactivation of *PRKARIA* 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 demonstrated by magnetic resonance imaging (MRI) upon investigating her head injury (Fig. 1A). She had increased serum levels of GH (11.0 µg/l) and IGF-1 (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 transnasal endoscopic adenomectomy at Toranomon Hospital, and the resected pituitary adenoma showed unusual unique histological feature. The adenoma was composed of extremely enlarged bizarre 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 1 α regulatory subunit of PKA (#610610; BD Biosciences, San Jose, CA, USA) compared to adjacent normal cells (Fig. 1C and 1D).

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 the ages of 52 and 68, respectively. An elder sister (III-1) underwent surgical operations for a gingival tumor at the age of 2 and eyelid tumors at the 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 the ethics committees of Toranomon Hospital and The University of Tokushima. Individual informed consents for genomic analyses and case presentations were obtained from the patients.

Gene mutation analysis

Gene mutation analysis for the *PRKAR1A* gene using polymerase chain reaction (PCR) and sequencing was performed as described previously (8). Genomic DNA

isolated from leukocytes and a frozen pituitary adenoma was subjected to PCR using TaKaRa Ex TaqTM 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, Foster City, CA, USA) using THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) with each primer set shown in supplementary Table. 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 -3.6 and -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 by using TaKaRa Ex TaqTM Polymerase with the following primers: forward: 5'-GGGACCATCCTGGCTAACACG-3', reverse: 5'-GGACATCTGACCTACAAACTGTGAGC-3'. Normal female DNA (Promega, Madison, WI) 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 performed.

Results

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

We examined mutations of the *PRKARIA* 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 *PRKARIA* gene in leukocyte genomic DNA from all subjects, but a somatic mutation of c.751_758del8 (p.S251LfsX16) in exon 8 of the *PRKARIA* gene in a pituitary adenoma (Fig. 2A). Intensities of wild-type 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 *PRKARIA* gene was measured by qPCR using 5 primer sets as shown in Fig. 2B (upper diagram). The DNA copy numbers of the *PRKARIA* gene were reduced by almost half in leukocyte 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 4 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 3C) 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 Figs. 1A-1C). 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 Figs. 1D and 1E), suggesting that each breakpoint

should be located in the overlapping region and the deletion size in genomic DNA was approximately 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, 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.

Discussion

The most common type of endocrine tumor in CNC is PPNAD, which was detected in 25-30% of the 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, IGF-1, or PRL level, acromegaly with pituitary adenomas occurs in a smaller population of the patients (approximately 10%) (9). Gigantism is frequent in CNC; in addition to two adolescents with gigantism published in individual cases reports (10, 11), 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 *PRKARIA* 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. Because of the expense of CGH array and the commercial unavailability of multiplex ligation-dependent probe amplification for the *PRKARIA* gene, qPCR of genomic DNA may be the first-line method for detecting a large deletion.

In approximately 75% of CNC patients, germline mutations in the *PRKARIA* gene have been reported (12). Large deletions of the gene loci at 17q24.2-24.3 have recently been detected in 7.7% (13) and 21.6% (7) of *PRKARIA* mutation-negative patients with CNC. The mutations and large deletions leading to *PRKARIA* haploinsufficiency have been considered to cause CNC. However, whether haploinsufficiency of *PRKARIA* is sufficient for the development of pituitary adenomas in CNC remains unknown. Because of the low frequency of pituitary adenomas, reports on biallelic inactivation of *PRKARIA* in CNC-related pituitary tumors have been

scarce. In the first report about inactivating mutations of the *PRKAR1A* gene in CNC, one of two GH-producing pituitary adenomas showed germline mutation and somatic loss of heterozygosity (LOH), while another adenoma was uninformative (12). Bossis *et al.* (14) and Takano *et al.* (15) reported LOH in pituitary adenomas with *PRKAR1A*-inactivating germline mutation. On the other hand, CGH revealed that loss of 17q was not observed in 4 pituitary adenomas from patients with CNC (16); however, this method may be inadequate to identify whether these tumors have inactivating mutations or a small deletion. In this study, we demonstrated that biallelic inactivation of *PRKAR1A* gene in a CNC-related pituitary adenoma and loss of the protein expression in the tumor cells (Fig. 1D).

Studies in mouse models have provided evidence that the development of a CNC-related pituitary tumor requires tumor-specific loss of the normal allele of the *Prkar1a* gene. Although *Prkar1a* knockout (KO) mice show embryonic lethality (17, 18), heterozygous *Prkar1a* KO mice develop sarcomas, hepatocellular carcinomas, and tumors in highly CNC-relevant tissue such as Schwann cells, osteoblasts, and thyrocytes, but not pituitary adenomas (18, 19). In contrast, pituitary-specific *Prkar1a*

KO mice develop pituitary tumors at a significantly higher frequency than that observed in control littermates (20).

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

Declaration of interest

The authors declare that there is no conflict of interest.

Funding

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant number 24591365) and a grant from the Foundation for Growth Science.

Acknowledgements

Many thanks to Dr. S Adachi and Dr. H Horikawa for the technical assistance in CGH array analysis and to Dr. A Hishida and Dr Y. Ohata for valuable discussion.

Patient consent

Informed consent has been obtained from the patient for publication of the case report and accompanying images.

