Human Cancer Biology

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Mechanisms of Inactivation of *PTCH1* Gene in Nevoid Basal Cell Carcinoma Syndrome: Modification of the Two-Hit Hypothesis

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Abstract

Purpose: *PTCH1* has been identified as the gene responsible for nevoid basal cell carcinoma syndrome (NBCCS). Keratocystic odontogenic tumors (KCOT) are aggressive jaw lesions that may occur in isolation or in association with NBCCS. The aim of this study was to investigate the genetic and/or epigenetic mechanisms of inactivation of the *PTCH1* gene in patients with NBCCS and related sporadic KCOTs.

Experimental Design: Loss of heterozygosity was analyzed in 44 patients (15 NBCCS-related and 29 sporadic KCOTs), all of whom were previously analyzed for *PTCH1* mutations. Allelic location was established in tumors carrying two coincident mutations. *PTCH1* mRNA expression and promoter methylation status were analyzed in a panel of KCOTs to define the possible role of epigenetic effects on *PTCH1* inactivation.

Results: Although mutations and loss of heterozygosity of *PTCH1* were frequently detected in both syndromic and nonsyndromic cases, hypermethylation of the *PTCH1* promoter was not identified in the present series. Of all the 44 cases examined, 13 were identified to fit the two-hit model, 14 to conform to a one-hit model, and the remaining 17 cases showing no alteration in *PTCH1*. The distribution of two-hit, one-hit, and non-hit cases was significantly different between syndrome and nonsyndrome patients ($P < 0.02$).

Conclusions: This study indicates that *PTCH1* gene alternation may play a significant role in the pathogenesis of NBCCS and the related sporadic tumors. Not only the standard two-hit model, but also haploinsufficiency or dominant-negative isoforms may be implicated in the inactivation of the *PTCH1* gene. *Clin Cancer Res; 16(2); 442–50. ©2010 AACR.*

Nevoid basal cell carcinoma syndrome (NBCCS, also known as Gorlin syndrome, OMIM NO.109400) is a rare autosomal dominant disorder (1) characterized by a spectrum of developmental defects and a predisposition to a number of different neoplasms, such as basal cell carcinomas, keratocystic odontogenic tumors (KCOT), medulloblastoma, ovarian fibroma, etc. (2). The human homologue of the Drosophila segment polarity gene, *PTCH1* (OMIM NO.601309), has been identified as the gene responsible for NBCCS as well as some related sporadic tumors (3). *PTCH1* has been mapped to 9q22.3-31, and consists of 23 exons spanning approximately 47 kb and encoding a 1447-amino acid transmembrane glycoprotein. It is involved in the Hedgehog signaling pathway, a key regulator of embryonic development and tumorigenesis controlling cellular proliferation and fate. Misregulation of the signaling caused by inactivation of *PTCH1* has been implicated in the development of NBCCS and some related sporadic tumors, supporting the hypothesis that *PTCH1* might act as a tumor suppressor gene in this syndrome (4).

According to Knudson's two-hit model of tumor suppressor genes (5), two mutations, one occurring in each of the two alleles of the gene, or one mutation in one tumor suppressor gene allele accompanied by another allelic loss of the remaining wild-type allele, are required to trigger neoplasm formation. Tumors in NBCCS patients are believed to develop according to the two-hit hypothesis (6). The first hit in NBCCS is a germline mutation in the *PTCH1* gene, and is followed by a second hit which is a somatic inactivating mutation or deletion. KCOTs are common aggressive jaw cystic tumors with a high growth potential and a propensity for recurrence (7, 8), which usually act as a feature of the inherited NBCCS. Like neoplasms in cancer predisposition syndromes, KCOTs in NBCCS are multiple and appear in a random pattern, but similar isolated defects are seen occasionally in the general population. They might also result from homozygous inactivation of the *PTCH1* gene in an early progenitor

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Translational Relevance

This study shows the possible mechanisms of *PTCH1* inactivation by mutation and/or loss of heterozygosity in nevoid basal cell carcinoma syndrome (NBCCS) and its related sporadic tumors. These findings imply that *PTCH1* will have a potential clinical application as a biomarker and a therapeutic target for NBCCS, as well as its related sporadic tumors. The frequent occurrence of *PTCH1* mutations in NBCCS suggests that prenatal diagnosis by genetic counseling is valuable for members in known syndrome family. As a key player in the Sonic Hedgehog pathway, PTCH1 acts to restrain the activity of SMO and inhibits signal transduction of the pathway. Inactivation of PTCH1 allows hedgehog ligand-independent activation of SMO, causing a downstream activation of the pathway that may lead to neoplastic growth. Therefore, future identification of inhibitors of the hedgehog signaling pathway as new drugs will offer new strategies for the treatment of NBCCS and its related sporadic tumors, including keratocystic odontogenic tumors.

cell of the relevant tissue. Our previous studies (9–12), as well as others (13–15), have revealed that >85% of NBCCS-associated KCOTs and nearly 30% of sporadic KCOTs harbored *PTCH1* mutations. In addition, loss of heterozygosity (LOH) at chromosome 9q22-31, the region to which the *PTCH1* gene maps, has been observed as a frequent event in NBCCS-associated tumors, although different estimates of the prevalence of LOH (16-75%) were reported (8, 16–18). Besides, research on NBCCS-related primitive neuroectodermal tumors has shown that *PTCH1* mutations occurred predominantly in tumors with LOH at 9q22 (17). These data propose a hypothesis that *PTCH1* might be inactivated through a two-hit mechanism in tumorigenesis.

