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Unbalanced overexpression of the mutant allele in murine *Patched* mutants

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Inherited mutations of *Patched* (*PTCH*) in the nevoid basal cell carcinoma syndrome (NBCCS) lead to several developmental defects and contribute to tumor formation in a variety of tissues. *PTCH* mutations have been also identified in sporadic tumors associated with NBCCS including basal cell carcinoma (BCC) and medulloblastoma. Mice heterozygous for *Ptch* recapitulate the typical developmental symptoms of NBCCS and develop rhabdomyosarcoma (RMS) and medulloblastoma. *PTCH* is assumed to act as a tumor suppressor gene although inactivation of both alleles has been demonstrated only in a fraction of tumors. We have investigated the status of *Ptch* in RMS of heterozygous $Ptch^{neo67/+}$ mice. Although the wild-type *Ptch* allele was retained in tumor tissue, the high levels of *Ptch* mRNA in these tumors result from overexpression of the mutant *Ptch* transcript. Our results suggest that the wild-type *Ptch* allele might be selectively silenced in RMS tissue or, alternatively, that haploinsufficiency of *Ptch* is sufficient to promote RMS formation in mice.

Introduction

NBCCS is an autosomal dominant disorder characterized by a combination of developmental defects with a predisposition to tumor formation (1). The clinical findings include generalized overgrowth of the body, closure defects of the neural tube and skeletal abnormalities such as bifid or missing ribs and polydactyly. Tumors present in patients with NBCCS are BCC, medulloblastoma, RMS, meningioma and fibroma (2). NBCCS results from germ-line mutations of the human homologue of the *Drosophila* segment polarity gene *PTCH* (3,4). *PTCH* is widely assumed to be a tumor suppressor gene. Tumor suppressor genes normally exert a negative control on cell growth and the paradigm is that inactivation of both alleles is required for tumor formation (5). Inactivation of tumor suppressor genes frequently occurs through point mutations of one allele and loss of the other allele by nondisjunction, mitotic recombination or by a *de novo* deletion. These mechanisms result in loss of heterozygosity (LOH) for polymorphisms in the chromosomal vicinity of the tumor suppressor gene (6). However, mutational inactivation of both alleles of *PTCH* has been detected only in a subset of tumors associated with its deficiency. LOH at the *PTCH* locus was reported in >50% of sporadic BCCs whereas SSCP analysis of tumor DNA identified mutations in the *PTCH* gene in only 30% of these tumors (7). The technical limitations of the SSCP technique and the existence of mutations in introns or regulatory elements not yet examined have been discussed as possible reasons for the failure to detect more mutations (8).

PTCH is predicted to contain 12 transmembrane-spanning domains and two large extracellular loops and thus does not resemble any known tumor suppressor gene (for review see ref. 9). Within the plasma membrane PTCH forms a receptor complex with its signaling partner Smoothened (SMO) that transduces hedgehog (HH) signaling (10,11). A main impact of this signaling pathway is on the control of cell differentiation and proliferation. A physiological activation of this pathway occurs during embryogenesis and is induced by HH. Binding of HH to PTCH suspends the inhibition of SMO, which leads to signal transduction and induction of target genes. Mutational inactivation of *PTCH* results in a pathological activation of this signaling pathway and is characterized by increased levels of *GLI 1* and *PTCH* mRNA (for review see ref. 12).

We have recently established a murine model of *Ptch* hemizygosity by replacement of exons 6 and 7 of the *Ptch* gene by a neomycin resistance cassette (13). Heterozygous $Ptch^{neo67/+}$ mice develop medulloblastoma and RMS, two frequent childhood tumors (13,14). As in human tumors associated with *PTCH* mutations, mutational inactivation of murine *Ptch* resulted in a pathological activation of its signaling pathway with consecutive expression of high levels of *Gli1 and Ptch* mRNA (12,13).

To determine if deletion of both copies of *Ptch* is a prerequisite in RMS development in heterozygous $Ptch^{neo67/+}$ mice, we examined whether the normal *Ptch* allele was deleted or inactivated by a mutation in these tumors. Furthermore, we tried to elucidate if both alleles contributed to the high *Ptch* mRNA expression previously found in RMS of these mice. Finally we examined the status of *PTCH* in human BCCs, allowing us to compare the role of *PTCH* in human *PTCH*-associated tumors with its role in murine *Ptch*-associated tumors.

