Acta Med. Okayama, 2014
Vol. 68, No. 3, pp. 163–170

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Biallelic Disruption of the *PTCH1* Gene in Multiple Basal Cell Carcinomas in Japanese Patients with Nevoid Basal Cell Carcinoma Syndrome

Genshu Tate*, Koji Kishimoto, and Toshiyuki Mitsuya

Department of Pathology, Showa University Fujigaoka Hospital, Yokohama 227–8501, Japan

The aim of the present study is to address whether the molecular pathogenesis is identical among multiple basal cell carcinomas (BCCs) present in the same nevoid basal cell carcinoma syndrome (NBCCS) patient. Patient 1 is a 61–year-old (yo) Japanese female whose clinical characteristics and findings of a genetic analysis of *PTCH1* have been previously described. Patient 2 is patient 1's 64–yo sister who also suffered from NBCCS with a single base deletion at nucleotide 2613 in exon 16 (c. 2613delC) in one *PTCH1* allele. Thirteen and 3 independent specimens of BCC were applied for a molecular analysis of loss of heterozygosity (LOH) in *PTCH1* in patients 1 and 2, respectively. Of particular note is that all BCC specimens examined showed a loss of the wild-type allele of exon 16 in *PTCH1*, thus indicating that LOH results in the biallelic disruption of *PTCH1* in multiple BCCs that develop in an age- and location-independent manner in the same patient. These results indicate that the germline single base deletion of *PTCH1* (c. 2613 delC) is a first hit and the LOH of the wild-type allele is a second hit, implying that all 16 BCCs detected in these NBCCS sisters fit the standard two-hit model.

Key words: biallelic disruption, hedgehog, nevoid basal cell carcinoma syndrome, Patched 1, two-hit model

Nevoid basal cell carcinoma syndrome (NBCCS, also known as Gorlin syndrome, OMIM 109400) is a rare autosomal dominant disorder characterized by a spectrum of developmental anomalies and a predisposition toward the development of a variety of neoplasms, including keratocystic odontogenic tumor (KCOT), basal cell carcinoma (BCC), ovarian fibroma, desmoplastic medulloblastoma and meningioma [1]. Congenital defects are associated with germline mutations of the human homologue of the *Drosophila* segment polarity gene, *Patched 1* (*PTCH1*, MIM 601309). The *PTCH1* gene has been mapped to 9q22. 3 and consists of 23 exons spanning approximately 74 kb encoding a 1447 amino acid transmembrane glycoprotein. PTCH1 is a receptor for Hedgehog (HH), and the HH signal is a key factor in embryonic development and tumorigenesis. Misregulation of signaling due to the inactivation of Sonic HH and PTCH1 has been implicated in the development of ciliopathy in patients with holoprosencephaly and NBCCS, respectively, thus supporting the hypothesis that *PTCH1* acts as a tumor suppressor gene in this syndrome [2, 3].

The inactivation of PTCH1 following the binding of Smoothened (SMO), a seven-pass transmembrane homologue of G-protein-coupled receptors, in turn activates a downstream signaling cascade involving GLI1, GLI2, GLI3 and SUFU to modulate the target

Received November 7, 2013; accepted February 18, 2014.
*Corresponding author. Phone: +81–45–971–1151; Fax: +81–45–972–6242
E-mail: gentate@yahoo.co.jp (G. Tate)

gene expression. The constitutive activation of the HH pathway via mutations is a key molecular event in the development of neoplasms, such as those involving the skin, prostate and alimentary tract [3–5].

According to Knudson's two-hit model of tumor suppressor genes [6], the occurrence of 2 mutations in both alleles of the tumor suppressor gene or one mutation in one tumor suppressor gene allele accompanied by the allelic loss of the remaining wild-type allele is required for neoplasm development. Tumors in NBCCS patients are believed to develop according to the two-hit hypothesis [2, 7]. The first fit in NBCCS is a germline mutation in the *PTCH1* gene, which is then followed by a second hit that involves a somatic inactivating mutation or deletion resulting in the constitutive activation of SHH signals.

Danaee *et al.* found a high prevalence (60%) of loss of heterozygosity (LOH) of the *PTCH1* gene in patients with sporadic BCC [8]. However, no study has documented how frequently the biallelic inactivation of *PTCH1* occurs among multiple BCCs in the same NBCCS patient, and nor has it been clearly demonstrated whether biallelic inactivation depends on the age of the patient or the location of the BCC. We herein demonstrate that all BCCs examined in two NBCCS patients exhibited the biallelic loss of the *PTCH1* gene and completely fit the two-hit theory.