Recently, however, more and more reports describe candidate tumor suppressors that do not conform to the standard two-hit model, including cancer-associated genes whose function can be abolished by inactivation of only one allele (19), and genes inactivated not only by mutation or LOH, but rather by epigenetic hypermethylation (20). Analysis of tumors taken from the $PTCH1^{+/-}$ mice revealed a retained wild-type allele of the gene and the absence of a second hit in these animals, indicating that haploinsufficiency of *PTCH1* may be sufficient for tumor development (21, 22). In addition, dominant-negative isoforms of *PTCH1* mutations, which caused only one single null allele, have been identified to contribute to tumorigenesis, probably due to improper multimer protein or stabilization of SMO, another transmembrane protein interacting with PTCH1 in the Hedgehog signaling pathway (23). This is in stark contrast to Knudson's two-hit model, which states that both alleles of the tumor suppressor gene must be inactivated.

To answer the question of whether *PTCH1* acts as a tumor suppressor gene through a two-hit model in NBCCS patients, we sought to identify the molecular mechanisms for the two hits inactivating this gene in NBCCS-associated and sporadic KCOTs. A range of *PTCH1* alteration profiles, including genetic mutation, LOH, and promoter hypermethylation, was investigated to dissect all possible genetic and epigenetic mechanisms.

Materials and Methods

Patients and samples. KCOT samples from 44 unrelated Chinese patients (15 NBCCS-related and 29 sporadic cases) were obtained from Peking University, Hospital and School of Stomatology, all of which were previously described for *PTCH1* mutations (27 mutations were detected in 9 of 29 sporadic and 13 of 15 NBCCS-associated KCOTs; Table 1). Diagnosis of NBCCS was established according to previously described clinical criteria (24). Fresh tissue specimens and corresponding peripheral blood samples were collected and stored at -80°C for subsequent analysis. As control, two normal gingival samples were obtained from healthy volunteers after tooth extraction. The protocol for the experiment was reviewed and approved by the Ethics Committee of Peking University Health Science Center. Informed consent was obtained from all patients and volunteers.

Sequencing analysis to determine allelic location of two coincident mutations. In the five tumors (NB9, NB11, NB19, KC19, and KC21) that were previously found to have two concomitant *PTCH1* mutations by intragenic mutation screening, cloning sequencing of the reverse transcriptase-PCR products containing both sites of the mutations was used to determine whether two variants were located in the same or different alleles. Total RNA was extracted from the five tumors by means of Trizol reagent (Invitrogen). cDNA was synthesized with Superscript Ø First-Strand synthesis system (Invitrogen) following the standard protocol. Primers designed to clone specific regions encompassing the two mutational changes were as follows: NB9-F, 5′-CAG CAC TGG AAA ACT CGT CA-3′, NB9-R, 5′-CTT TGT CGT GGA CCC ATT CT-3′; NB11-F, 5′-CGC CAG AAG ATT GGA GAA GA-3′, NB11-R, 5′-CCA GGA GTT TGT AGG CAA GG-3′; NB19-F, 5′-TGG GAT TAA AAG CAG CGA AC-3′, NB19-R, 5′-AGC CCC AGG CTC GTA TAG TT-3′; KC19-F, 5′-ACA AAC TTC GAC CCT TTG GA-3′, KC19-R, 5′-CAG CAT GGT TAG ACA GGC ATA G-3′; KC21-F, 5′-TCG CCT ATG CCT GTC TAA CC-3′, KC21-R, 5′-CTT TGT CGT GGA CCC ATT CT-3′. The products were purified on 2% agarose gels and ligated into pCR 2.1 vectors (Invitrogen). Resulting clones were sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

LOH assay. In the search for possible allelic loss of *PTCH1* in KCOTs, six microsatellite markers, including D9S253, D9S197, D9S196, D9S287, and D9S127, which

covered chromosome region 9q22.3-31, as well as an intragenic microsatellite marker within the *PTCH1* gene (*PTCH1* intra, exon1a; ref. 25), were examined. The epithelium of KCOTs from frozen samples was carefully microdissected on routinely H&E-stained slides to obtain pure tumor tissue. Genomic DNA was isolated from tumor epithelial cells and the corresponding peripheral blood samples using QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's protocol. PCR was done in 44 tumor-blood DNA pairs, using fluorescent forward primer. Primer sequences of the microsatellite markers were obtained from the National Center for Biotechnology Information UniSTS database. Genomic instability was detected with ABI PRISM 3770 Genetic Analyser (Applied Biosystems), and analyzed with GeneScan version 1.7 software. A given informative marker was considered to display LOH when a ≥1.5-fold difference was seen in the relative allele height ratio between tumor and peripheral blood. Tumors with at least two loci loss were considered as LOH.

