Human Cancer Biology

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

Shuang Pan1,2, Qing Dong1, Li-Sha Sun1, and Tie-Jun Li1

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.
isoforms of development (21, 22). In addition, dominant-negative 9q22 (17). These data propose a hypothesis that mutations occurred predominantly in tumors with LOH at (20). Analysis of tumors taken from the tion or LOH, but rather by epigenetic hypermethylation standard two-hit model, including cancer-associated genes candidate tumor suppressors that do not conform to the stan-
tumorigenesis.

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.

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.

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 genet-
ic 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 ob-
tained 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 pa-
tients 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 O 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′-CTT TGT CGT GGA CCC ATT CT-3′; NB19-F, 5′-ACA AAC TTC GAC CCT TTG GA-3′, NB19-R, 5′-AGC CCC AGG CTC GTA TAG TT-3′; KB19-F, 5′-ACA AAC TTC GAC CCT TTG GA-3′, KB19-R, 5′-AGC CCC AGG CTC GTA TAG TT-3′; KC21-F, 5′-CTG CCT ATG CCT GTC TAA CC-3′, KB21-R, 5′-CTT TGT CGT CCA CCC ATT CT-3′. The products were pu-
rified on 2% agarose gels and ligated into pCR 2.1 vec-
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LOH assay. In the search for possible allelic loss of PTCH1 in KCOTs, six microsatellite markers, including D9S253, D9S197, D9S196, D9S287, and D9S127, which

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covered chromosome region 9q22.3-31, as well as an intragenic microsatellite marker within the \textit{PTCH1} gene (\textit{PTCH1} intra, exon 1a; 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 $\pm1.5$-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 \textit{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 \textit{PTCH1} and GAPDH (internal control). The primers sequences were: \textit{PTCH1}, (upstream) 5′-CAC TGG CAG GAG GAG TTG ATT-3′ and (downstream) 5′-TTG CTT CAC TAC AAA AAA AAC CAA C-3′; GAPDH, (upstream) 5′-ATG GGG AAGGTGAGGTAATG-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 \textit{PTCH1} mRNA in individual tumors was internally normalized to GAPDH expression, quantitated relative to normal gingiva as tissue controls, and represented the average of at least three independent experiments.

**Bisulfite-modified DNA sequencing of \textit{PTCH1} promoter**. Genomic DNA from tumors with low \textit{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 Methylinecode Bisulfite Conversion Kit (Invitrogen) according to the manufacturer's recommendations. The CpG island-rich promoter region (~834 to +203) relative to \textit{PTCH1} alternative exon 1b was selected for analysis. Bisulfite sequencing PCR primers were designed to clone the CpG island of \textit{PTCH1} promoter into two regions: region 1 (~834 to ~411), (upstream) 5′-TTT AIT GAA TTA AGT TGT TG-3′, (downstream) 5′-CTT CAC TAA AAA AAC CAA C-3′; region 2 (~432 to +203), (upstream) 5′-TGT TTG TTT TTT GTT AAG G-3′, (downstream) 5′-ACC TTA AAA TCT 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 $\chi^2$ 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 \textit{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 \textit{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.2619G>A) and a somatic variant splicing (c.1504-1G>A) located in different \textit{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 \textit{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 \textit{PTCH1} in KCOTs**. All 44 tumors were informative for LOH assay using six markers on chromosome 9q22.3-31. 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 $\chi^2 = 1.602; P = 0.206$).

**\textit{PTCH1} mRNA expression**. \textit{PTCH1} mRNA expression level was analyzed in a total of 14 KCOT samples from which high-quality RNA could be obtained. \textit{PTCH1} expression was elevated in most KCOT samples (10 of 14, 71.4%), whereas 4 tumors (28.6%) expressed relative lower levels of \textit{PTCH1} mRNA in comparison with normal controls (Fig. 3). In cases that underwent two hits through mutations or LOH, \textit{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 \textit{PTCH1} mRNA expression, were selected for later epigenetic analysis of promoter methylation.