Patients and Methods

The previous clinical characteristics of patient 1 have already been described [9]. Table 1 summarizes the recent clinical characteristics of patient 1 and the clinical features of patient 2. Their father died because of skin cancer probably associated with NBCCS, whereas their mother has no history of skin cancer. Genomic DNA of basal cell carcinomas was extracted from formalin-fixed, paraffin-embedded tissues according to phenol-chloroform extraction and ethanol precipitation methods and was used to detect LOH of the *PTCH1* gene. The peripheral blood mononuclear cells (PBMCs) were used as a DNA source for germline sequence analysis. RT-PCR fragments amplified by a pair of primers, PTCHex15 and PTCHex17 were cloned by pGEM-T easy vector systems according to the manufacturer's technical protocol (Promega, Madison, WI, USA) and sequenced by primer extension methods. PBMCs were used as an RNA source. The primers used in the present study, PTCHex15, PTCHex16, PTCHex17, rs10512248 and ivs19+1210 are described in Table 2. Amplified DNA fragments were recovered from a low melting temperature agarose gel (2%) and subjected to a direct sequencing analysis using an automated DNA sequencing system

Table 1 Clinical history of patients 1 and 2 with NBCCS

	Patient 1	Patient 2
Age of onset of BCC	42-year-old (yo)	60 yo
Multiple BCC, excision	More than 20 times	8 times
Location of BCC	Head and face	Head and face
Skull invasion of BCC	61 yo	−
Dura invasion of BCC	61 yo	−
Meningioma (meningothelial)	41 yo	−
Epidermal cyst	Excised twice	NA
Calcified falx cerebri	+	NA
Palmar pits	+	NA
Scoliosis	+	NA
Hypertelorism	+	NA
Rectal cancer (advanced)	−	48 yo
Uterine leiomyoma	−	48 yo

NBCCS, nevoid basal cell carcinoma syndrome; BCC, basal cell carcinoma; NA, not available.

Table 2 Primers used in the present study

Primer	Sequences	Product size (bp)
rs10512248F	5'-AAGCCTGGAAAAGCTGATGGGTTG-3'	143
rs10512248R	5'-TCATTAGCAGACTTCTTACTTCAG-3'	
PTCHivs19F3–2	5'-GCCAATAATATGGCAACAGTATTC-3'	300
PTCHivs19R3	5'-CTAGAGTTTAGAGACTGCAGTTAG-3'	
PTCHex16F2	5'-AGGGTCCTTCTGGCTGCGAG-3'	219
PTCHex16R2	5'-TCAGTGCCCAGCAGCTGGAGTA-3'	
PTCHex15	5'-ACCCGAATATCCAGCACTTAC-3'	351
PTCHex17	5'-TGCTGACCCAAGCCGTCAGG-3'	

ivs, intervening sequence.

(Model 377, Applied Biosystems, Foster City, CA, USA) [10]. Written informed consent for the gene analysis was obtained from both patients. This study complied with the Declaration of Helsinki revised in 2008 and was conducted according to the ethical guidelines presented annually by the Committee for Medical Experiment at Showa University.

Results

We screened age- and location-independent BCC specimens in patient 1 using direct sequencing of exon 16 of the *PTCH1* gene in order to analyze the heterozygosity of c. 2613delC. A representative case of BCC-9 is shown in Fig. 1. The germline sequence shows c. 2613delC (Fig. 1A), whereas no wild-type sequence is observed in BCC-9, indicating LOH (Fig. 1B). None of the 13 specimens examined in the present study exhibited any wild-type sequences. Only the c. 2613delC mutation was identified, implying that the *PTCH1* gene was biallelically inactivated in all BCCs in patient 1.

Direct germline sequencing identified c. 2678G/A, resulting in p. 893R/H, in patient 1 (Fig. 2A). In order to examine whether c. 2678G/A is an SNP or a mutation, we next analyzed the restriction fragment length polymorphism. A restriction enzyme, *Acc*II, cut CGCG but not the sequence, CACG (c. 2678A). After we cut a fragment amplified with 2 primers, *PTCH* ex16F2 and *PTCH* ex16R2 (219 bp), using *Acc*II, a fragment of 171 bp was obtained in the amplicon with c. 2678G, although a 48-bp fragment was invisible, while a 219-bp fragment remained in the amplicon with c. 2678A (Fig. 2C). N2 in Fig. 2c indicates a healthy individual who shows the same restriction fragment length polymorphism as that in patient 1 (Fig. 2C). Among 96 healthy chromosomes, 2 were c. 2678A (data not shown), thus indicating that c. 2678G/A is an SNP. The germline sequence in patient 1 shows c. 2678G/A (Fig. 2A), whereas only c. 2678G is observed in BCC-9 of patient 1 (Fig. 2B). Cloning of the RT-PCR fragment using the primer pair PTCHex15 and PTCHex17 followed by the sequenceing revealed that the wild type sequence of exon 16 was segregated with p. 893H/ c. 2678A (Fig. 3A), and that p. 893R/c. 2678G was segregated with c. 2613delC (Fig. 3B), thereby indicating that c. 2678A (p. 893H) was derived from the patient's mother. These results indicate that the lost allele was the mother's wild type allele and that it retained a c. 2613delC-containing allele, which causes a frame shift, in BCC-9 of patient 1. Other BCCs in patient 1 showed the same results as those of BCC-9 (Table 3).