References

1. Carney JA, Gordon H, Carpenter PC, Shenoy BV & Go VL. The complex of myxomas, spotty pigmentation, and endocrine overactivity. *Medicine (Baltimore)* 1985 **64** 270-283.
2. Rothenbuhler A & Stratakis CA. Clinical and molecular genetics of Carney complex. *Best Practice and Research Clinical Endocrinology and Metabolism* 2010 **24** 389-399.
3. Casey M, Vaughan CJ, He J, Hatcher CJ, Winter JM, Weremowicz S, Montgomery K, Kucherlapati R, Morton CC & Basson CT. Mutations in the protein kinase A R1alpha regulatory subunit cause familial cardiac myxomas and Carney complex. *Journal of Clinical Investigation* 2000 **106** R31-38.
4. Kirschner LS, Carney JA, Pack SD, Taymans SE, Giatzakis C, Cho YS, Cho-Chung YS & Stratakis CA. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex. *Nature Genetics* 2000 **26** 89-92.
5. Bertherat J, Horvath A, Groussin L, Grabar S, Boikos S, Cazabat L, Libe R,

- Rene-Corail F, Stergiopoulos S, Bourdeau I *et al.* Mutations in regulatory subunit type 1A of cyclic adenosine 5'-monophosphate-dependent protein kinase (PRKAR1A): phenotype analysis in 353 patients and 80 different genotypes. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 2085-2091.
6. Matyakhina L, Pack S, Kirschner LS, Pak E, Mannan P, Jaikumar J, Taymans SE, Sandrini F, Carney JA & Stratakis CA. Chromosome 2 (2p16) abnormalities in Carney complex tumours. *Journal of Medical Genetics* 2003 **40** 268-277.
7. Salpea P, Horvath A, London E, Faucz FR, Vetro A, Levy I, Gourgari E, Dauber A, Holm IA, Morrison PJ *et al.* Deletions of the PRKAR1A locus at 17q24.2-q24.3 in Carney complex: genotype-phenotype correlations and implications for genetic testing. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** E183-188.
8. Yamasaki H, Mizusawa N, Nagahiro S, Yamada S, Sano T, Itakura M & Yoshimoto K. GH-secreting pituitary adenomas infrequently contain inactivating mutations of PRKAR1A and LOH of 17q23-24. *Clinical Endocrinology (Oxf)* 2003 **58** 464-470.

9. Pack SD, Kirschner LS, Pak E, Zhuang Z, Carney JA & Stratakis CA. Genetic and histologic studies of somatomammotrophic pituitary tumors in patients with the "complex of spotty skin pigmentation, myxomas, endocrine overactivity and schwannomas" (Carney complex). *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 3860-3865.
10. Proppe KH & Scully RE. Large-cell calcifying Sertoli cell tumor of the testis. *American Journal of Clinical Pathology* 1980 **74** 607-619.
11. Rosenzweig JL, Lawrence DA, Vogel DL, Costa J & Gorden P. Adrenocorticotropin-independent hypercortisolism and testicular tumors in a patient with a pituitary tumor and gigantism. *Journal of Clinical Endocrinology and Metabolism* 1982 **55** 421-427.
12. Kirschner LS, Sandrini F, Monbo J, Lin JP, Carney JA & Stratakis CA. Genetic heterogeneity and spectrum of mutations of the PRKAR1A gene in patients with the carney complex. *Human Molecular Genetics* 2000 **9** 3037-3046.
13. Guillaud Bataille M, Rhayem Y, Sousa SB, Libe R, Dambrun M, Chevalier C, Nigou M, Auzan C, North MO *et al.* Systematic screening for PRKAR1A gene

- rearrangement in Carney complex: identification and functional characterization of a new in-frame deletion. *European Journal of Endocrinology* 2014 **170** 151-160.
14. Bossis I, Voutetakis A, Matyakhina L, Pack S, Abu-Asab M, Bourdeau I, Griffin KJ, Courcoutsakis N, Stergiopoulos S, Batista D *et al.* A pleiomorphic GH pituitary adenoma from a Carney complex patient displays universal allelic loss at the protein kinase A regulatory subunit 1A (PRKARIA) locus. *Journal of Medical Genetics* 2004 **41** 596-600.
15. Takano K, Yasufuku-Takano J, Morita K, Mori S, Takei M, Osamura RY, Teramoto A & Fujita T. Evidence that PKA activity is constitutively activated in human GH-secreting adenoma cells in a patient with Carney complex harbouring a PRKAR1A mutation. *Clinical Endocrinology (Oxf)* 2009 **70** 769-775.
16. Pack SD, Qin LX, Pak E, Wang Y, Ault DO, Mannan P, Jaikumar S, Stratakis CA, Oldfield EH, Zhuang Z *et al.* Common genetic changes in hereditary and sporadic pituitary adenomas detected by comparative genomic hybridization.

- Genes Chromosomes and Cancer* 2005 **43** 72-82.
17. Amieux PS, Howe DG, Knickerbocker H, Lee DC, Su T, Laszlo GS, Idzerda RL & McKnight GS. Increased basal cAMP-dependent protein kinase activity inhibits the formation of mesoderm-derived structures in the developing mouse embryo. *Journal of Biological Chemistry* 2002 **277** 27294-27304.
18. Kirschner LS, Kusewitt DF, Matyakhina L, Towns WH, 2nd, Carney JA, Westphal H & Stratakis CA. A mouse model for the Carney complex tumor syndrome develops neoplasia in cyclic AMP-responsive tissues. *Cancer Research* 2005 **65** 4506-4514.
19. Veugelers M, Wilkes D, Burton K, McDermott DA, Song Y, Goldstein MM, La Perle K, Vaughan CJ, O'Hagan A, Bennett KR *et al.* Comparative PRKAR1A genotype-phenotype analyses in humans with Carney complex and prkar1a haploinsufficient mice. *Proceedings of National Academy of Science of the United States of America* 2004 **101** 14222-14227.
20. Yin Z, Williams-Simons L, Parlow AF, Asa S & Kirschner LS. Pituitary-specific knockout of the Carney complex gene Prkar1a leads to

pituitary tumorigenesis. *Molecular Endocrinology* 2008 **22** 380-387.

