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Involvement of *PTCH* gene in various noninflammatory cysts

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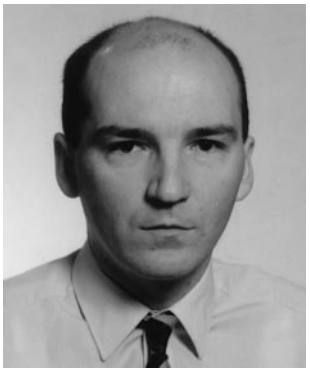

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Abstract Constitutional hemizygous inactivation of *PTCH*, the *Shh* signaling pathway gene that moderates the signal, manifests itself as nevoid basal cell carcinoma syndrome or Gorlin syndrome, a condition variably characterized by a number of developmental disorders and malformations, and by predisposition to some malignancies, basal cell carcinoma in particular. Loss of heterozygosity for the *PTCH* region was found several years ago in the epithelial lining of odontogenic keratocysts, the cyst type with highly increased incidence in nevoid basal cell carcinoma syndrome. This finding confirmed the expectations that the gene responsible for the syndrome would have a decisive role in the genesis of these cysts even when they are not syndrome related. Suggestive temporal distribution of *Shh* signaling, recently observed during tooth development, lead us to investigate *PTCH* association with dentigerous cysts, the other major noninflammatory cyst of odontogenic origin. We report here that *PTCH* appears to be inactivated in dentigerous cysts, suggesting that it is responsible for their genesis as well. More generally, if our similar observations of incomplete heterozygosity in this region for dermoid cysts can be interpreted as loss of heterozygosity, *PTCH* alterations may prove to be a necessary, and perhaps the initiating event, in formation and growth of various noninflammatory cysts. This would be consistent with our view that local *PTCH* inactivation can, under favorable circumstances, lead to persistent though not by itself truly aggressive cell proliferation.

Key words *PTCH* gene · Loss of heterozygosity · Dentigerous cysts · *Shh* signaling · Noninflammatory cysts

Abbreviations *DC:* Dentigerous cysts · *LOH:* Loss of heterozygosity · *NBCCS:* Nevoid basal cell carcinoma syndrome · *OKC:* Odontogenic keratocysts · *PCR:* Polymerase chain reaction · *RC:* Radicular cysts

Introduction

Underlying genetic alterations are most readily suspected for disorders which have an increased incidence in heritable syndromes. Nevoid basal cell carcinoma syndrome (NBCCS) or Gorlin syndrome is a heritable condition [1] associated with constitutional hemizygous inactivation of the recently cloned *PTCH* gene [2], which is a human homologue of *ptc*, *Drosophila* segment polarity gene (patched). The gene appears to have a tumor suppressor role at least for some of the malignancies associated with the syndrome, as has been most convincingly demonstrated for basal cell carcinoma [3].

Even before *PTCH* was cloned, loss of heterozygosity (LOH) had been reported in the 9q22.3 region (to which the gene was mapped) for odontogenic keratocysts (OKC) [4], a lesion of the jaws with highly increased incidence in NBCCS. This finding confirmed the expectations that the gene responsible for the syndrome would have a decisive role in the genesis of these cysts even when they are not syndrome related. The LOH in NBCCS-related and sporadic OKC was used for speculation about the mechanisms through which the suspected gene might be involved in a number of developmental disorders and malformations variably found in the syndrome. In particular, it led to the suggestion that the genesis of OKC may be explained by the two-hit hypothesis proposed by Crosby et al. [5] for a certain class of developmental malformations, which is similar to the better known Knudson's [6] hypothesis for tumor suppressor inactivation in carcinogenesis.

The idea can now be examined in some more detail in the context of *Shh* signaling, through which the *PTCH* gene has been shown to play an important role in development and organogenesis [7]. Furthermore, several recent accounts of *Shh* signaling have lead us to investigate other developmental cysts for *PTCH* alterations.

More than half of all jaw cysts in adults are of inflammatory origin, and therefore unlikely to be associated with genetic alterations. OKC belong to the next largest group, that of odontogenic developmental cysts, which are believed to arise as hamartias from epithelial tissues that normally are or could be involved in tooth development [8]. In addition to OKC, the other major subgroup is that of dentigerous cysts (DC), which actually have a somewhat greater incidence in the general population than do OKC. Although OKC and DC share some common features (which is reflected in notorious clinical difficulties with differential diagnosis), they originate from distinctly different subtypes of odontogenic epithelium, belonging to different stages of tooth development [9].