Exon 16 of the *PTCH1* gene showed the same c. 2613delC in all three BCC specimens in patient 2 as that observed in patient 1 (Fig. 1). We next searched for SNPs to determine the region of LOH in the *PTCH1* gene, and found two SNPs, rs10512248 and ivs19+1210, in patient 2. Although the germline sequence showed an SNP (Fig. 4A), all three BCCs exhibited only T in the ivs19+1210, indicating the loss of the mother's wild type allele of the *PTCH1*

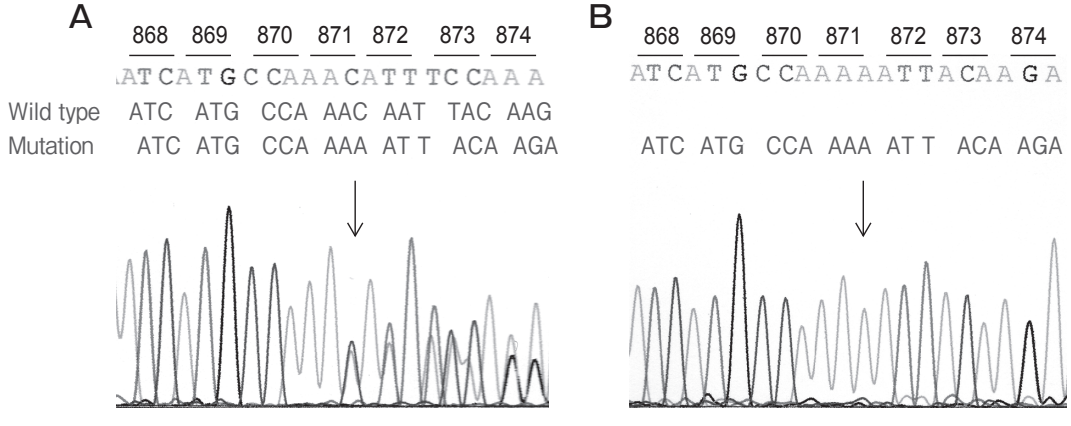

Fig. 1 A representative case of the biallelic disruption of the *PTCH1* gene in BCC-9 in patient 1. **A**, Germline sequence of exon 16 of the *PTCH1* gene. The codon numbers are designated above the bars. The arrow indicates the position of c.2613; **B**, Sequence of exon 16 of *PTCH1* in BCC-9. The arrow indicates the position of c.2613delC.