Materials and methods

Animals

The $Ptch^{neo67/+}$ mutation, originally established in 129Sv/Pas-derived MPI II ES cells, has been maintained on CD-1 or C57BL/6 backgrounds for 10 generations. The F10 progenies were scored for the presence of tumors. Cumulative tumor incidences were calculated taking into account confounding causes of death that appeared during the time course of the experiment.

Abbreviations: BCC, basal cell carcinoma; LOH, loss of heterozygosity; NBCCS, nevoid basal cell carcinoma syndrome; RMS, rhabdomyosarcoma.

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Table I. Oligonucleotides for amplification of murine *Ptch*

Primer	Sequence (5′–3′ orientation)
1	GGG AGG GGA TTT CAG CAG AAT GTT (PstK)
2	CTG CCT GTT ATG TGG TTC AAA CCG (mPTC11f)
3	AGT GCC AGC GGG GCT GCT AAA (neol)
4	ATG GCC TCG GCT GGT AAC GCC (mPTC1)
5	GTA CCC ATG GCC AAC TTC GGC TTT (mPTC11R)
6	AAA GCC GAA GTT GGC CAT GGG TAC (mPTC11)
7	AAG GGA GGC TGA TGT CTG GAG T (mPTCgen6R)
8	ACT CCA GAC ATC AGC CTC CCT T (mPTCgen6F)
9	TCT CCT CAC ATT CCA CGT (mPTC22R)
10	TAC AGT CCG GGA CAG CAT ACC (mPTC10)
11	ATA CTT CTT TGC CGT CCT GGC (mPTCgen4F)
12	TTT AGG CCA TTG GCT GGA GAC A (mPTCgen4R)
13	AGG TAA GCC TCC TTT ACG GT (ex6/7F)
14	AGT GGC ATT CTT GAC GGT AC (ex6/7R)
15	CAA TGG CTA CCC CTT CCT GTT CT (mPTCgen1F)
16	GAG GAA GAC TGC GCA CAC TAG AAA (mPTCgen1R)
17	ACC GTT GAG CTC TTT GGC ATG ATG (mPTCgen2F)
18	GCC AAC AGA TGC AAT CAG GA (mPTCgen2R)
19	ACA AGA ACC ACA GGG CTA TGC TC (mPTCgen3F)
20	TAC ACC CAG CAG AGT GGA CAC A (mPTCgen3R.2)
21	TGT CTC CAG CCA ATG GCC TAA A (mPTCgen5F)
22	ACC CAT TGT TCG TGT GAC CAG GA (mPTCgen5R)

Samples

RMS and non-cancerous skeletal muscle (SM) taken as a reference were excised from heterozygous *Ptch*$^{neo67/+}$ mice maintained on a CD-1 background. Human nodular, solid BCCs were obtained at surgery. All tissues were snap-frozen and stored in liquid nitrogen for later extraction of RNA and DNA. The identity of the tumors as murine RMS or human BCCs was confirmed on haematoxylin and eosin (H&E) stained sections by a trained pathologist.

Tissue preparation and microdissection

Murine RMS were examined prior to extraction of DNA or RNA on H&E stained sections and only tumor tissue that consisted of a pure population of 90–95% of tumor cell was used to obtain tumor DNA or RNA samples.

BCCs were sectioned at 10 µm in a cryostat, mounted on glass slides and non-neoplastic epidermis and neoplastic tissue was microdissected using the Palm Laser-MicroBeam System (P.A.L.M., Bernried, Germany). After selecting the cells of interest, adjacent cells were photolysed by the microbeam. At least 1000 selected cells were picked from the slides using conventional sterile needles and transferred into reaction tubes containing 200 µl STE buffer (20 mM Tris–HCl pH 8.0, 10 mM NaCl, 10 mM EDTA, 0.5% w/v sodium dodecyl sulfate and 1 mg/ml proteinase K) for isolation of DNA or 200 µl guanidine isothiocyanate (GITC) Solution (Life Technologies, Rockville, MD, USA) and 1.6 µl β-mercaptoethanol for isolation of total RNA.