Figure legends

Figure 1. (A) Preoperative coronal T1-weighted enhanced MRI of the proband and histopathology of pituitary tumor. MRI demonstrates a pituitary tumor (15 mm in diameter) located in the left wing. (B-D) Hematoxylin and eosin staining (B) and immunostaining using antibodies against GH (C) and type 1 α regulatory subunit of PKA (D) in serial sections of pituitary adenoma with adjacent normal pituitary cells (left upper side). (E) A pedigree of the CNC family and histological findings of tumors. The proband is indicated by an arrow. Family members are indicated by generation (Roman numerals) and individuals (Arabic numerals). Individuals are represented as male (squares) and female (circles). Filled symbols denote patients with CNC.

Figure 2. Mutation and deletion analyses of the *PRKARIA* 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 wild type (black) and a frameshift mutation (red) are indicated. (B) Validation of

PRKARIA deletion by qPCR. Upper diagram shows the genomic structure of the *PRKARIA* 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 *PRKARIA* 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.

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 *PRKARIA* gene compared with the *TBP* gene. Using the set of 4 primers (P1-P4) indicated in the upper diagram, relative DNA copy number at 4 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 4 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. Estimated size of PCR products amplified from a wild-type allele and an allele with the deletion using a primer set (arrow) to detect the deletion (upper diagram). Representative electropherogram of PCR products for leukocytes 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 performed. NC, no template control. C, control genomic DNA. PC, a plasmid including DNA encompassing a deletion junction.

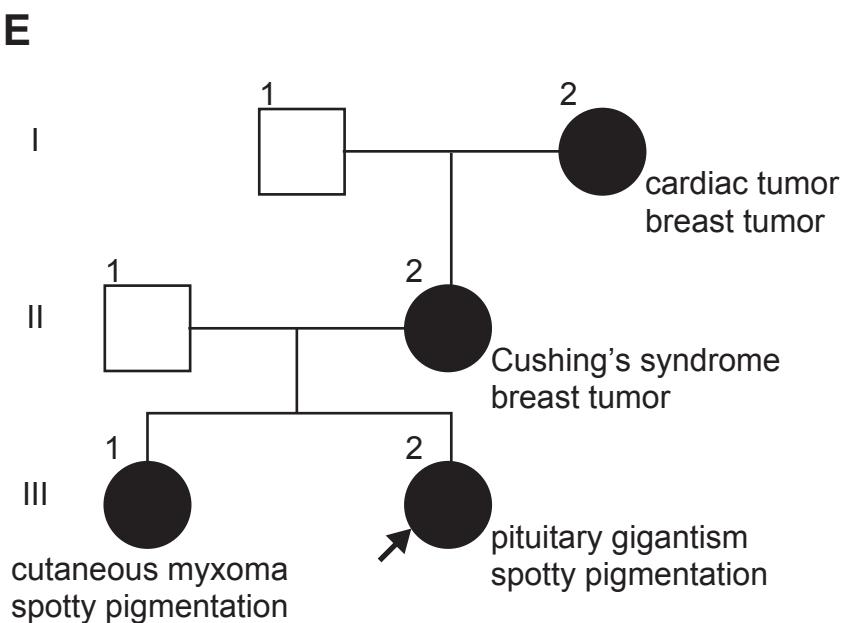
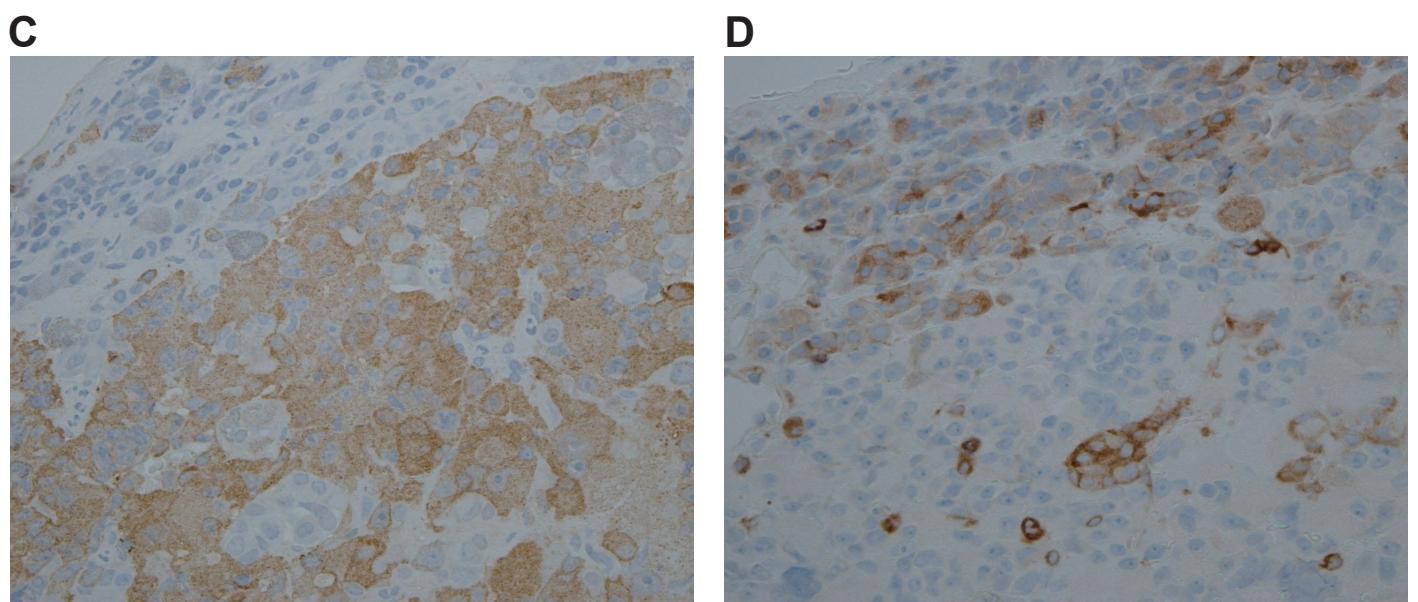
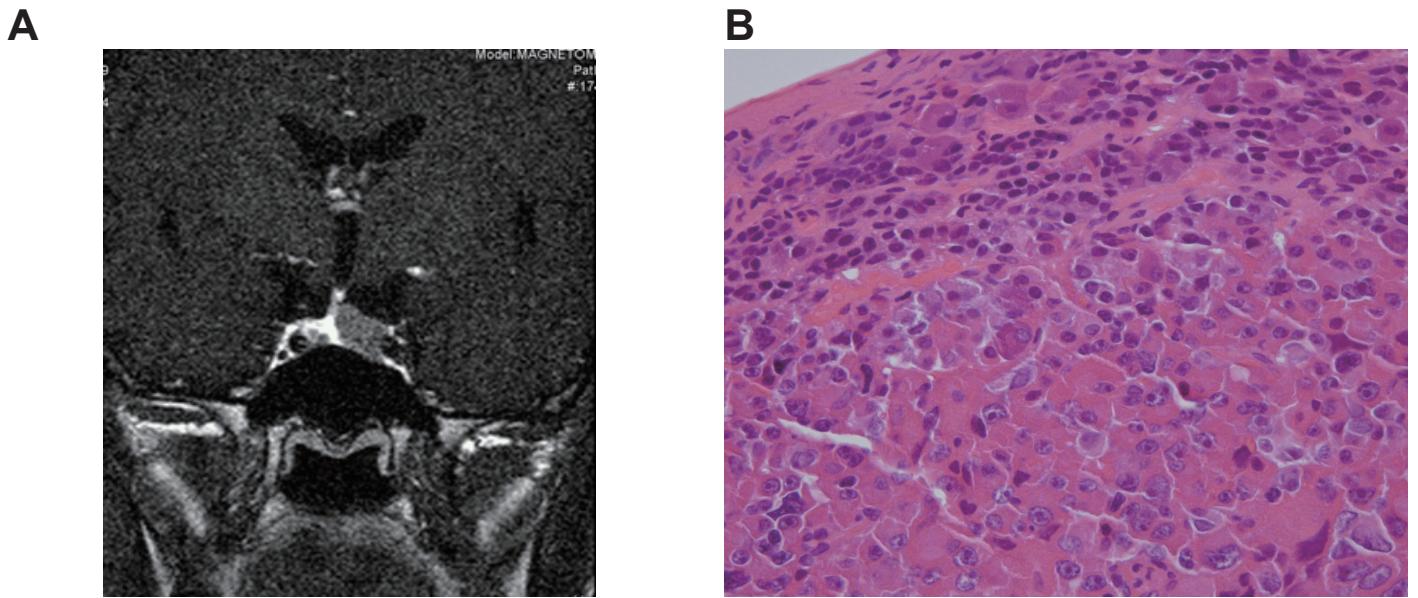