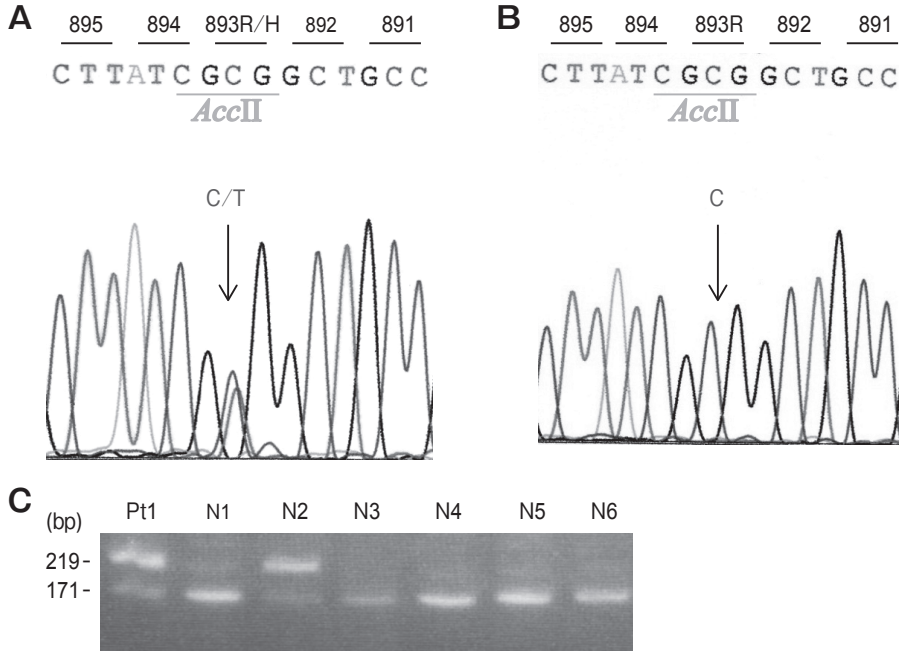

Fig. 2 LOH of *PTCH1* in BCC-9 in patient 1. **A**, Germline sequence of exon 16 of the *PTCH1* gene. The codon numbers are designated above the bars. The reverse complementary sequence is indicated. The arrow indicates the position of c.2678G/A (p.893R/H); **B**, Sequence of exon 16 of *PTCH1* in BCC-9. The arrow indicates the position of c.2678; **C**, The agarose gel of exon 16 amplicons following digestion with *Acc*II. DNA extracted from peripheral blood mononuclear cells was used for the amplification. Band 219 bp is the uncut amplicon; 171 bp is the cut band. Pt1 indicates patient 1, and N indicates a healthy individual.

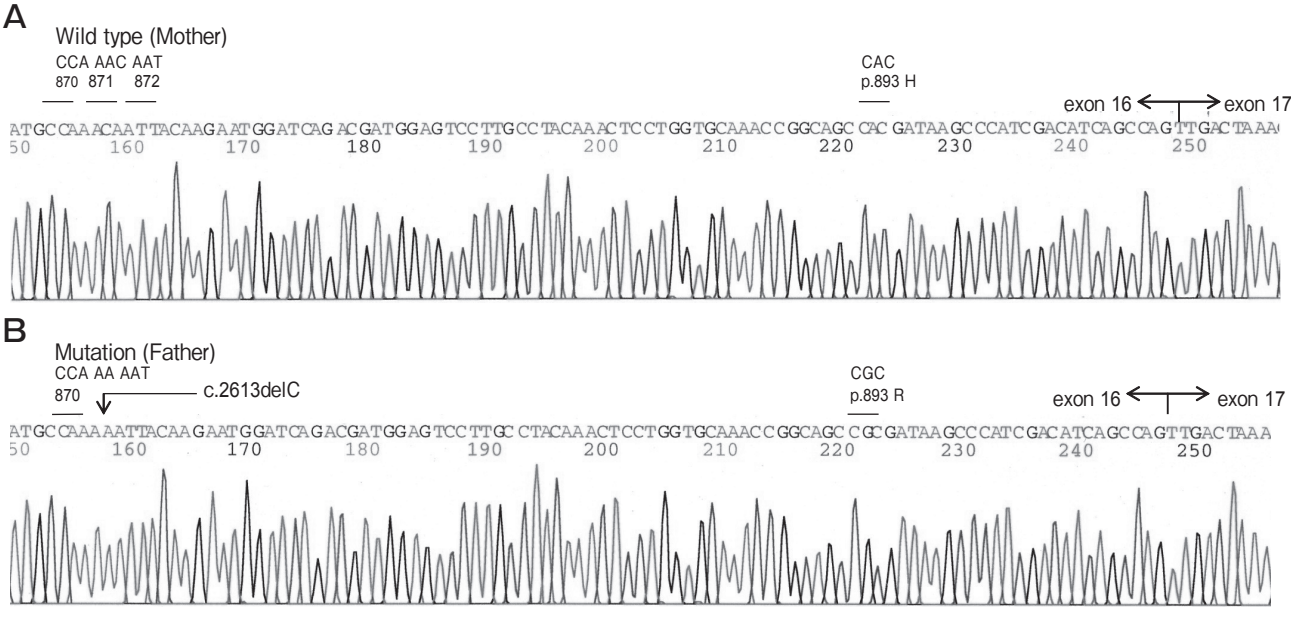

Fig. 3 DNA sequencing of the RT-PCR fragment. (**A**) TA cloning of the RT-PCR fragment following the DNA sequencing revealed that the wild type sequence in codon 871 is segregated with 893H, whereas c.2613delC is associated with 893R (**B**).