Real-time reverse transcriptase-PCR to detect mRNA expression of PTCH1. Total RNA isolated from 14 tumor tissues (available for high-quality RNA) was reverse-transcribed. Amplification of cDNA was done using primers specific for *PTCH1* and GAPDH (internal control). The primers sequences were: PTCH1, (upstream) 5′-CAC TGG CAG GAG GAG TTG ATT-3′ and (downstream) 5′-TTG CTT GGG AGT CAT TAA CTG G-3′; GADPH, (upstream) 5′-ATG GGG AAG GTG AAG GTC G-3′ and (downstream) 5′-GGG GTC ATT GAT GGC AAC AAT-3′. Quantitative reverse transcriptase-PCR was carried out on a 7300 Real-Time PCR System (Applied Biosystems) over 40 cycles, with denaturation for 15 s at 95°C and combined annealing/extension at 60°C for 1 min. The expression level of *PTCH1* mRNA in individual tumors was internally normalized to GADPH expression, quantitated relative to normal gingiva as tissue controls, and represented the average of at least three independent experiments.

Bisulfite-modified DNA sequencing of PTCH1 promoter. Genomic DNA from tumors with low *PTCH1* mRNA expression, as well as normal gingiva controls, was extracted using QIAamp DNA Mini Kit (Qiagen). Bisulfite modification of DNA was done with the Methylcode Bisulfite Conversion Kit (Invitrogen) according to the manufacturer's recommendations. The CpG island-rich promoter region (−834 to +203) relative to *PTCH1* alternative exon 1b was selected for analysis. Bisulfite sequencing PCR primers were designed to clone the CpG island of *PTCH1* promoter into two regions: region 1 (−834 to −411), (upstream) 5′-TTT ATT GAA TTA AGG AGT TGT TG-3′, (downstream) 5′-CTT CAC TAC AAA AAA AAC CAA C-3′; region 2 (−432 to +203), (upstream) 5′-TTG GTT TTT TTT GTA GTG AAG G-3′, (downstream) 5′-ACC TTA AAA ATC TAC TCC AAA AC-3′. For sequencing assay, the PCR products were subcloned into the pCR 2.1 TOPO vector. At least four clones were picked for sequencing of each PCR product to ensure consistency for each sample.

Statistical analysis. Differences in distributions between variables were calculated using the χ^2 test or Fisher's exact test, as appropriate. All statistical analyses were carried out using the SPSS 13.0 software and probability values <0.05 were considered statistically significant.

Results

Allelic location of mutations in KCOTs carrying two coincident mutations. Forty-four KCOTs were screened previously by our lab for *PTCH1* mutations and 27 mutations were detected in 9 of 29 sporadic and 13 of 15 NBCCS-associated KCOTs (Table 1). Among the cases, five patients (NB9, NB11, NB19, KC19, and KC21) carrying two concomitant mutations were analyzed to determine the allelic location of the mutations. Three patients (KC19, KC21, and NB9) had abolished PTCH1 with two null alleles carrying biallelic mutations respectively. Two sporadic cases [KC19 (c.983delA, c.1325dupT) and KC21 (c.1559-1575del, c.2635delG)] carried two biallelic somatic mutations respectively (Fig. 1), and one syndromic case (NB9) carried a germline nonsense mutation (c.2619C>A) and a somatic variant splicing (c.1504-1G>A) located in different *PTCH1* alleles. In the other two syndrome patients (NB11 and NB19), two mutations were detected to occur in the same allele: NB11 carried two germline mutations, one missense (c.863G>A) inherited from her father and another frameshift mutation (c.2196-2197del) found to be a *de novo* mutation (Fig. 2). NB19 carried one germline frameshift (c.2824delC) and one somatic nonsense mutation (c.403C>T) occurred simultaneously. No other hit in these two patients was found in the following analysis.

LOH of PTCH1 in KCOTs. All 44 tumors were informative for LOH assay using six markers on chromosome 9q22-31. Overall, 15 tumors showed LOH of at least two loci at a high prevalence (34.1%; Table 1). Nearly half of the syndrome-associated KCOTs (7 of 15, 46.7%) and one third of sporadic KCOTs (8 of 29, 27.6%) presented LOH, respectively, whereas no difference was detected between these two groups (Pearson $\chi^2 = 1.602$; $P = 0.206$).

PTCH1 mRNA expression. *PTCH1* mRNA expression level was analyzed in a total of 14 KCOT samples from which high-quality RNA could be obtained. *PTCH1* expression was elevated in most KCOT samples (10 of 14, 71.4%), whereas 4 tumors (28.6%) expressed relative lower levels of *PTCH1* mRNA in comparison with normal controls (Fig. 3). In cases that underwent two hits through mutations or LOH, *PTCH1* gene expression seemed to be relatively higher compared with cases with only one hit or with no hit. The five cases (three sporadic and two syndromic KCOTs) with no hit, exhibiting relatively low *PTCH1* mRNA expression, were selected for later epigenetic analysis of promoter methylation.