OKC are clinically aggressive lesions which develop from dental lamina or its remnants (glands of Serres) [10], and perhaps can also arise from extensions of basal cells from the overlying oral epithelium [9, 11]. Even before LOH findings, the proliferative activity of the epithelial lining was thought to play a significant role in OKC expansion and possibly in recurrence [12]. Proliferation status of keratocysts in Gorlin syndrome is higher than in sporadic cases [13], consistent with their early manifestation and their high incidence in the syndrome.

DC are odontogenic cysts that develop around the crown of an impacted (unerupted) tooth by expansion of its follicle. This happens to only about 1% of impacted teeth [14]. The lining of DC develops from reduced enamel epithelium which envelops the crown before eruption. Although mitotic rates in DC epithelial lining appear to be lower than in OKC, dentigerous lesions several centimeters in diameter have been reported to develop within 3–4 years [15]. This is comparable to the growth rate of 2–14 mm per year that has been estimated for OKC [16].

Our decision to examine DC for *PTCH* alterations was based on suggestive temporal distribution of *Shh* signaling during tooth development. Expression of *Shh* in odontogenic epithelium was reported first for the early stage of tooth germ initiation [17] and later in the enamel knot [18]. These two stages of tooth formation can be associated approximately with the respective epithelial tissue forms implicated as possible precursors for OKC and DC. Therefore we hypothesized that both types of cysts are induced by malfunctioning of the same mechanism.

The simplest way to test this hypothesis was to screen DC for LOH in the *PTCH* region. Along with DC, we included two control groups of equal size. One group consisted of OKC and was intended for comparison of LOH incidence. For the other control we selected radicular cysts (RC), a major subgroup of inflammatory cysts, since they are also of odontogenic origin and develop a true epithelial lining. It is generally agreed that their linings are derived from the rests of Malassez, remnants of the final form of odontogenic epithelium, which remain in the periodontal ligament after tooth growth has been completed [9]. It is not yet clear how these cell rests are stimulated to proliferate, but it has been hypothesized that some product of a dead and often infected pulp initiates the process.

In addition, we decided to include in the LOH screening a nonodontogenic type of developmental cysts. Previous investigations of NBCCS features related to the ovaries lead us to select ovarian dermoid cysts. Dermoid cysts are generally believed to arise from primitive germ cells, not only in the ovaries and male gonads but also anywhere in the organism where these cells may have accidentally migrated during early development [19].

Materials and methods

Jaw cyst specimens in paraffin blocks were obtained from 52 patients: OKC (n=15) including cases associated with NBCCS (n=5), DC (n=19), and RC (n=20). These were from the Department of Pathology, School of Medicine, University of Zagreb, Croatia; School of Dental Medicine, University of Zagreb; and Division of Oral Surgery and Pathological Institute, University of Wurzburg, Germany. The cysts were evaluated histologically by a pathologist (S.M.). Thick sections (5–25 μm) were cut for microdissection and DNA extraction. All cyst types have very thin and fragile lining, and some samples were not suitable for microdissection. Only well defined sections were used, avoiding areas of

Table 1 Screening of various cyst types for LOH (loss of heterozygosity *DC* dentigerous cysts, *OKC* odontogenic keratocysts, *RC* radicular cysts, *ODC* Ovarian dermoid cysts, *Het* clearly heterozygous, – not informative)

	Age (years)	Sex	Marker			
			D9S196	D9S287	D9S180	D9S127
DC						
1	69	M	Het	Het	Het	LOH
2	29	M	–	LOH	LOH	–
3	40	F	Het	Het	Het	–
4	36	F	–	Het	LOH	–
5	57	M	–	Het	Het	–
6	40	M	Het	Het	Het	Het
7	34	M	–	Het	Het	Het
OKC						
1	13	M	–	–	Het	Het
2	34	M	–	LOH	Het	–
3	69	F	–	–	Het	Het
4	31	M	Het	LOH	–	LOH
5	74	F	–	–	LOH	–
6	20	F	Het	–	LOH	Het
7	34	M	Het	Het	Het	Het
RC						
1	37	F	–	Het	–	Het
2	36	F	Het	Het	–	Het
3	26	M	–	Het	Het	Het
4	30	M	–	Het	Het	Het
5	37	M	–	Het	–	Het
6	66	F	–	Het	–	Het
7	42	M	Het	Het	–	Het
ODC						
1	24	F	Het	Het	Het	Het
2	43	F	–	–	–	–
3	39	F	–	LOH	–	Het
4	30	F	–	–	–	Het
5	53	F	LOH	LOH	–	Het
6	29	F	Het	LOH	–	Het
7	28	F	–	LOH	–	Het