Table 3 Clinical characterictics and heterozygosity of *PTCH1* gene in multiple basal cell carcinoma developed in NBCCS patients

Patients	age/location	rs10512248	c.2613delC exon 16	c.2678G/A p.893R/H	ivs19+1210
Patient 1					
BCC-1	45 (yo)/head		LOH	LOH	
BCC-2	46/head		LOH	LOH	
BCC-3	49/head		LOH	LOH	
BCC-4	49/face		LOH	LOH	
BCC-5	50/face		LOH	LOH	
BCC-6	51/face		LOH	LOH	
BCC-7	52/head		LOH	LOH	
BCC-8	52/head		LOH	LOH	
BCC-9	55/head		LOH	LOH	
BCC-10	55/face		LOH	LOH	
BCC-11	57/head		LOH	LOH	
BCC-12	61/head		LOH	LOH	
BCC-13	61/head		LOH	LOH	
Patient 2					
BCC-1	61/head (L)	LOH	LOH	NA	LOH
BCC-2	61/head (R)	ROH	LOH	NA	LOH
BCC-3	61/head (L)	ROH	LOH	NA	LOH

BCC, basal cell carcinoma; LOH, loss of heterozygosity; ROH, retention of heterozygosity; NBCCS, nevoid basal cell carcinoma syndrome; ivs, intervening sequence; yo, year-old; NA, not available.

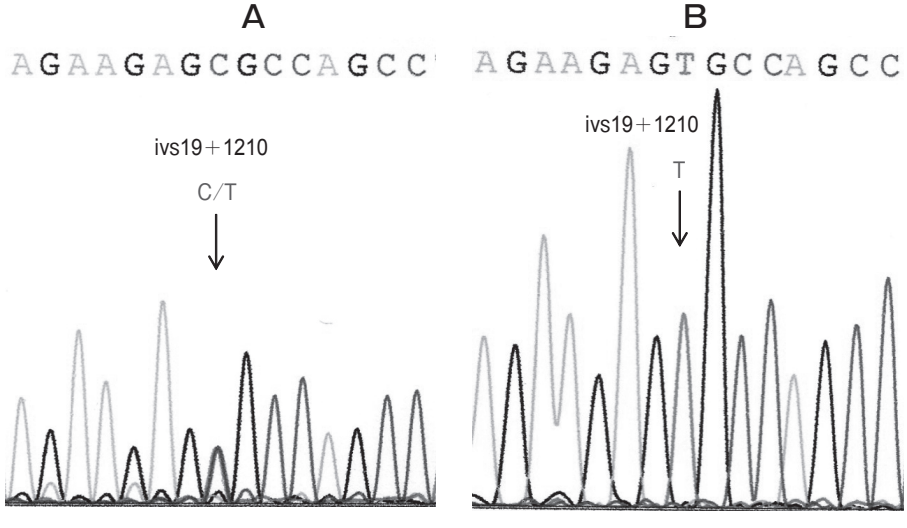

Fig. 4 Allelic loss of ivs19+1210 in *PTCH1* in BCC-1 in patient 2. (**A**) Germline sequence of ivs19+1210 of the *PTCH1* gene. The arrow indicates the position of ivs19+1210. (**B**) Sequence of BCC-1.

gene and thus LOH (Fig. 4B). Such evidence, in addition to c. 2613delC of the father's allele, revealed the biallelic inactivation of the *PTCH1* gene. Despite of the SNP at 10512248 (Fig. 5A), 2 cases of BCC, BCC-2 (Fig. 5B) and BCC-3, demonstrated retention of heterozygosity at rs10512248. We next examined whether the histology of three BCCs, BCC-1, BCC-2 and BCC-3 in patient 2 differed and whether the histology of BCC differed between patients 1 and 2. Fig. 6 exhibits the BCC histologies of patients 1 and 2. All BCCs (A-E) exhibit nests of basaloid cell proliferation with peripheral palisading.

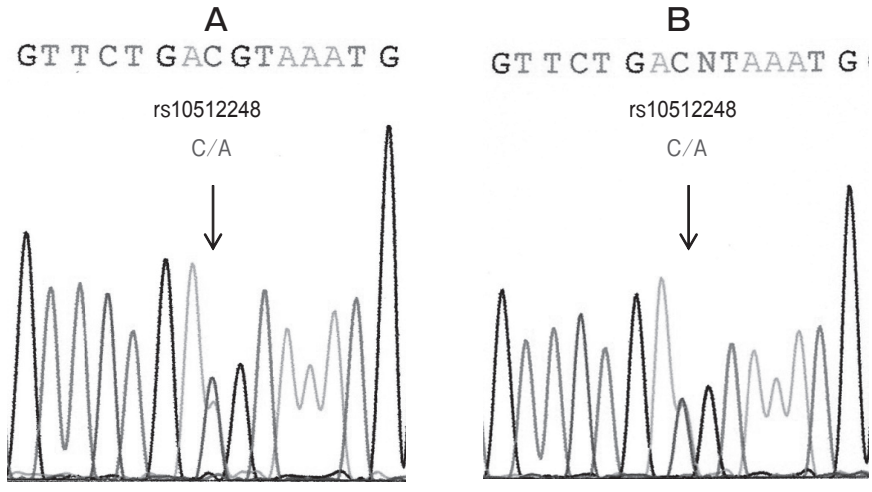

Fig. 5 The retention of heterozygosity of rs10512248 in *PTCH1* in BCC-2 in patient 2; **A**, Germline sequence of rs10512248 in the *PTCH1* gene. The arrow indicates the position of rs10512248; **B**, Sequence of BCC-2.

