Trichoepitheliomas Contain Somatic Mutations in the Overexpressed PTCH Gene: Support for a Gatekeeper Mechanism in Skin Tumorigenesis

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Abstract

The nevoid basal cell carcinoma (Gorlin) syndrome (NBCCS) is an autosomal dominant disorder characterized by multiple developmental defects and cancer susceptibility. NBCCS is caused by mutations in the human homologue (PTCH) of the Drosophila patched gene, a developmental regulator implicated in signaling of hedgehog and smoothened. The PTCH gene was found to contain somatic mutations also in sporadic basal cell carcinomas and medulloblastomas, tumors seen in NBCCS, consistent with PTCH acting as a tumor suppressor. Because basal cell carcinomas have been observed to develop in association with benign trichoepitheliomas (TEs) in the same lesions, patients, and families and may share the same cell of origin, we have analyzed PTCH for mutations and expression in TEs. We report frameshift and in-frame somatic deletions in this gene and a consistent overexpression of PTCH mRNA in TEs. These findings provide the first evidence of a gene mutation in TEs and identify a common pathogenic pathway for histopathologically similar but prognostically distinct skin tumors. Moreover, these results support the presence of a gatekeeper mechanism in multistep skin tumorigenesis exerted by the altered PTCH signaling pathway.

Introduction

The NBCCS4 (McKusick number MIM 109400; for a review see Ref. 1) is an autosomal dominant disorder characterized by multiple developmental abnormalities and susceptibility to a variety of tumors, in particular to BCCs. BCC of the skin is the most common cancer in humans, and its annual incidence has increased considerably in the last 2 decades (2). BCCs seen in NBCCS develop earlier in life and are often seen in large numbers at multiple sites, consistent with the NBCCS gene acting as a tumor suppressor. The NBCCS locus was mapped to the long arm of chromosome 9 by genetic linkage analysis, supported by frequent loss of constitutional heterozygosity at 9q (3–5), and the defective gene was found by positional cloning (6–8). The gene was identified as PTCH, a human homologue of ptc, an important developmental regulator, initially found as a segment polarity gene (9). ptc was implicated in the transcriptional repression of shh (10–13). Recently, Oro et al. (21) reported a putative activating missense mutation (H133T) in human shh in several tumors, suggesting that PTCH may be a part of an important cancer pathway. The inactivation of the PTCH gene product was proposed to be virtually necessary for passing the genetic threshold of the neoplastic process in the skin (22).

TEs are benign skin tumors with follicular differentiation, most commonly seen on the face and sometimes urging cosmetic interventions. They appear as skin-colored nodules or papules with few telangiectasias. If they present as multiple lesions, they are usually smaller and are commonly found on the nasolabial folds and upper lips (23, 24). These tumors are occasionally familial, usually transmitted from one generation to another with no clear sex preference, compatible with autosomal dominant inheritance. Interestingly, BCCs have been reported in such families and both tumor types have been suggested to have a common origin in hair follicle keratinocytes (23, 24). Although there is a defined set of histopathological and clinical diagnostic criteria, TEs can, in some cases, be mistaken for BCCs and vice versa (23, 24). To test PTCH as a candidate gene involved in the development of TEs, we have analyzed this gene for mutations using SSCP analysis of genomic DNA extracted from tumor cells dissected from TEs and for expression by in situ hybridization.

Materials and Methods

Tumor Samples. A total of 21 different TEs derived from 17 unrelated patients were analyzed for PTCH mRNA expression and CD34 staining. Nine tumor samples from unrelated Swedish patients with TEs were included in the mutational analysis. The samples were obtained from the Karolinska and Danderyd Hospitals (Stockholm, Sweden). The mean age of patients at diagnosis was 55 years and ranged from 25 to 82 years. With the exception of two patients with multiple TEs (TR4 and TR8 in Table 1), none of the patients had a known positive family history of TEs. Patient TR4 had an affected brother with multiple TEs, and patient TR8 had a sister and a daughter with multiple TEs. The patients and tumors are described in Table 1. All tumors were histologically reviewed and classified according to established criteria (23). All tumors were reevaluated independently, and only clinically and histopathologically typical TEs were included. All TEs were small, skin-colored, nonulcerated papules. They were epithelial-mesenchymal lesions with frond-like epithelial growth patterns, surrounded by fibroblastic stromas forming abortive or...
Table 1 Clinical characterization of TEs and in situ PTC mRNA expression