Figure 1

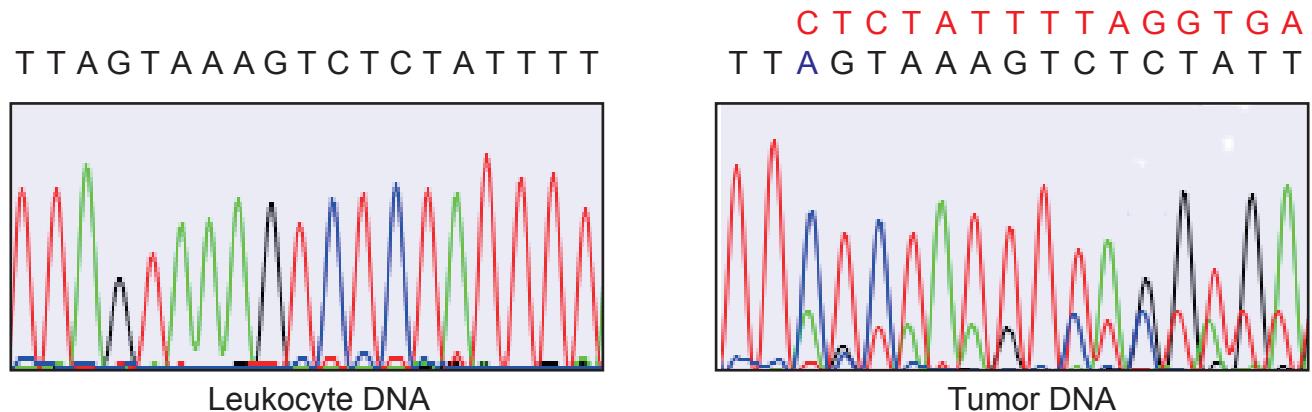
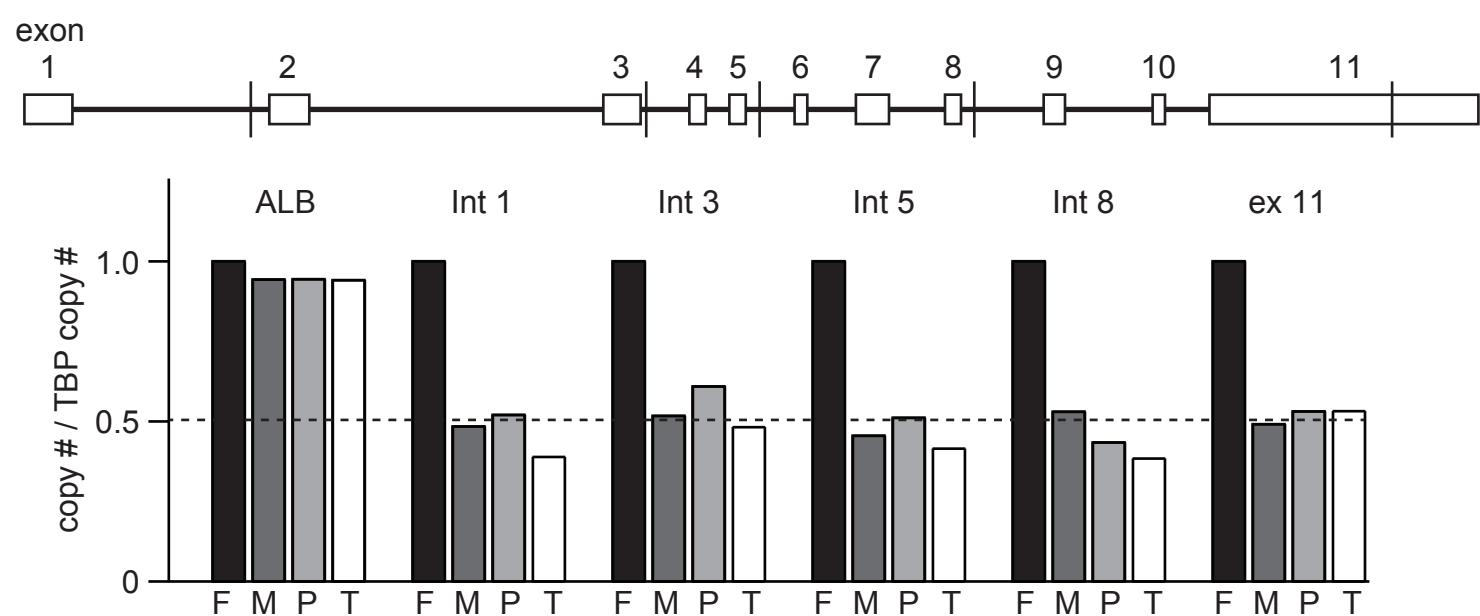
A**B**