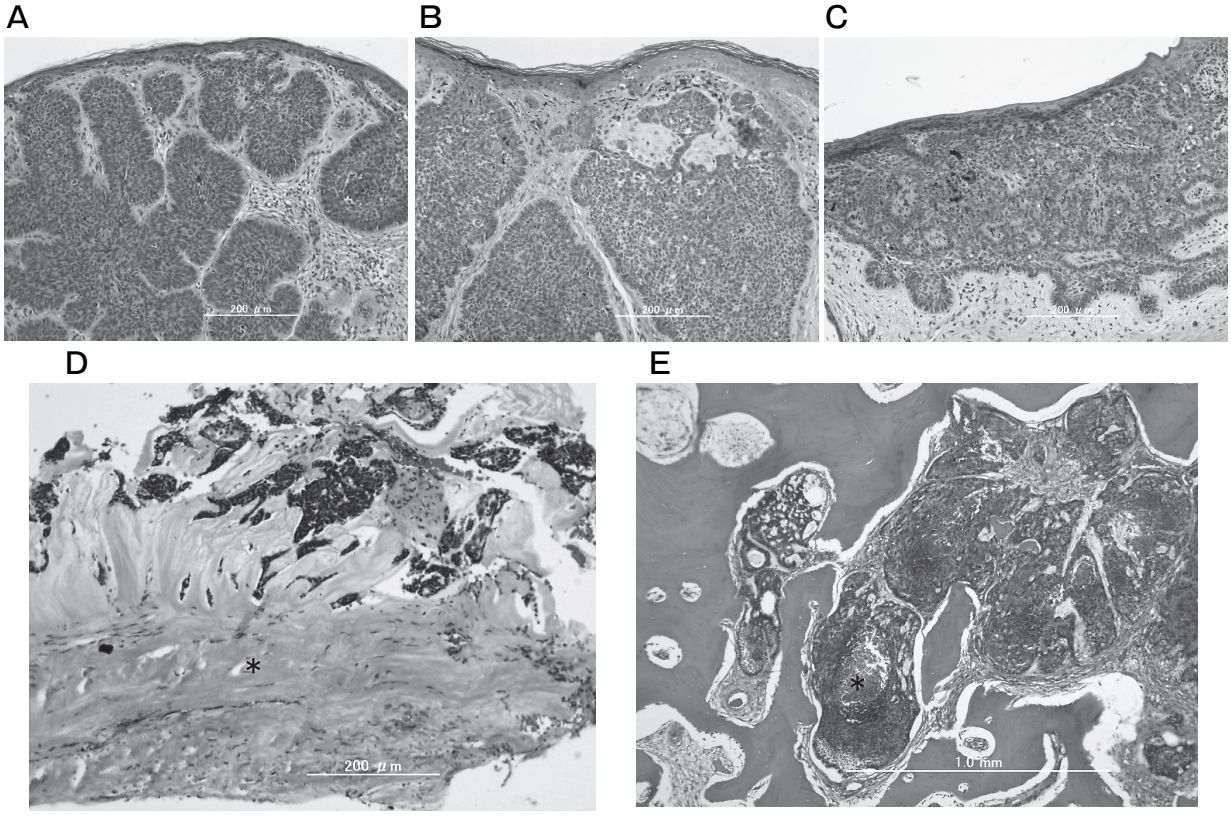

Fig. 6 Histopathology of BCC in patient 2 (**A**–**C**) and patient 1 (**D**, **E**). (**A**–**C**) Histology of BCC-1, -2 and -3 in patient 2 is shown in A, B and C, respectively. Dural invasion of BCC and skull invasion of BCC in patient 1 are shown in **D** and **E**, respectively. An asterisk indicates dura (**D**) and central necrosis (**E**) of BCC.

In patient 2, all BCCs are small, measuring approximately 2 mm in diameter, and they show no histological differences among them (Fig. 6A–C). The histology of BCCs in these patients was indistinguishable from that of nonsyndromic BCCs. However, central necrosis was observed in the nest of BCCs that invaded the skull in patient 1 (E).