Table 2 Primer pairs used in this study

Polymorphic marker	Locus	Amplimer size (bp)	Primer sequence
D9S127	9q31	149–159	5-AGATTGATTGATACAAGGATTTG 3′-CCCTCAAATTTGCTGCTGTCTAT
D9S180	9q22.3	220–230	5′-AGCTATTTTTGGGGGCTGAG 3′-CAGTGGTTTGGAATCGAACC
D9S287	9q22.3	168–180	5′-AGGATGCTCCTCACGC 3′-ACCACTACATTGTTCAAGGG
D9S196	9q22.3	254–260	5′-GGGATTACACCTCAAAACCA 3′-ACCACACTGCGGGACTT

folding and irregularity. Finally, seven cysts of each type were selected for LOH screening.

Selected DC (Table 1) displayed typical variations in appearance: cysts 1, 6, and 7 were surrounded by squamous epithelium and fibrosclerosis; cyst 2 had well expressed basal membranes and weak nonspecific inflammation; cyst 3 contained chronic ruptures and cholesterol granuloma; cyst 4 had no keratin; and cyst 5 had pronounced collagen sheets. None of them showed any sign of malignancy. The two control groups (OKC and RC; Table 1) were also selected to be representative of the larger samples. RC were fully or partly lined with nonkeratinized, stratified squamous epithelium supported by chronically inflamed fibrous tissue capsule and were associated with apices of nonvital teeth. Ovarian dermoid cysts (Table 1) were obtained as fresh tissue with accompanying blood samples from the Department of Obstetrics and Gynecology, School of Medicine, University of Zagreb. The samples were evaluated by a pathologist (S.O.), and showed no evidence of malignancy.

DNA extraction from dissected tissue

Archival, paraffin-embedded tissue was stained in hematoxilin/eosin and used as a control for microdissection of unstained paraffin embedded tissues slices. Microdissected paraffin-embedded cyst lining was collected free of surrounding tissue (1×1×2 mm) into a

500-µl microcentrifuge tube with 36 µl digestion buffer (0.9% Tween 20, 0.9% Triton X-100, 5 mM EDTA, 2 mM DTT, 10 mM TrisHCl, pH 7.5) and 4 µl proteinase K, 20 mg/ml (Boehringer-Mannheim). Digestion solution was overlaid with mineral oil, centrifuged briefly to ensure submersion of the tissue flakes in the digestion buffer and incubated at 65°C overnight. Then the tubes were boiled for 10 min [20]. The DNA was extracted by the standard phenol-chloroform method [21]. Microdissection and polymerase chain reaction (PCR) amplification of material from paraffin-embedded specimen was performed on unstained tissue slices. DNA was isolated from dermoid tissue by the standard phenol-chloroform method [21], and constitutional DNA was obtained from peripheral blood leukocytes. The red blood cells were lysed in a buffer containing 1.6 M NH_4Cl, 0.1 $MKHCO_3$ and 1 mM EDTA, pH 8. DNA of the leukocytes was extracted by the guanidine hydrochloride method.

Polymerase chain reaction

PCR analysis was performed in 50-µl volumes containing 100 ng template DNA or crude extract from paraffin-embedded tissue prepared after microdissection; 200 µM dNTPs; 1.5 mM $MgCl_2$; 0.25 mM spermidine; 10 pM of each primer; 1 µl ^{32}P dCTP (Amersham); 1.25 U *Taq* polymerase (Promega) and 2.5 U *Taq* Extender (Stratagene) in Promega buffer (10 mM TrisHCl, pH 9; 50 mM KCl, 0.1% Triton X-100). An Ericomb Dual Block thermocycler was programmed with the following parameters for 25–35 cycles [22]:

- D9S287: 98°C for 5 min plus 35× (95°C for 30 s; 55°C for 30 s; 72°C for 40 s) plus 72°C for 10 min
- D9S196: 96°C for 5 min plus 30× (94°C for 45 s; 55°C for 45 s; 72°C for 1 min) plus 72°C for 10 min
- D9S127: 35× (94°C for 1 min, 56°C for 1 min, 72°C for 2 min) plus 72°C for 5 min
- D9S180: 35× (94°C for 30 s, 55°C for 30 s, 72°C for 40 s) plus 72°C for 10 min