Methylation status of PTCH1 promoter. The methylation status of the *PTCH1* promoter in five selected KCOTs and two normal controls was investigated following bisulfite conversion, specific PCR amplification, and sequencing. The average methylation level of region 1 (424 bp,

Table 1. Mutations and LOH of *PTCH1* in 44 KCOTs

Patient	Age/Sex	Mutation*	LOH	Phenotype
NB1	60/F	c.1939A>T	Positive	Multiple KCOTs, multiple epidermal cysts, calcification of falx cerebri
NB2	16/M	c.317T>G	Negative	Multiple KCOTs, palmar/planter pits, calcification of falx cerebri, multiple skin naevi, telecanthus, multiple epidermal cysts
NB3	15/M	c.331delG	Positive	Multiple KCOTs, bifid rib, calcification of falx cerebri
NB4	47/F	c.361insGAGC	Positive	Multiple KCOTs, multiple basal cell carcinomas, calcification of falx cerebri, ovarian fibromas
NB6	37/M	c.1338-1339insGCG	Negative	Multiple KCOTs, calcification of falx cerebri, multiple skin naevi
NB9	9/M	c.2619C>A c.1504-1G>A	Negative	Multiple KCOTs, palmar/planter pits, calcification of falx cerebri, telecanthus
NB11	14/F	c.863G>A c.2196-2197del	Negative	Multiple KCOTs, palmar/planter pits, frontal bossing, telecanthus
NB12	43/M	c.1247C>G	Positive	Multiple KCOTs, palmar/planter pits, telecanthus
NB13	22/M	c.3440T>G	Positive	Multiple KCOTs, palmar/planter pits, calcification of falx cerebri, frontal bossing, telecanthus
NB15	14/M	Negative	Negative	Multiple KCOTs, bifid rib, calcification of falx cerebri
NB16	42/M	c.3499G>A	Negative	Multiple KCOTs, multiple basal cell carcinomas, palmar/planter pits
NB17	53/M	c.1012C>T	Positive	Sporadic KCOT, multiple basal cell carcinomas, calcification of falx cerebri, bifid rib
NB18	15/F	c.2179delT	Positive	Multiple KCOTs, bifid rib, multiple skin naevi
NB19	26/F	c.403C>T c.2824delC	Negative	Multiple KCOTs, palmar/planter pits, calcification of falx cerebri, bridged sella, frontal bossing, multiple skin naevi
NB20	34/F	Negative	Negative	Multiple KCOTs, palmar/planter pits, bifid rib, telecanthus, frontal bossing, multiple skin naevi
KC1	26/M	c.3068-3074dup	Positive	Sporadic KCOT
KC4	18/F	Negative	Negative	Sporadic KCOT
KC5	21/F	Negative	Negative	Sporadic KCOT
KC6	20/F	c.3124-3129dup	Positive	Sporadic KCOT
KC8	17/M	Negative	Negative	Sporadic KCOT
KC9	29/M	Negative	Negative	Sporadic KCOT
KC16	29/M	Negative	Negative	Sporadic KCOT
KC17	25/F	Negative	Negative	Sporadic KCOT
KC18	21/M	Negative	Positive	Sporadic KCOT
KC19	56/M	c.983delA c.1325dupT	Negative	Sporadic KCOT
KC20	30/M	Negative	Positive	Sporadic KCOT
KC21	29/M	c.1558-1574del c.2635delG	Negative	Sporadic KCOT
KC22	73/F	c.1247C>G	Negative	Sporadic KCOT
KC24	30/M	Negative	Negative	Sporadic KCOT
KC26	45/F	Negative	Negative	Sporadic KCOT
KC27	35/F	Negative	Negative	Sporadic KCOT
KC29	28/M	Negative	Negative	Sporadic KCOT
KC30	10/F	c.403C>T	Negative	Sporadic KCOT
KC32	42/M	Negative	Positive	Sporadic KCOT
KC33	33/F	c.3162dupG	Positive	Sporadic KCOT
KC34	56/M	Negative	Negative	Sporadic KCOT

(*Continued on the following page*)

Table 1. Mutations and LOH of *PTCH1* in 44 KCOTs (Cont'd)

Patient	Age/Sex	Mutation*	LOH	Phenotype
KC35	24/M	Negative	Negative	Sporadic KCOT
KC36	72/F	Negative	Positive	Sporadic KCOT
KC37	75/F	Negative	Positive	Sporadic KCOT
KC38	46/M	c.3162dupG	Negative	Sporadic KCOT
KC39	16/M	c.1362-1374dup	Negative	Sporadic KCOT
KC40	64/M	Negative	Negative	Sporadic KCOT
KC41	34/M	Negative	Negative	Sporadic KCOT
KC42	21/F	Negative	Negative	Sporadic KCOT

NOTE: NB, NBCCS-associated KCOT; KC, sporadic KCOT.
*Data derived from refs. 9–12.