No SNPs for the *PTCH1* gene, rs2297697, rs10988802, rs357564, rs10512248 or ivs19+1210 were observed (rs10512248 was A and ivs19+1210 was T). These markers could not be used to identify the lost allele of *PTCH1*. Hence, the region of allelic loss of *PTCH1* was not available in patient 1. Rs10512248 was only A and ivs19+1210 was only T in patient 1, implying that the mutation allele derived from her father is A and T, respectively. The mutation allele in patient 2 is the same as that in patient 1, suggesting that rs10512248 is C and ivs19+1210 is C in the wild-type allele derived from her mother in patient 2. These results are summarized in Fig. 7 and Table 3. Patients 1 and 2 each inherited a different *PTCH1* allele from their mother (Fig. 7).

Discussion

Keratocystic odontogenic tumors (KCOTs) are aggressive jaw neoplasms that occur sporadically or in association with NBCCs. Levanat *et al.* originally reported that the tumorigenesis of KCOT fits a two-hit model in patients with Gorlin syndrome [7]. Recently, Pan *et al.* examined the LOH in 44 patients with KCOT (15 NBCCS-related cases and 29 sporadic cases). Among these, 13 were identified to fit the two-hit model, 14 conformed to the one-hit model and the remaining 17 showed no alterations in *PTCH1*. Of particular note is that the distributions of the two-hit, one-hit and non-hit cases differed significantly between the NBCCS and nonsyndromic patients. The incidences for two-hit, one-hit and non-hit inactivation of *PTCH1* were 53.3%, 33.3% and 13.3% of the NBCCS-associated KCOT patients, respectively. In contrast, these mechanisms were observed in 17.2%, 31% and 51.7% of the sporadic KCOT patients, respectively. The two-hit inactivation of *PTCH1* is highly prevalent in patients with NBCCS-associated KCOT, although cases of KCOT that lack any *PTCH1*

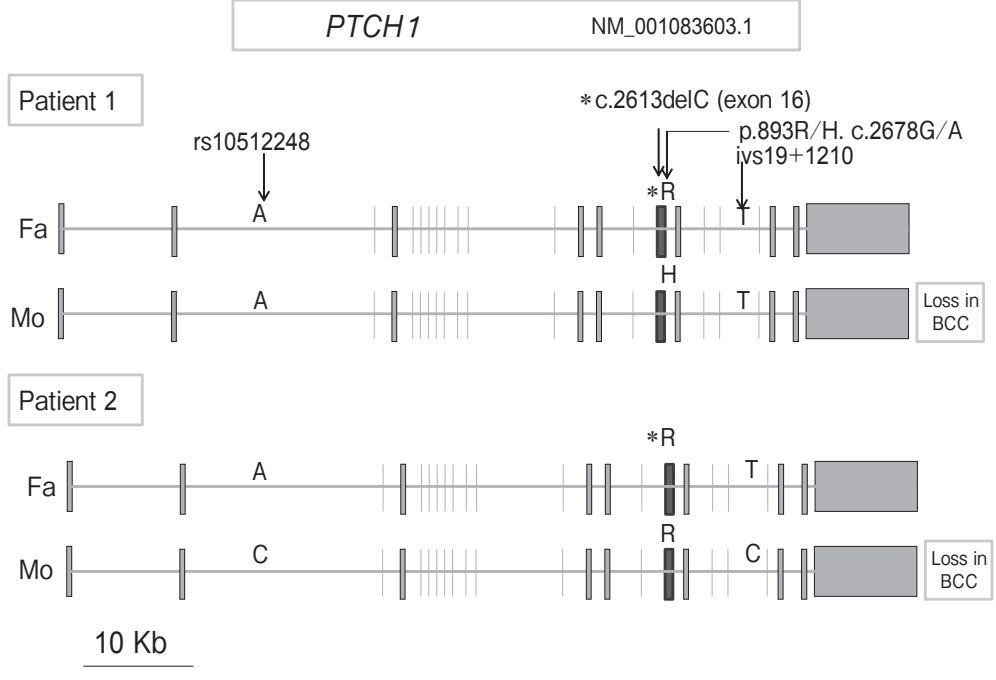

Fig. 7 An integrated germline map of the *PTCH1* gene in patients 1 and 2. Fa and Mo indicate haplotypes derived from the father and mother, respectively. The asterisk denotes the c.2613delC mutation in exon 16 (shown in blue), and the box designated as a loss in BCC is a deleted haplotype in BCC.

hits do exist [11]. The standard two-hit hypothesis has been demonstrated not only in patients with KCOT but also in those with NBCCS-associated meningioma. Kijima *et al.* reported biallelic mutations, c. 290dupA (germline) and c. 307delG (somatic), in meningioma that developed in an NBCCS patient [12]. Koch *et al.* reported that the Gorlin syndrome-associated hepatic mesenchymal tumor fits the two-hit model [13]. However, many types of tumors develop in patients with NBCCS. Therefore, it remains to be addressed whether the biallelic inactivation of *PTCH1* is involved in the molecular pathogenesis of all NBCCS-associated tumors. The NBCCS patients evaluated in the present study exhibited not only multiple BCCs but also meningioma in patient 1 and rectal cancer and uterine leiomyoma in patient 2. Molecular analyses for heterozygosity of the *PTCH1* gene in these tumors might provide important evidence for molecular tumorigenesis; however, the results were inadequate due to the low quality of DNA in these tumors.