Polymorphic markers from NBCCS region

Table 2 displays the sequences of the primer sets used. Of these four markers, three belong to the 9q22.3 region (D9S196 on the proximal side), while D9S127 flanks it from the distal side (in 9q31). Deletions throughout the 9q22–9q31 region have often been found in tumors of Gorlin syndrome patients [23, 24, 25, 26], and D9S127 has been an informative polymorphic marker for NBCCS-related disorders, although it is not nearly as close to *PTCH* gene as the other three markers. Contribution from this marker to LOH score in our screening is comparable to that in other LOH studies of the syndrome. By its inclusion, "the NBCCS region" extends over more than 8 cM [4], but all the genes mapped to this interval (e.g., *XPAC*, *FACC*, *ESS1*, *ZN169*) [22, 27], except the *PTCH* gene, have generally been excluded from linkage to NBCCS [2].

Data analysis

From each PCR reaction 5 µl was added to 15 µl of loading buffer (95% deionized formamide; 20 mM EDTA; 0.05% bromphenol blue; 0.05% xylene cyanol), heated to 95°C for 2 min and electrophoresed in 6–8% denatured gel at 1750 V or 8–12% native gel at 500 V. Gels were autoradiographed for 24–36 h at –20°C or silver stained [28]. Allelic loss (denoted as "LOH" in the Table 1) was scored when DNA in surrounding normal tissue was heterozygous

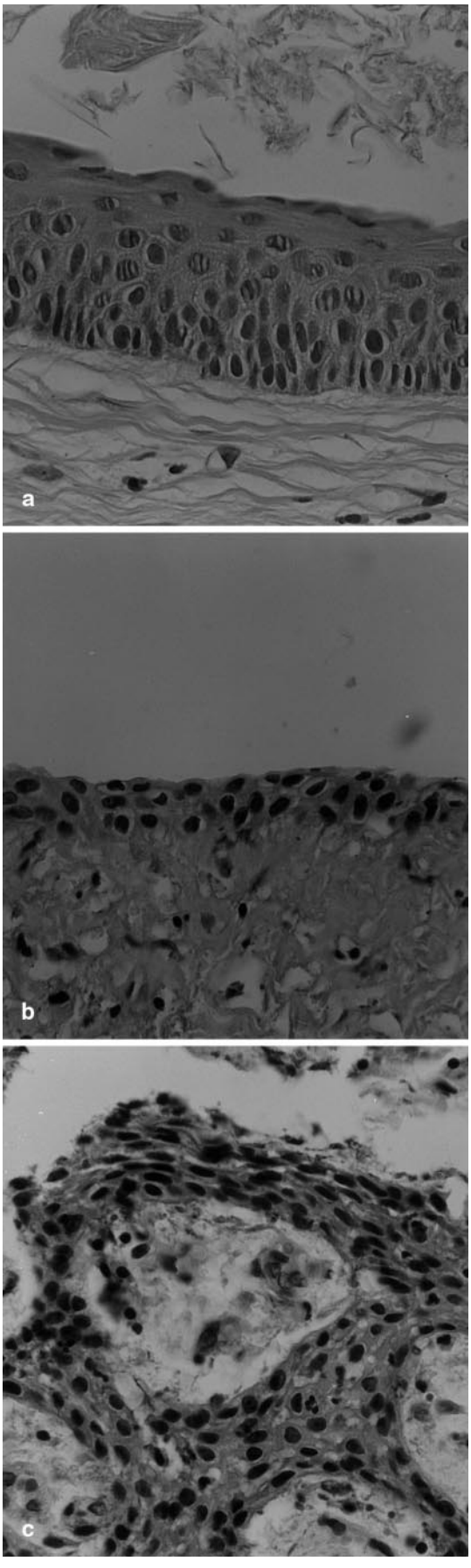

Fig. 1a–c Histology of jaw cysts; hematoxylin and eosin stain, ×200. **a** An odontogenic keratocyst. **b** A dentigerous cyst. **c** A radicular cyst