Figure 2

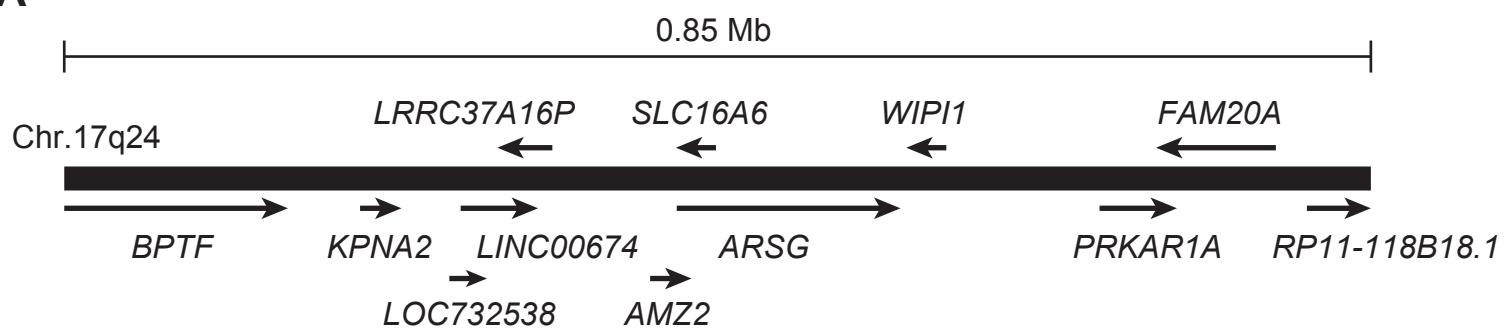
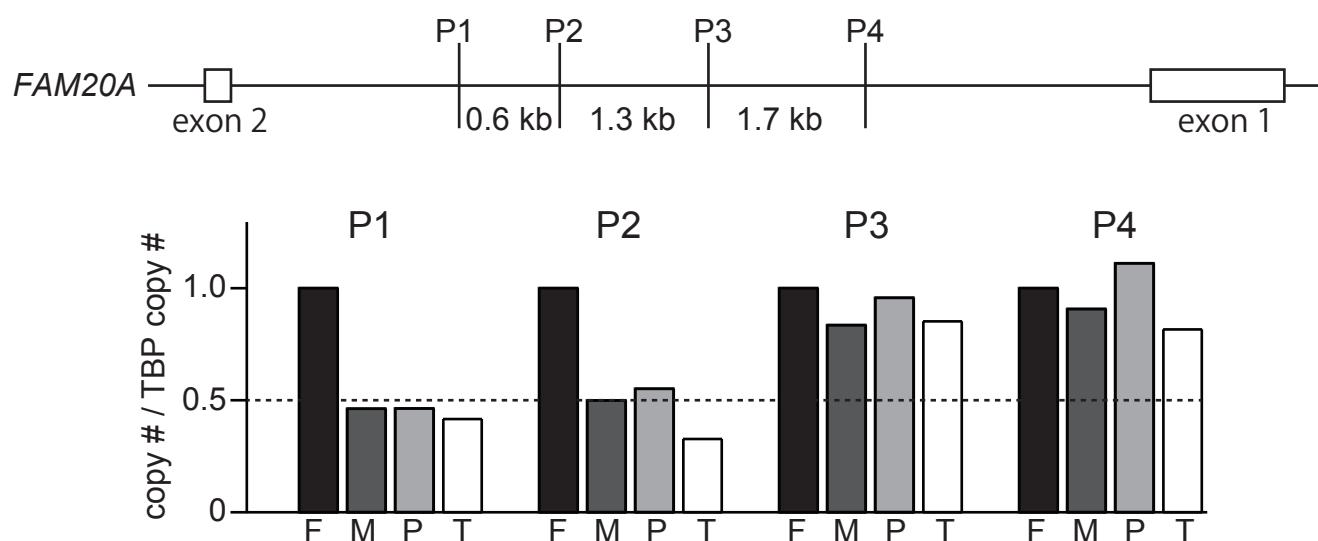
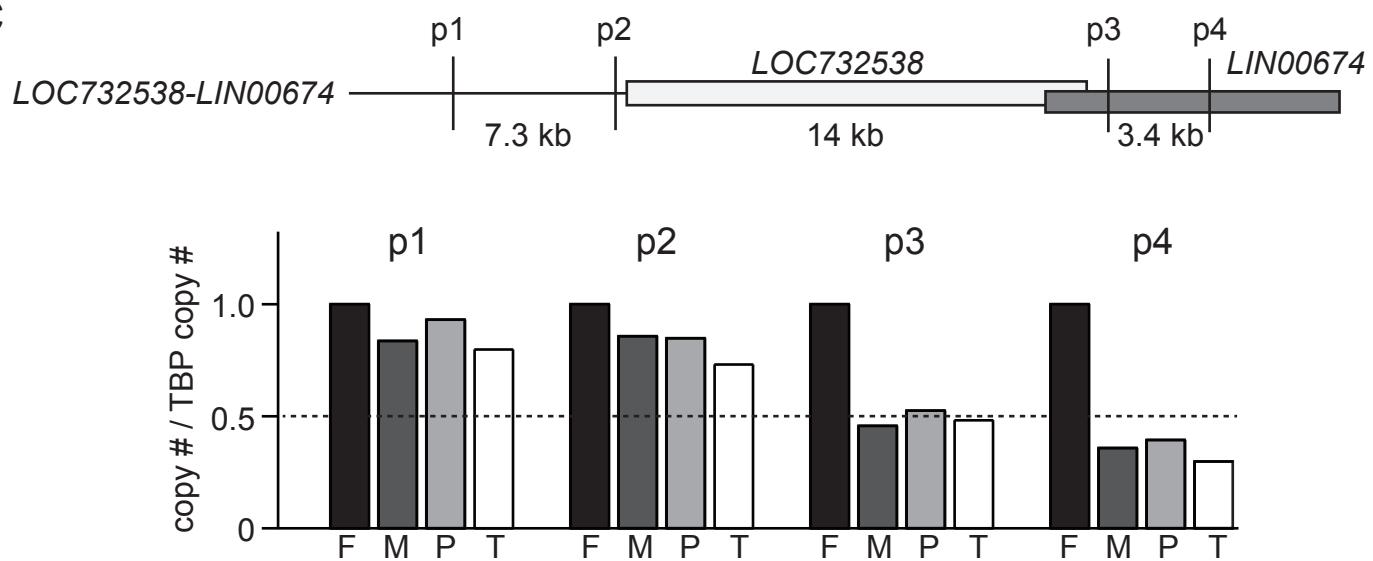
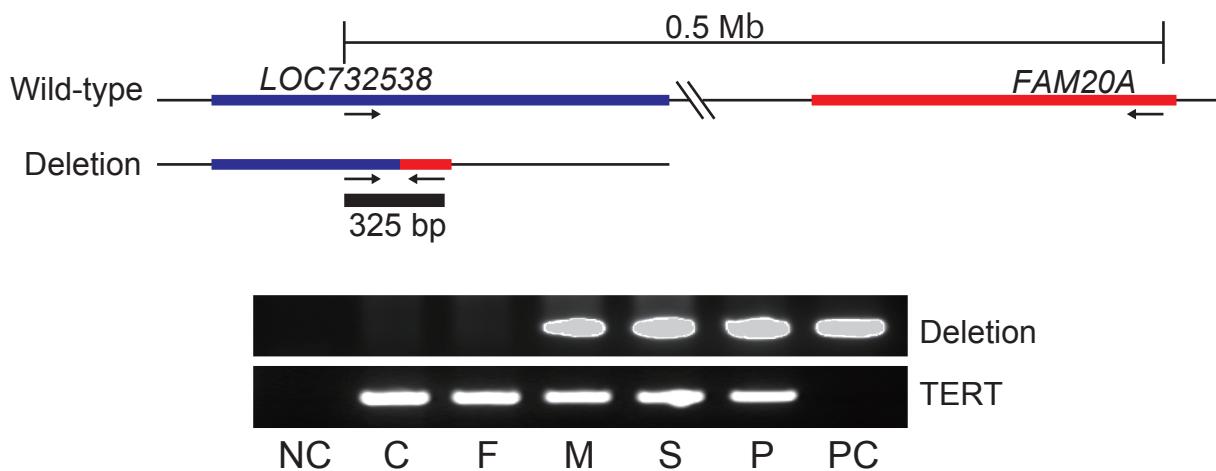
A**B****C****D**

Figure 3

Supplementary data

Methods

Cloning of DNA fragments encompassing a deletion junction

DNA encompassing a deletion junction on 17q24 was amplified by using leukocyte-derived genomic DNA from the proband and KOD FX DNA polymerase (Toyobo) with the following primers: forward: 5'-GCTGAGCTCCAGTGATCTCC-3', reverse: 5'-CTCACCCACACGTTCCCTTT-3'. PCR products were subcloned into pCR4-Blunt plasmid vector (Invitrogen). Then, the approximately 1.3 kb fragment obtained by digestion of the plasmid with *Hind*III and *Eco*RI was subcloned into pBluescript II SK+ followed by sequencing analysis.