**Methylation status of \textit{PTCH1} promoter**. The methylation status of the \textit{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

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

(Continued on the following page)
41 CpGs) in KCOTs was 3.58% of successfully analyzed CpGs, slightly lower than 4.76% in normal tissues. In region 2 (635 bp, 91 CpGs), 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. 5) 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 χ² = 8.03; P = 0.013; Table 2).

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

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<td>KC35</td>
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<td>KC36</td>
<td>72/F</td>
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<td>KC37</td>
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<td>46/M</td>
<td>c.3162dupG</td>
<td>Negative</td>
<td>Sporadic KCOT</td>
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<td>KC39</td>
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<td>Sporadic KCOT</td>
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<td>KC40</td>
<td>64/M</td>
<td>Negative</td>
<td>Negative</td>
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</tr>
<tr>
<td>KC42</td>
<td>21/F</td>
<td>Negative</td>
<td>Negative</td>
<td>Sporadic KCOT</td>
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</tbody>
</table>

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

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

![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 (600bp) 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.](image-url)
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
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>
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<th>Table 2. Summary of PTCH1 allelic alterations in KCOTs</th>
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<td>Two-hit</td>
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<td><strong>NBCCS-KCOT</strong></td>
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<td><strong>Sporadic-KCOT</strong></td>
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<tr>
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(38.6%) failed to show any \textit{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 \textit{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 \textit{PTCH1} \textsuperscript{–/–} mouse (21, 22, 31). Mice heterozygous for \textit{PTCH1} recapitulate the typical developmental symptoms of NBCCS and develop thalidomidosarcoma and medulloblastoma, indicating that haploinsufficiency of \textit{PTCH1} is sufficient to promote tumor formation in mice. One explanation for this phenomenon is that the low level of protein produced by monoallelic \textit{PTCH1} makes it unable to fulfill its function as a tumor suppressor gene, and a dose-dependent effect of \textit{PTCH1} might be involved in Hedgehog signaling (19). However, haploinsufficiency is not the only possible mechanism for a one-hit tumorigenesis model. \textit{In vivo} studies have shown that several mutant \textit{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 \textit{PTCH1} (23, 32, 33). \textit{PTCH1} may normally form a multimer in its active state, and the mutant \textit{PTCH1} might interact with wild-type \textit{PTCH1} to block its function or proper localization within the cells. Or, alternatively, mutant \textit{PTCH1} might associate nonproductively with SMO and thereby shield it from interaction with wild-type \textit{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 tumors (38), including KCOTs.

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 \textit{PTCH1} has been identified to play a confirmative role in NBCCS, other genes, such as \textit{PTCH2} and \textit{SUFI}, might also be involved (35, 36). We previously identified two novel missense \textit{PTCH2} mutations in 15 NBCCS patients. Interestingly, one \textit{PTCH2} mutation occurred in a patient carrying no \textit{PTCH1} mutation, but the other case carried both \textit{PTCH1} and \textit{PTCH2} mutations (35). Although not as frequent as \textit{PTCH1} mutation, \textit{PTCH2} germline mutations were detectable in a subset of NBCCS patients. The pathogenetic role of these \textit{PTCH2} mutations is yet to be clarified.

In summary, the present study indicates that \textit{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 \textit{PTCH1} gene. As a key player in the Sonic Hedgehog pathway, \textit{PTCH1} acts to restrain the activity of the G-protein coupled receptor, SMO, and thus inhibits signal transduction of the pathway. Inactivation of \textit{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.

\section*{Disclosure of Potential Conflicts of Interest}

No potential conflicts of interest were disclosed.

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\section*{References}

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Mechanisms of Inactivation of \textit{PTCH1} Gene in Nevoid Basal Cell Carcinoma Syndrome: Modification of the Two-Hit Hypothesis

Shuang Pan, Qing Dong, Li-Sha Sun, et al.


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