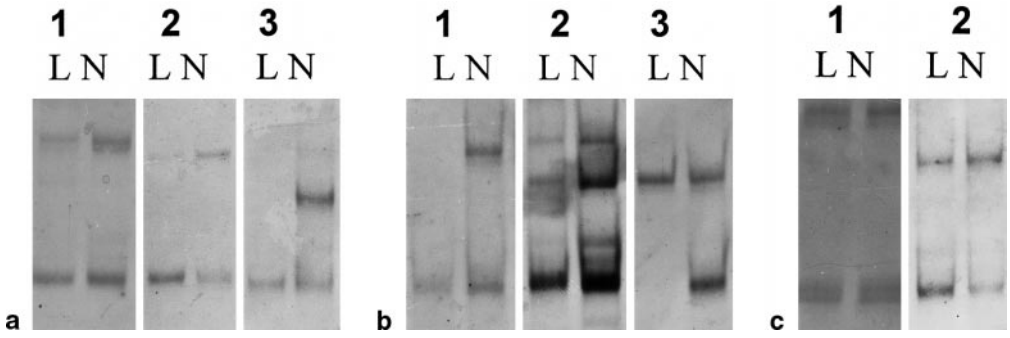

Fig. 2a–c Illustration of LOH analysis at NBCCS region; several cases from Table 1. Nonradioactive PCR, silver stained gels; *L* cyst lining; *N* surrounding tissue. **a** Odontogenic keratocysts for polymorphic marker D9S180. *1* – Patient 4; *2* – patient 5; 3 – patient 6. **b** Dentigerous cysts for polymorphic marker D9S180. *1* – Patient 2; *2* – patient 3; *3* – patient 4. **c** Radicular cysts; patient 3. *1* D9S287; *2* D9S180

and one of the expected bands in DNA from the cyst's lining was completely absent or markedly reduced in intensity ("het" denotes clear heterozygosity, while "–" means not informative).

Results

The results presented in Table 1 confirm our expectations that *PTCH* would be implicated in DC in a similar manner as in OKC. Clear LOH was scored in three DC and in four OKC, which is about one-half of the screened cysts in each group. Also, one cyst in each group showed LOH for two markers, and, similarly, each group had only one cyst clearly heterozygous for all markers used. No LOH was scored with D9S196 in either of these two groups, and this marker was most uninformative. D9S127 scored one LOH in each group, with several inconclusive readings, most of them in the DC group. OKC showed more LOH for D9S287 (two versus one in DC), and each group had two LOH scores for D9S180. While the LOH observed in OKC was consistent with the previously published data [4], this is the first evidence of *PTCH* inactivation in DC. From the relatively high percentage of LOH, *PTCH* alterations may be inferred for all DC and OKC in the screened arrays. Therefore a preliminary conclusion can be drawn, even before further analysis of genetic material is undertaken, that *PTCH* is inactivated in both types of developmental jaw cysts. On the other hand, RC gave no indication that the gene should be implicated in inflammatory cysts. Table 1 shows that overall LOH for dermoid cysts is similar to DC and OKC results. LOH was found in four of seven screened cysts, in one of them for two markers, and only one cyst was clearly heterozygous for all markers used. Nearly all cases of LOH were scored for D9S287, while D8S180 gave mostly inconclusive readings, but D9S127 showed high heterozygosity. Again, this would generally suggest that *PTCH* did not have normal alleles in any of the screened cysts. Figure 1 illustrates histology of the three types of jaw cysts under consideration, while Fig. 2 illustrates LOH for one of the polymorphic markers in OKC and DC, as well as heterozygosity characteristic for RC.

Discussion

The LOH observed in the epithelial lining of DC suggest that a decisive initiating event in their development, just as in development of OKC, is *PTCH* inactivation in a progenitor epithelial cell from which the whole lining is later cloned. Before such an hypothesis can be taken seriously, however, the difference in the reported incidences of OKC and DC in NBCCS must be explained.

The high incidence of OKC has been attributed to the increased likelihood of *PTCH* inactivation in NBCCS individuals, where only a second hit in a potential cyst progenitor cell is needed due to the constitutional haploinsufficiency. The question must be raised, however, why only OKC have been reported as a characteristic feature of the syndrome, if DC are initiated through a similar mechanism, simply in a different type of progenitor cell. This might be explained by the requirement that DC develop around impacted teeth. Such teeth are relatively rare both in the general population and in NBCCS individuals, and become a limiting condition for DC incidence in NBCCS. Even if DC accompanied every impacted tooth in the syndrome (instead of 1% as in the general population), this would hardly be noticed due to the very small number of individuals affected by the two conditions, NBCCS and impacted teeth.

Similar preconditions may also preclude pronounced expression of some other *PTCH* related aberrations in NBCCS. Therefore we did not think that a search for *PTCH* alterations should necessarily be confined to disorders for which markedly higher incidence has been reported in the syndrome.