41CpGs) in KCOTs was 3.58% of successfully analyzed CpGs, slightly lower than 4.76% in normal tissues. In region 2 (635 bp, 91CpGs), the methylation level in KCOTs (11.93%) was also slightly lower than that in normal controls (13.4%). However, no statistical significance was identified between these two groups (tumors versus controls) by independent *t*-test (*P* values of 0.834 and 0.874 separately in these two regions). Thus, hypermethylation in the *PTCH1* promoter was not detected in these five KCOTs exhibiting a low level of *PTCH1* mRNA expression.

Summary and statistical analysis. Taken together, of all the 44 samples tested, 13 cases (30%) were identified to fit the standard model of two-hit, 10 of which contained two inactivating alleles by LOH and mutation separately (Fig. 4) and 3 obtained disrupted alleles through two mutations in each of the alleles. Fourteen KCOTs (32%) were found to conform to a one-hit model by LOH or mutation in a single allele; of these, five lost a normal allele in *PTCH1* locus, seven tumors carried only one mutation, and two tumors harbored two coincident mutations in the same allele of *PTCH1*. Among the 15 NBCCS-associated KCOTs, 8 cases (53.3%) harbored two hits, 5 (33.3%) harbored only one hit, and the remaining 2 cases carried no hit. In the 29 sporadic cases, however, only 5 (17.2%) contained two hits, 9 (31%) carried one hit, and 15 cases (51.7%) showed no alteration of *PTCH1*. Significant differences were detected in the percentage of two-hit, one-hit, and non-hit cases between syndrome-related and sporadic KCOTs (Pearson $\chi^2 = 8.03$; $P = 0.013$; Table 2).

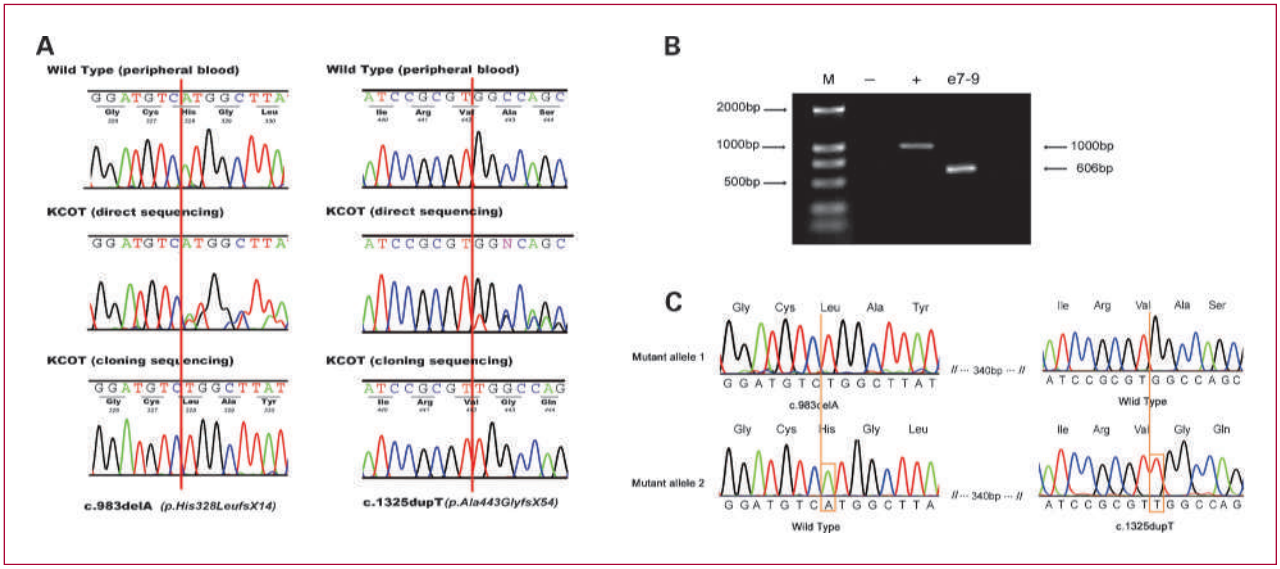

Fig. 1. Biallelic somatic mutations identified in KC19. *A,* two somatic mutations (c.983delA and c.1325dupT) were confirmed in KC19 by direct and cloning sequence. *B,* reverse transcriptase-PCR products of cDNA fragment (606bp) containing both somatic mutant loci were amplified, cloned, and sequenced. *C,* cloning sequences of the two mutant *PTCH1* alleles. Mutant allele 1 carried a c.983delA mutation while being wild type for c.1325; mutant allele 2 is wild type for c.983, but carried a somatic c.1325dupT mutation.