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sex</th>
<th>Tumor location</th>
<th>Ages at diagnosis/sampling (yr)</th>
<th>Signal intensity by ISHa</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR1b</td>
<td>M</td>
<td>Periorbital</td>
<td>59/59</td>
<td>++</td>
</tr>
<tr>
<td>TR4</td>
<td>M</td>
<td>Forehead</td>
<td>30/79</td>
<td>+</td>
</tr>
<tr>
<td>TR5</td>
<td>F</td>
<td>Upper eyelid</td>
<td>64/64</td>
<td>+</td>
</tr>
<tr>
<td>TR6</td>
<td>M</td>
<td>Forehead</td>
<td>82/82</td>
<td>+</td>
</tr>
<tr>
<td>TR7</td>
<td>F</td>
<td>Periorbital</td>
<td>50/50</td>
<td>+</td>
</tr>
<tr>
<td>TR8</td>
<td>F</td>
<td>Nasolabial</td>
<td>25/49</td>
<td>+</td>
</tr>
<tr>
<td>TR10</td>
<td>F</td>
<td>Nasolabial</td>
<td>49/49</td>
<td>+</td>
</tr>
<tr>
<td>TR11b</td>
<td>M</td>
<td>Forehead</td>
<td>41/41</td>
<td>+</td>
</tr>
<tr>
<td>TR12</td>
<td>F</td>
<td>Nose</td>
<td>44/44</td>
<td>+</td>
</tr>
</tbody>
</table>

a Signal intensity was estimated using a semiquantitative scale from 0 to +++. b Tumors with PTC mutations.

d denotes samples from patients with multiple and familial TEs; the remaining tumors were solitary and sporadic.

Table 2 Summary of PTC mutations in TE DNA samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Primers</th>
<th>PTC Exon</th>
<th>Nucleotide changea</th>
<th>Protein changea</th>
<th>Presence of the wild-type allele</th>
<th>Mutation in the germ line</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR11</td>
<td>PTC20</td>
<td>20</td>
<td>3509del26, 3539del11</td>
<td>L1170W, delL1171—G1179, P1180T</td>
<td>45%</td>
<td>Absent</td>
</tr>
<tr>
<td>TR1</td>
<td>PTC7</td>
<td>8</td>
<td>1108delAAGC</td>
<td>Frameshift</td>
<td>&lt;1%</td>
<td>Absent</td>
</tr>
</tbody>
</table>

a The numbering of nucleotides and codons is as in Ref. 8 (GenBank accession no. U59464).

b The deleted sequence was 5'-TGCTCGGAGGTTTCTTCTTT-3', followed by 5393delC.

developed hair bulb structures. All TEs contained small keratinocytes. Cellular material was punched out from macro- and microscopically well-defined nodules in the paraffin blocks to minimize contamination with normal cells.

**DNA Extraction.** DNA was extracted from paraffin-embedded material using methods described previously (25, 26). Constitutional DNA was isolated either from patients' blood samples or normal fixed tissue using the same methods.

**PCR-SSCP Analysis and Sequencing.** The sequence of oligonucleotide primers used for PCR-SSCP were as described previously (7, 16). The sequences of the forward and reverse primers flanking the shh H1331 mutation (21) were 5'-GAG GCC TGG GAC GAA GAT TG-3' and 5'-CTG CGG TCG CGG TCA GAG G-3', respectively. PCRs contained 100 ng of DNA, 0.25 μM each primer, 100 μM each dNTP, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 0.2 μCi of [α-33P]dCTP (3000 Ci/mmol), and 0.5 units of Taq polymerase (Perkin-Elmer) in a volume of 20 μl. Amplifications were performed for 30–35 cycles, with denaturation at 94°C for 30 s, annealing at 52–58°C for 45 s, and extension at 72°C for 30 s in a Perkin-Elmer thermocycler (GeneAmp System 9600). The samples were mixed with 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, and 50 mM NaOH, and autoradiographed for 1–2 days at —70°C. PTCH segments identified as having excess of hedgehog function can have an effect similar to loss of ptc, which is caused by loss of one or both copies of the ptc gene. The results revealed no copy of the mutated allele in the germ line, normal DNA from patients demonstrating multiple TEs.

**Immunohistochemistry.** A mouse monoclonal antibody directed against CD34 (QBEND/10; Unipath, Basingstoke, United Kingdom) was used. Sections from each tumor sample were incubated with antibody for 16 h at room temperature and processed using a standard avidin-biotin immunoperoxidase/diaminobenzidine detection system. Control serial sections were used with no primary antibody.