PCR conditions

PCR condition for gene mutation analysis: 94°C for 5 min; followed by 35 cycles of 94°C for 45 sec, 62°C (for the exon 9 primer set) or 57°C (for the others) for 30 sec; 72°C for 30 sec; and then 72°C for 5 min. For cloning of DNA fragments encompassing a deletion junction: 94°C for 2 min; followed by 35 cycles of 98°C for 10

sec, 60°C for 30 sec, 68°C for 7 min; and then 68°C for 5 min. For detection of deleted allele: 94°C for 5 min; followed by 37 cycles of 94°C for 30 sec, 66°C for 30 sec, 72°C for 30 sec; and then 72°C for 5 min.

CGH array analysis

CGH array analysis of genomic DNA from the tumor sample was performed using Affymetrix CytoScan HD arrays (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's recommended protocol. Images were acquired using the GeneChip Scanner 3000 7G (Affymetrix) and analyzed using Chromosome Analysis Suite v2.1 software (Affymetrix). This analysis was supported by the Support Center for Advanced Medical Sciences, Institute of Health Biosciences, the University of Tokushima Graduate School.

Figure legends

Supplementary Figure 1

Identification of breakpoints on 17q24 in the CNC family. (A) Schematic diagrams of deleted allele (her mother, II-2, and the proband, III-2) and wild-type allele (her father, II-1) in the family. To amplify the genomic DNA fragment including a deletion junction, a forward primer of p2 shown in Fig. 3C and a reverse primer of P4 shown in Fig. 3B were used. The size of the fragment amplified by PCR was expected to be between 1.7 kb and 17 kb. (B) A representative image of PCR products on agarose gel electrophoresis. Approximately 8.7 kb fragments were amplified in leukocytes DNA from the proband (III-2, P) and her mother (II-2, M), but not her father (II-1, F). (C) Restriction enzyme mapping of the PCR fragment of approximately 8.7 kb. Solid and dashed vertical lines indicate *Eco*RI and *Hind*III recognition sites, respectively. The deletion junction was predicted to be contained in a 1.3 kb fragment digested with *Eco*RI and *Hind*III. (D) A scanned segment of a sequencing electropherogram of the PCR product encompassing the deletion junction. At the top of the panel, nucleotide

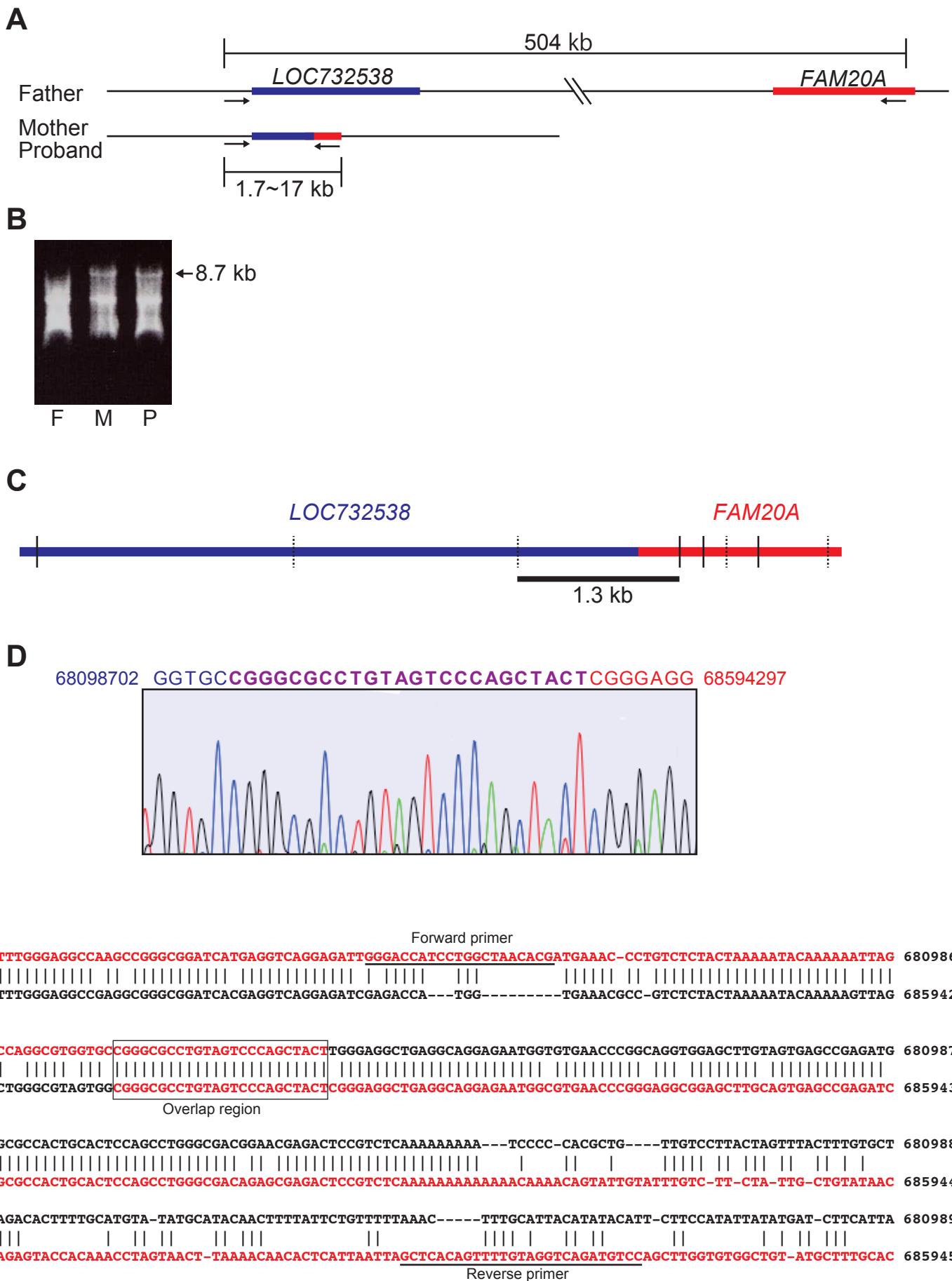
sequences derived from *LOC732538* (blue) and *FAM20A* (red) and the overlapping sequences (purple) are shown. (E) Alignment of sequences of *LOC732538* (upper: the position from 68098595 to 68098977 on chromosome 17) and *FAM20A* (lower: the position from 68594167 to 68594547 on chromosome 17). Red color indicates sequences of the PCR products encompassing the deletion junction. Positions of PCR primers used to identify the deletion are underlined. A box denotes the 24 bp region of overlap between the *FAM20A* gene and the *LOC732538* gene in the PCR product.