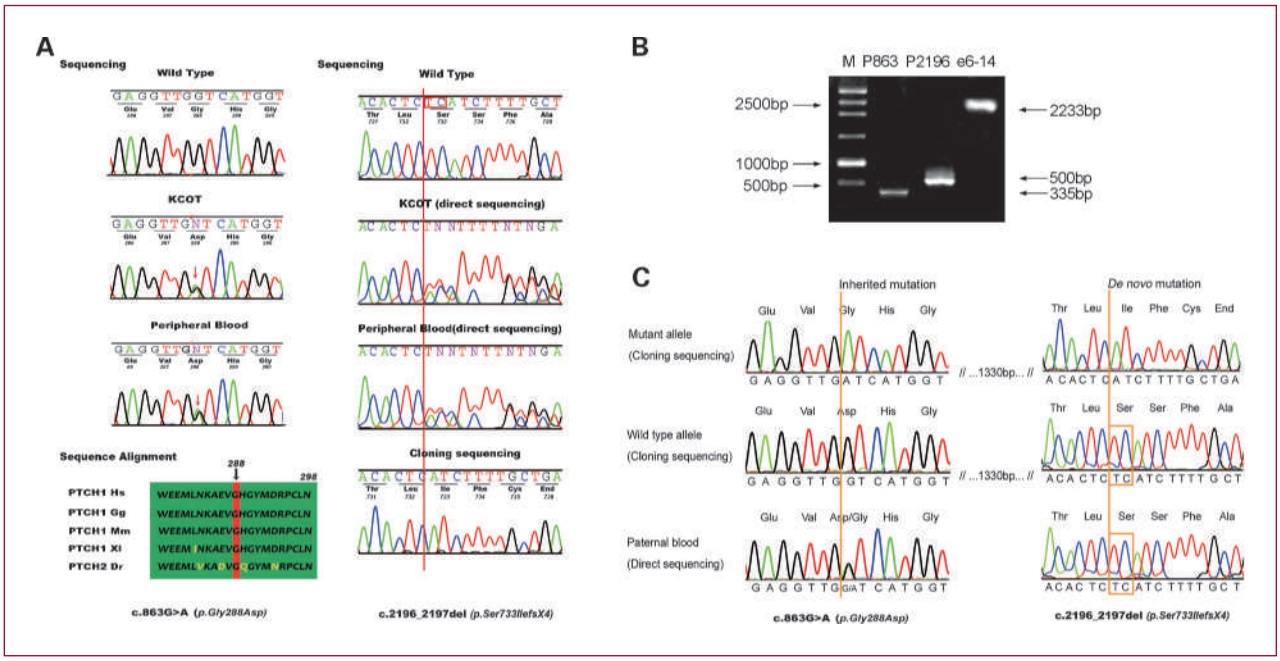

Fig. 2. Monoallelic mutations identified in NB11. *A,* two germline *PTCH1* mutations were detected in patient NB11: c.863G>A (*left*), resulting in amino acid substitutes in a highly conserved domain through sequence alignment; c.2196-2197del (*right*), which is expected to introduce a stop codon at amino acids 736. *B,* PCR products amplifying exon 6 and exon 14 of peripheral blood–derived DNA from the father of patient NB11 (*lanes P863 and P2196*) and reverse transcriptase-PCR product from the KCOT lesion of NB11 spanning exon 6 to 14 (*lane e6-14*). *C,* cloning sequencing of the reverse transcriptase-PCR products reveals two types of alleles in NB11: wild-type allele and mutant allele carrying both mutations (c. 863G>A and c.2196-2197del). Only c.863G>A was identified in paternal peripheral blood–derived DNA. The absence of c.2196-2197del indicates this germline mutation is a *de novo* mutation in NB11.

Discussion

NBCCS is a rare autosomal dominant disorder characterized by a predisposition to a number of different neoplasms, such as basal cell carcinomas, KCOTs, medulloblastomas, and ovarian fibromas, as well as a spectrum of developmental defects including pits of the palms and soles, calcification of falx cerebri, bifid rib, and so on (2). Apart from these anomalies that are inaccessible by biopsy or excision for medical reasons, keratocystic odontogenic tumors in the jaws are clinically apparent at the early stage, and usually act as the onset symptom of NBCCS patients. Removal of the tumor is medically indicated and provides an opportunity to obtain tissue samples. KCOT was previously known as odontogenic keratocyst. It was recently classified as a benign neoplasm in the new WHO classification for head and neck tumors (26) due to its locally aggressive behavior and high prevalence of *PTCH1* mutations. Multiple KCOTs are the most common feature of NBCCS, in which multiple basal cell carcinomas of skin