**Results**

**Identification of PTC Mutations in TEs.** The PTC coding region was amplified from tumor DNA samples using oligonucleotide primer pairs flanking each exon. The PCR products were analyzed on SSCP gels, and those with altered mobility were sequenced. Mutations were found in two patients (Table 2 and Fig. 1). Although one sample (TR1 in Table 1 and Fig. 1A) had a frameshift deletion with no detectable wild-type PTC allele, the other sample with a mutation was found to contain significant amounts of the normal copy of PTC (patient TR11 in Table 1 and Fig. 1B). The latter mutation was a larger deletion in exon 20 and would be predicted to lead to a removal of the COOH-terminal part of the 12th transmembrane domain. This domain was previously shown to be deleted and mutated in tumor DNA extracted from BCCs and medulloblastomas (16, 20) and is likely to be essential for downstream signaling by PTC. The ratio of the signal intensity from the wild-type and mutated allele was estimated at about 45% (Fig. 1B).

**Somatic Origin of PTC Mutations.** To determine whether these mutations are present in the germ line, normal DNA samples from patients TR1 and TR11 were analyzed in parallel with tumor DNA and controls. The results revealed no copy of the mutated allele in the germ line (data not shown), indicating a somatic origin of the mutations. Thus, the significant amounts of normal copy observed in sample TR11 was due to the presence of cells not containing the alteration, presumably normal stromal cells. No mutations were found in tumor or normal DNA from patients demonstrating multiple TEs (samples TR4 and TR8 in Table 1).

**Screening for the H133T Mutation in SHH.** In Drosophila, an excess of hedgehog function can have an effect similar to loss of ptc function, and this relationship appears to be conserved in vertebrates. Transgenic mice overexpressing shh in the skin develop many features of NBCCS, including BCC-like tumors (21). One candidate activating mutation (H133T) in the human shh was recently found in 5 of 43 BCCs, 1 of 14 medulloblastomas, and 1 of 6 breast cancers (21). We have examined TE tumor DNA for this particular change; however, the mutation was not found in any samples, nor was it present in any tumor DNA extracted from 32 unrelated patients with primitive neuroectodermal tumors or in 5 medulloblastoma cell lines.5

**Expression of PTC mRNA.** The results of in situ hybridization experiments are shown in Table 1 and Fig. 2. All TEs included in the mutation screening showed CD34 positivity (Fig. 2F), confirming

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5 I. Voitechovsky, O. Tingby, M. Nistér, V. P. Collins, and R. Toftgård, unpublished data.
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Fig. 1. SSCP alterations in tumor DNA samples of two patients with TEa and a corresponding nucleotide sequence. A, patient TR1, exon 8; B, patient TR11, exon 20. @, abnormal SSCP patterns. Arrowheads, deletion breakpoints in patients (top); bars, missing nucleotides in controls (bottom). Second arrowhead in exon 20 of patient TR11 (B), a cytosine deletion, which restores the reading frame.

Fig. 2. Expression of PTCH mRNA and CD34 staining in a solitary TE. A, bright-field photomicrograph of TE counterstained with H&E. Tu, tumor cells. Arrow, area shown in E under high magnification. B, dark-field photomicrograph of the same section. The tumor sections were hybridized with 35S-labeled antisense PTCH probe. Abundant autoradiographic signal is seen in tumor cells, whereas no expression is observed in normal epidermis (Ep). C, dark-field photomicrograph of an adjacent area of the same tumor. In contrast to the strong signal for PTCH mRNA in tumor nests (Tu), no signal is detected in normal follicular epithelium (arrow). D, sections of the same tumor hybridized with the sense probe showed no signal. E, tumor nests under high power demonstrate specific signal manifested as black grains in tumor cells. F, positive immunoreactivity for anti-CD34 antibody in stromal cells adjacent to tumor cells. Bars, 150 μm (A–D and F) and 15 μm (E).
previous findings (27) and, together with clinical and histopathological criteria, indicating that the tumors analyzed were TEs and not BCCs. The analysis of 21 TEs revealed a strong PTCH signal confined to tumor cells in 19 TE sections, whereas two TEs of the desmoplastic variant lacked overexpression. No detectable signal could be seen in normal epidermis or normal follicular epithelium (Fig. 2, B and C). All samples included in mutation screening showed an overexpression, and the mutated tumor samples showed a relatively strong signal intensity (Tables 1 and 2). The average signal intensity from TE tumor cells was comparable to that previously observed in BCCs (17, 18). These results present the first evidence of a consistently overexpressed gene in benign tumors of the skin and suggest that PTCH overexpression reflects a failure in the negative feedback mechanism due to mutations in PTCH or possibly in other genes in the signaling cascade.