In conclusion, the current study showed the biallelic disruption of the *PTCH1* gene in all 16 BCCs examined in two NBCCS patients. Our results provide interesting results indicating that the molecular pathogenesis of BCC completely fits the two-hit theory. The first hit is a germline mutation of c. 2613delC and the second hit is a somatic loss of wild-type allele in *PTCH1*, resulting in the lack of expression of normal PTCH1 protein, then leading to the activation of the HH pathway and finally the formation of BCC. In the present study, immunohistochemical analysis of PTCH1 in BCC could not be conducted because no adequate antibodies were available. Thus it remains to be addressed whether PTCH1 is actually synthesized and expressed on the cell surface.

Acknowledgments. We would like to thank the patients for their valuable cooperation.

References

1. Gorlin RJ and Goltz RW: Multiple nevoid basal-cell epithelioma, jaw cysts and bifid rib. A syndrome. New Eng J Med (1960) 262: 908–912.
2. Hahn H, Wicking C, Zaphiropoulos PG, Gailani MR, Shanley S, Chidambaram A, Vorechovsky I, Holmberg E, Unden AB, Gillies S, Negus K, Smyth I, Pressman C, Leffell DJ, Gerrard B, Goldstein AM, Dean M, Toftgard R, Chenevix-Trench G, Wainerght B and Bale AE: Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell (1996) 85: 841–851.
3. Bale AE and Yu KP: The hedgehog pathway and basal cell carcinomas. Hum Mol Genet (2001) 10: 757–762.
4. Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, Parker AR, Shimada Y, Eshleman JR, Watkins DN and Beachy PA: Widespread requirement for hedgehog ligand stimulation in growth of digestive tract tumours. Nature (2003) 425: 846–851.
5. Karhadkar SS, Bova GS, Abdallah N. Dhara S, Gardner D, Maitra A, Issacs JT, Berman DM and Beachy PA: Hedgehog signaling in prostate regeneration, neoplasia and metastasis. Nature (2004) 431: 707–712.
6. Knudson AG Jr: Mutation and cancer; statistical study of retinoblastoma. Proc Natl Acad Sci USA (1971) 68: 820–823.
7. Levanat S, Gorlin RJ, Fallet S, Johnson DR, Fantasia JE and Bale AE: A two-hit model for developmental defects in Gorlin syndrome. Nat Genet (1996) 12: 85–87.
8. Danaee H, Karagas MR, Kelsey KT, Perry AE and Nelson HH: Allelic loss at Drosophila Patched gene is highly prevalent in basal and squamous cell carcinomas. J Invest Dermatol (2006) 126: 1152–1158.
9. Tate G, Li M, Suzuki T and Mitsuya T: A new germline mutation of the PTCH gene in a Japanese patient with nevoid basal cell carcinoma syndrome associated with meningioma. Jpn J Clin Oncol (2003) 33: 47–50.
10. Tate G, Suzuki T, Kishimoto K and Mitsuya T: A c-KIT codon 816 mutation, D816H, in the testicular germ cell tumor: case report of a Japanese patient with bilateral testicular seminomas. Acta Med Okayama (2005) 59: 33–36.
11. Pan S, Dong Q, Sun LS and Li TJ: Mechanisms of inactivation of PTCH1 gene in nevoid basal cell carcinoma syndrome: Modification of the two-hit hypothesis. Clin Cancer Res (2010) 16: 442–450.
12. Kijima C, Miyashita T, Suzuki M, Oka H and Fujii K: Two cases of nevoid basal cell carcinoma syndrome associated with meningioma caused by a PTCH or SUFU germline mutation. Fam Cancer (2012) 11: 565–570.
13. Koch CA, Chrousos GP, Chandra R, Evangelista RS, Gilbert JC, Nobuhara K, Zhuang Z and Vortmeyer AO: Two-hit model for tumorigenesis of nevoid basal cell carcinoma (Gorlin) syndrome-associated hepatic mesenchymal tumor. Am J Med Genet (2002) 109: 74–76.