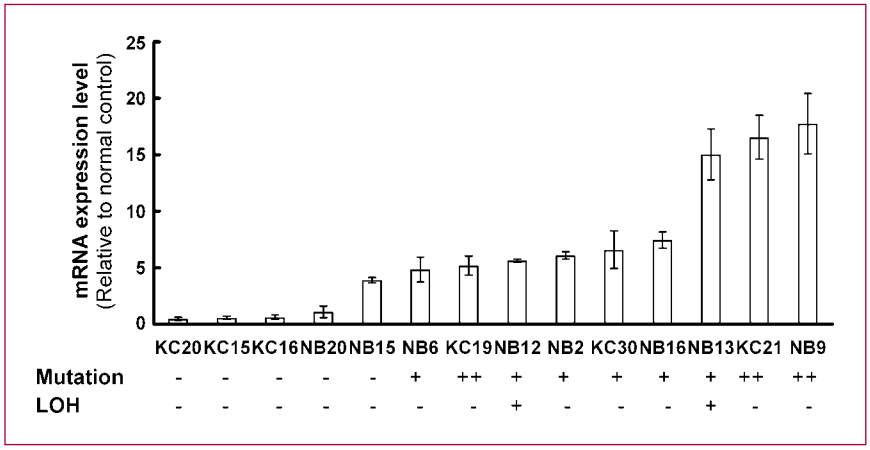

Fig. 3. Relative mRNA expression levels of *PTCH1* in 14 KCOT samples. Four KCOTs (KC20, KC15, KC20, and NB20) showed relative lower level of *PTCH1*, and no genetics alterations were found after analysis. The other 10 KCOTs, showing high mRNA level of *PTCH1*, were all found to have at least one hit of *PTCH1* in each of them (except for NB15). –, negative; +, one mutation or detectable LOH; ++, two biallelic mutations.

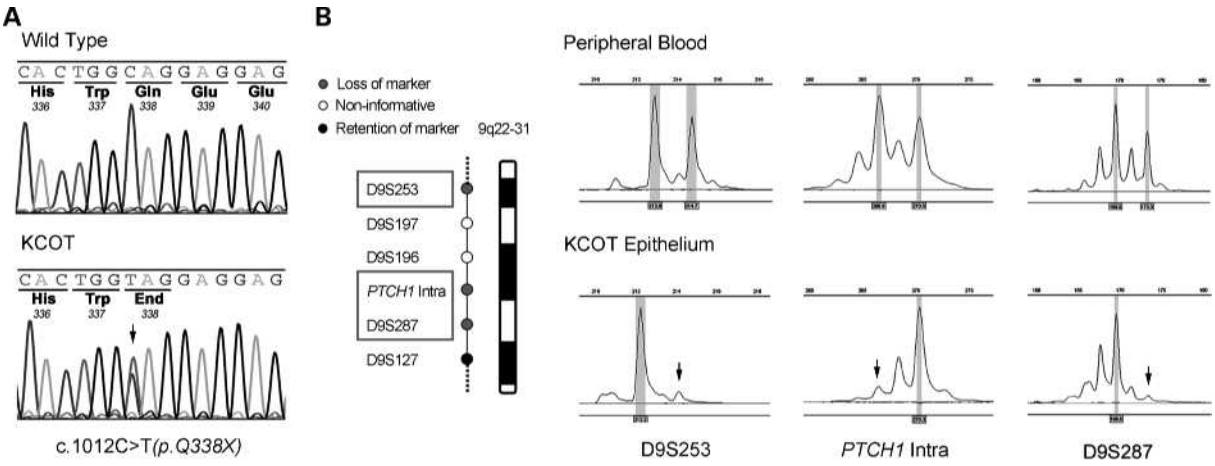

Fig. 4. Illustration of a typical two-hit model through LOH and mutation in NB17. *A,* one *PTCH1* allele was inactivated by a nonsense mutation (c.1012T), resulting in a premature stop at codon 338 (*arrow*). *B,* LOH assay showed loss of three DNA markers (D9S253, *PTCH1* intra, and D9S287) in *PTCH1* locus when a ≥1.5-fold difference was seen in the relative allele height ration between the peripheral blood and tumors (*arrows*).

and skeletal abnormalities may also develop. Some authors have also argued that sporadic KCOT might represent the syndrome in its least expressed form (1, 8).

In the present study, we investigated the mechanisms of *PTCH1* inactivation in a panel of NBCCS-associated and sporadic KCOTs which had been previously screened for *PTCH1* mutations by our group (9–12). Of the five cases harboring two heterozygous *PTCH1* mutations, three were identified to have two null *PTCH1* alleles and fit the two-hit hypothesis for tumor suppressor genes. However, the other two cases showed two coincident mutations in the same allele, contradicting the classic two-hit model.

To detect another possible hit caused by allelic loss of *PTCH1*, LOH assay was done in all samples. We found a high frequency (34.1%) of allelic loss of *PTCH1* in KCOTs. Although a higher frequency of LOH was detected in NBCCS-associated KCOTs than in sporadic ones, no statistical difference was found between these two groups. LOH is believed to be a frequent event in the early stage of tumorigenesis. The heterozygous cells caused by LOH would undergo a clonal expansion and thus increase the population of target cells available for further hits in the multistep tumorigenesis pathway (27). Therefore, most mutations were found predominantly in tumors exhibiting LOH at loci flanking *PTCH1* (17). Of all 13 tumors in which we identified two hits, 10 showed abolished alleles as a combination of intragenic mutation and LOH. This result suggests that inactivation of both copies of the gene is required for progression of a proportion of KCOTs, and supports the criterion of the two-hit concept defining tumor suppressor genes.