Supplementary Figure 2

Identification of a large deletion at 17q24 by CGH array analysis. A deletion was detected on chromosome 17q24. Each dot represents weighted log₂ ratio of each marker in the vicinity of the deleted region. The deletion was estimated to be approximately 0.5 Mb between *LOC732538* and intron 1 of the *FAM20A*.

Supplementary Table. Sequences of primers used for qPCR

Gene (location)		Sequence
TBP	F	5'-CTGTTCTGGCGTGTGAAG-3'
(chr.6: 170556927-170557029)	R	5'-CGCTGGAACTCGTCTCACTA-3'
ALB	F	5'-TGAAACATACGTTCCCAAAGAGTTT-3'
(chr.4: 73418213-73418293)	R	5'-AGCAGGTTGCTGAAATGCT-3'
TERT	F	5'-CTCTCCTCTCAGAAAGTGTGCATAT-3'
(chr.5: 1291864-1292015)	R	5'-AAGGAAGCTGGAGCACAAAA-3'
PRKAR1A Intron 1	F	5'-GTTGACTTTGTGCCCTGGT-3'
(chr.17: 68514986-68515151)	R	5'-TCAGCTCCTGGGAGGAAGTA-3'
PRKAR1A Intron 3	F	5'-GACCCACTTGGTGGTCATCT-3'
(chr.17: 68522965-68523172)	R	5'-CTGCAAAACAACGTCAAGGA-3'
PRKAR1A Intron 5	F	5'-ATTTTGTGGCAGCCTGATA-3'
(chr.17: 68524581-68524781)	R	5'-GGGGAAAGAAAATCAAGCAA-3'
PRKAR1A exon 8/Intron 8	F	5'-GGGAAGCACACTGAGAAAGC-3'
(chr.17: 68527839-68528081)	R	5'-AGACGGGAAGTCACAGAGGA-3'
PRKAR1A exon 11	F	5'-GGGCATGAGATTGGAAGA-3'
(chr.17: 68531519-68531674)	R	5'-AAGCAGGACTTCAGCAGGA-3'
FAM20A Intron 1 primer set-1 (P1)	F	5'-ACCACCCCTGCTGAGACATT-3'
(chr.17: 68593312-68593551)	R	5'-CAGAGCAGGGATAGCTTGG-3'
FAM20A Intron 1 primer set-2 (P2)	F	5'-CCTTCCCAAAGGAGGAAAAG-3'
(chr.17: 68593928-68594174)	R	5'-CTCCCCAAAGTGTGGGATTA-3'
FAM20A Intron 1 primer set-3 (P3)	F	5'-GCAGGAAGTGCCTGTCTTC-3'
(chr.17: 68595265-68595458)	R	5'-GGAGGAAACCTCTCCACTCC-3'
FAM20A Intron 1 primer set-4 (P4)	F	5'-GGTTTGAGAAAGCTTCAGG-3'
(chr.17: 68596910-68597107)	R	5'-CTCACCCACACGTTCTTTT-3'
LOC732538 primer set-1 (p1)	F	5'-GGTGTGCTGGGTAATGAAC-3'
(chr.17: 68085662-68085849)	R	5'-CAGCCCAGTGGCTGAATAC-3'
LOC732538 primer set-2 (p2)	F	5'-GCTGAGCTCCAGTGATCTCC-3'
(chr.17: 68092953-68092953)	R	5'-AGCTGGATGTGGTAGCAGGT-3'
LOC732538 primer set-3 (p3)	F	5'-GAGGATCTCATTAGGGCTGT-3'
(chr.17: 68106871-68107022)	R	5'-GCGTATTCAAGGGTTCAGC-3'
LOC732538 primer set-4 (p4)	F	5'-AATCTTAAGTTGATTGGCAGTTG-3'
(chr.17: 68110265-68110414)	R	5'-GGAGGTTGTGGTGAGCAGAG-3'

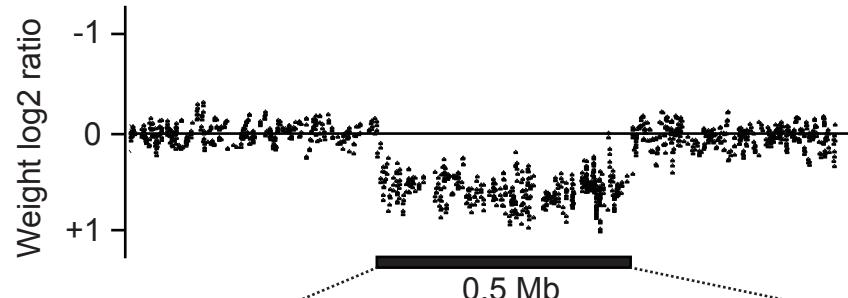


Supplement Figure 1

A



B



C



Supplement Figure 2