For dermoid cysts we believe that results in Table 1 indicate LOH for *PTCH* region rather than a haploid segment with an occasional crossing-over (as hypotheses of arrested meiosis would suggest [19]), due to relatively high and alternating incidence of heterozygosity for such a region. However, as these cysts have not been associated with NBCCS [2], a plausible reason for their limited incidence must be identified. A distinguishing feature for progenitor cells is that they normally should *not* be prone to mitotic division. This allows the hypothesis that aberrant germ cells can be found in only a small proportion of both the general and the NBCCS populations which could be induced to proliferate autonomously. With such a limiting

hypothesis, paralleling the precondition of unerupted teeth for DC, the NBCCS-predisposing potential for dermoid cysts would be equally hard to notice. Admittedly, this would introduce another (and unknown) "aberration" before *PTCH* can be implicated in genesis of these cysts.

Even if *PTCH* inactivation alone is not sufficient, our results suggest that it is a necessary condition for development of not only keratocysts, but also of other noninflammatory cysts. Such conclusion is particularly convincing in the case of DC, where *Shh* signaling in the enamel knot may be taken as a supporting indication of *PTCH* involvement in regulation of the epithelial tissue proliferation.

Developmental cysts and Shh signaling

In the *Shh* signaling pathway PTCH protein normally prevents transmembrane protein Smo from expressing the pathway target genes. When and if Shh protein reaches the cell membrane to relieve Smo, *PTCH* eventually prevents overexpression of these target genes – being one of them and therefore producing new protein to suppress Smo [29]. Details of the process and roles of all the target genes in the pathway are far from being fully understood. Nevertheless, their functions can be assumed to include a programmed and limited cell proliferation during intensive *Shh* signaling. For example, it has been demonstrated [30] that the addition of exogenous Shh protein directly into or adjacent to tooth germs results in abnormal epithelial invagination, mimicking cell proliferation at the early stage of tooth initiation.

Similarly, nonprogrammed and unlimited cell proliferation in cystic growth could be attributed to the same pathway target genes when their overexpression is made possible by an accidental *PTCH* inactivation in a potential progenitor cell. Even a small quantity of Shh arriving at the cell membrane thereafter might be sufficient to trigger the process of proliferation. Once the overexpression of the pathway genes has caused the first cell division, and the new cells replicate the impaired *PTCH*, which cannot balance their normal content of Smo, the cells continue to divide without further Shh stimuli.

It should be noted that neither cysts nor less benign instances of cell proliferation attributable to *PTCH* inactivation, such as basal cell carcinoma, are very dramatic and aggressive processes. Therefore some other predisposing circumstances may also be required for their onset and/or propagation. All the putative progenitor cell types for the cysts described here, in which *PTCH* was impaired, are in some way associated with a potential for proliferation in normal development (of a tooth or of a whole organism), so that the cystic growth is not an extremely distant aberration from the normal course for which the cells were programmed. Truly aggressive malignant growth probably requires involvement of some other genes. (In ovarian dermoid cysts, for instance, malignant transformations occur in about 2% and may be associated with p53 alterations [31].)

Cyst genesis versus developmental malformations

It is possible to speculate that the role of constitutional haploinsufficiency in NBCCS is not limited to predisposing for full *PTCH* inactivation but manifests in a lower level of the gene expression. If this lower expression actually does occur, it clearly has no dramatic effects on the whole organism since NBCCS individuals develop almost normally. Still, it may be possible that PTCH occasionally does not build up fast enough after some *Shh* signal to prevent a few nonprogammed cell divisions, but the daughter cells would not continue to proliferate indefinitely (as in the case of full *PTCH* inactivation). Such an accident could not explain cystic growth, but it might be a plausible initiating event for some developmental malformations found in NBCCS, provided that it occurs early in development. The only likely direct impact of constitutional *PTCH* haploinsufficiency on cystic growth could be their higher proliferation status in NBCCS than in sporadic cases.

While it is obvious initiating events for developmental malformations must occur early in development, it is not clear that the same requirement should be associated with cyst initiation. Although their incidence decreases at advanced age, all the cyst types analyzed here can occur at various times in life. There is no convincing evidence that cystic growth is triggered during embryonic or fetal development. On the contrary, their growth rates suggest that they become clinically manifest within several years after the proliferation has begun. If the immediate triggering event for this proliferation is assumed to be an *Shh* signal reaching a progenitor cell (probably intended for the adjacent tissue), *PTCH* inactivation in this cell could have happened at any time before the Shh protein arrived at its membrane.

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