Discussion

Here, we provide the first demonstration of the involvement of the important tumor suppressor gene PTCH in benign skin tumorigenesis. Our finding of two of nine mutated TEs suggests that altered PTCH or, more generally, alterations in the signaling pathway leading to overexpression of PTCH may be required for the initiation of both BCCs and TEs. One of the tumors (TR1 in Table 2) exhibited the frameshift mutation and virtual absence of normal copy of PTCH, indicating somatic inactivation of the gene in this tumor, which is consistent with PTCH acting as tumor suppressor. Our results are compatible with previously formulated hypothesis postulating the existence of a gatekeeper mechanism in skin tumor development (22), originally suggested for the inactivated APC gene in the initiation of colorectal tumors (28). As in dominantly inherited familial adenomatous polyposis, which typically leads to the development of hundreds to thousands of adenomas, NBCCS alleles confer extremely high risks of developing similar numbers of BCCs. The Knudson’s “second hit” in a cell already containing a single NBCCS allele seems to be most commonly represented by a mitotic recombination event but can also be achieved by a somatic mutation, loss of large chromosomal regions (16, 20), or other mechanisms. This event may be rate limiting in the development of these and other epithelial tumors. By contrast, squamous cell carcinomas of the skin, lungs, and esophagus have not been found to contain PTCH mutations (29, 30), nor have they been found in tumors of mesenchymal origin (31). Recent data show PTCH mutations also in extracutaneous ectodermal tumors and in additional tumors seen in NBCCS (32).

Consistent with our previous observations in BCCs (17, 18), an increased expression of PTCH at the level of RNA seen in all but two TEs analyzed, including tumors with somatic mutations, suggests that deregulation of the SHH-PTCH signaling pathway may be an early and necessary event in TE development. Interestingly, two TEs that did not show PTCH mRNA overexpression were of the desmoplastic type. Because a susceptibility to multiple desmoplastic TEs has been described as a separate hereditary syndrome (McKusick number MIM 190345; Ref. 33), the development of desmoplastic TEs may result from accumulating distinct genetic events.

There has been a controversy as to the origin of BCCs and TEs. Multiple hereditary TEs were reported independently by Brooke (34) and Fordyce (35) as epithelioma adenoides cysticum. Previously reported cases of malignant transformation of TEs have later been considered BCCs in NBCCS (36). Association of BCCs and multiple hereditary TEs without other signs of NBCCS was described, but the occurrence of BCCs and TEs in the same patient has only been well documented in a few reports (37–39). However, Johnson and Bennett (38) suggested that the association of BCCs and TEs is not unusual, and histopathological characteristics of the two tumor types support the notion that they are related. The absence of PTCH mutations and overexpression in squamous cell carcinomas (29, 30) and their presence in BCCs and TEs may argue in favor of their common histogenetic origin, perhaps supporting the follicular hair cells as a source of malignant transformation. In addition to common PTCH alterations, further genetic changes may be present in BCCs or TEs or both that would account for distinct biological potential of the two tumor types.

The development of hair follicle-derived tumors, including TEs and tricholemmomas, was reported in a nonclassical Li-Fraumeni syndrome patient with a constitutional missense mutation in exon 8 (R273C) of the p53 gene (40). Positive nuclear immunostaining for p53 was observed in tumor cells but not in surrounding normal cells, suggesting a possible presence of an additional somatic mutation in p53. However, to our knowledge, TEs have not been analyzed for mutations in this gene.

Using two African-American families, the tentative disease locus for multiple familial TE was recently mapped to 9p21, reporting a maximum LOD score of 3.31 at zero recombination fraction for D9S171, whereas the analysis of four markers at 9q22-q31 did not show a significant evidence for linkage (41). Although the number of genotyped family members was limited, these results, if confirmed, would support the existence of a TE susceptibility locus on chromosome 9p. Therefore, samples from well-documented multigenerational families should be collected, followed by genetic linkage analysis. In this context, it is worth noting that TEs have not been reported in NBCCS and that we did not find any PTCH mutations in the two patients with familial multiple TEs (Tables 1 and 2).

In addition to BCCs, TEs have been observed in the same patients together with multiple CCs, an autosomal dominant disorder known as the Brooke-Spiegler syndrome (42). A recent review of the literature on CC revealed that these tumors often appear concomitantly with TEs in sporadic patients (43). The same report documented a large family with a dominant transmission of these lesions, involving 20 family members with CCs only, 3 patients with TEs only, and 4 patients with the concomitant presence of both TEs and CCs (43). Furthermore, familial cases of CCs observed in the literature had lesions on the face, a typical location of TEs (43). CCs and TEs have even been reported together in a single congenital plaque lesion (42). Also, co-occurrence of multiple TEs and multiple eccrine spiradenomas have been observed in the same patients (44, 45). These observations strongly suggest a common genetic pathway in the development of TEs, spiradenomas, and CCs, and it will be interesting to examine DNA from these skin tumors for PTCH alterations. It is tempting to speculate that the genes altered in these tumor types will play a role in the SHH-PTCH signaling pathway.

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References


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