Epigenetic mechanisms such as promoter methylation have recently been recognized as another possible hit of inactivating tumor suppressor genes (19). Methylation of the *PTCH1* promoter has been documented as a mechanism responsible for breast cancer (28), ovarian dermoids, and fibromas (29). In the present study, hypermethylation of the *PTCH1* promoter, an alternative cause to mutation and LOH, was not detected in tumors with relatively low *PTCH1* mRNA expression. This result is consistent with other reports in basal cell carcinomas and medulloblastomas (29, 30), suggesting different methylation status of *PTCH1* promoter in NBCCS-related tumors.

Taken together, 13 cases (30%) of the present series were identified to fit the standard model of two-hit, whereas 14 (32%) fit a one-hit model, exhibiting atypical tumor suppressor gene behavior. In addition, the remaining 17 cases

Table 2. Summary of *PTCH1* allelic alterations in KCOTs

	Two-hit		One-hit		Non-hit	
	No.	% of total	No.	% of total	No.	% of total
NBCCS-KCOT	8	53.55	5	33.33	2	13.33
Sporadic-KCOT	5	17.24	9	31.03	15	51.72
Total	13	29.55	14	31.82	17	38.64

(38.6%) failed to show any *PTCH1* alteration after careful analysis. These results prompt us to propose that in KCOTs, a number of mechanisms might be implicated in inactivation of the *PTCH1* gene in addition to the two-hit hypothesis. Perhaps the most contested exception to the two-hit hypothesis is haploinsufficiency (absent or reduced function due to the loss or inactivation of a single allele). Evidence for the haploinsufficiency model arises from studies of the $PTCH1^{+/-}$ mouse (21, 22, 31). Mice heterozygous for *PTCH1* recapitulate the typical developmental symptoms of NBCCS and develop rhabdomyosarcoma and medulloblastoma, indicating that haploinsufficiency of *PTCH1* is sufficient to promote tumor formation in mice. One explanation for this phenomenon is that the low level of protein produced by monoallelic *PTCH1* makes it unable to fulfill its function as a tumor suppressor gene, and a dose-dependent effect of *PTCH1* might be involved in Hedgehog signaling (19). However, haploinsufficiency is not the only possible mechanism for a one-hit tumorigenesis model. *In vivo* studies have shown that several mutant PTCH1 proteins (Ptc1130X, PtcG509V, and PtcD584N) could result in the activation of Hedgehog signaling through a dominant-negative mechanism despite the production of wild-type PTCH1 (23, 32, 33). PTCH1 may normally form a multimer in its active state, and the mutant PTCH1 might interact with wild-type PTCH1 to block its function or proper localization within the cells. Or alternatively, mutant PTCH1 might associate nonproductively with SMO and thereby shield it from interaction with wild-type PTCH1, resulting in activation of the downstream signaling (33).

It is long believed that tumors arise from a multistep progression. The more advanced the tumor, the more hits it has accumulated. In the present study, we showed that the percentage of two-hit cases in NBCCS-associated KCOTs (53.3%) was significantly higher than that in sporadic tumors (17.2%). Our results, showing two-hit was significantly more common in syndrome cases than in sporadic cases, indicate that more destructive hits accumulated due to decreased DNA repair efficiency and/or increased genetic instability in syndrome-related lesions. The cases with only one hit at the time might represent an early stage of tumor progression, and a second hit might eventually occur with a more severe presenting phenotype (34).

The failure to detect any hit in about one third of the cases examined (including two syndrome cases) might be explained by the presence of a multigene tumorigenesis model. Although *PTCH1* has been identified to play a confirmative role in NBCCS, other genes, such as *PTCH2* and *SUFU*, might also be involved (35, 36). We previously identified two novel missense *PTCH2* mutations in 15 NBCCS patients. Interestingly, one *PTCH2* mutation occurred in a patient carrying no *PTCH1* mutation, but the other case carried both *PTCH1* and *PTCH2* mutations (35). Although not as frequent as *PTCH1* mutation, *PTCH2* germline mutations were detectable in a subset of NBCCS patients. The pathogenetic role of these *PTCH2* mutations is yet to be clarified.

In summary, the present study indicates that *PTCH1* gene alternation may play a significant role in the pathogenesis of NBCCS-related and sporadic KCOTs. Not only the standard two-hit model, but also the one-hit model exhibiting haploinsufficiency or dominant-negative isoforms might be implicated in the inactivation of the *PTCH1* gene. As a key player in the Sonic Hedghog pathway, PTCH1 acts to restrain the activity of the G-protein coupled receptor, SMO, and thus inhibits signal transduction of the pathway. Inactivation of PTCH1 allows hedgehog ligand-independent activation of SMO, causing a downstream activation of the pathway that may lead to neoplastic growth (37). Therefore, future identification of inhibitors of the hedgehog signaling pathway as new drugs will offer new strategies for the treatment of NBCCS and its related sporadic tumors (38), including KCOTs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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