Isolation of DNA and total RNA

DNA from fresh frozen microdissected human BCCs, from macrodissected murine RMS and from murine tails was isolated from STE buffer by phenol–chloroform extraction according to standard procedures.

Total RNA of fresh frozen murine RMS was isolated using Trizol (Life Technologies) and digested with DNase (Roche Diagnostics, Mannhein, Germany) according to the manufacturer's instructions.

Total RNA from microdissected BCC tissue was lysed in GITC buffer and isolated by addition of 0.1 volume 2 M sodium acetate (pH 4.0), 1 volume water-saturated phenol and 0.3 volumes chloroform followed by precipitation with an equal volume of isopropanol in the presence of 10 µg carrier glycogen. The RNA pellet was washed once in 70% ethanol, air-dried and resuspended in RNase-free water.

Northern blot analysis

For analysis of expression of wild-type and mutant *Ptch* transcripts of *Ptch*$^{neo67/+}$ heterozygous mice 10 µg of total RNA isolated from RMS and other non-neoplastic tissue of *Ptch*$^{neo67/+}$ heterozygous CD-1 mice were electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane and hybridized with ^{32}P-labeled probes according to standard protocols. The cDNA probes used for detection of mutant or wild-type *Ptch* transcripts corresponded to nucleotides 705–1011 and 3509–4279 of the murine *Ptch* cDNA, respectively (amplified with primer pairs 13/14 and 21/9, Figure 2; Table I). Filters were washed and exposed to a film at –80°C.

Table II. Oligonucleotides for amplification of human *PTCH*

Primer	Sequence (5′–3′ orientation)
12F[a]	GAC CAT GTC CAG TGC AGC TC
12R[a]	CGT TCA GGA TCA CCA CAG CC
23F[a]	CCC TTC TAA CCC ACC CTC AC
23R2	AAA CAG GCC GTG GTC AGT CTC A
Ex12F	AAT CCC TTT TGA GGA CAG GAC C
Ex12R	GTC CTC GCG TCG ATA TAA ATC C
Ex23F	GTG ATC GTG GAA GCC ACA GAA A
Ex23R	GCC AGA ATG CCC TTC AGT AGA A
Ex7F	TTG ATT GTG GGT GGC ACA GT
Ex8R	GCT TGG GAG TCA TTA ACT GGA AC
Ex7/Ex8	ACT GGA AAA CTC GTC AGC GCC CAT

[a]The primer sequences are the same as described in Xie *et al.* (20).

Reverse transcription

Two µg of total RNA extracted from murine RMS or SM were reverse-transcribed in a 20 µl reaction using random hexamers and SuperScriptII reverse transcriptase (Life Technologies) according to the manufacturer's instruction.

RNA isolated from microdissected BCC samples was reverse-transcribed in a final volume of 20 µl using SuperScriptII reverse transcriptase (Life Technologies) in the manufacturer's buffer containing 1 mM dNTPs, 10 U of RNase inhibitor (Life Technologies), 250 ng of random hexamers (Roche Diagnostics) and 500 ng of oligo(dT)$_{15}$ primers (Roche Diagnostics). After digestion with DNase, the cDNAs were diluted with DNase free water to a total volume of 90 µl and stored at –20°C until use.

Real-time quantitative RT–PCR

Real-time quantitative RT–PCR analysis for human *PTCH* in BCC was carried out using an ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA). The *PTCH*-specific primers spanning exon 7 and 8 of the human *PTCH*-cDNA were Ex7F and Ex8R and the fluorogenic intron-spanning probe was Ex7/Ex8 (Table II). PCR amplifications were carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 10 µl of the diluted BCC cDNA (see reverse transcription), 100 nmol/l of the probe and 300 nmol/l forward primer and reverse primer in a 30 µl final reaction mixture. After 2 min incubation at 50°C, AmpliTaq Gold was activated by incubation for 10 min at 95°C. Each of the 40 PCR cycles consisted of 15 s denaturation at 95°C and hybridization of probe and primers for 1 min at 60°C. Amplification of *TBP* was performed as an endogenous control. The *TBP* primers, the *TBP* probe and amplification conditions are described elsewhere (15). For each experimental sample, the amount of target and endogenous reference was determined from standard curves constructed by fivefold serial dilution of plasmids (1000 pg to 0.32 pg) containing fragments of either human *PTCH* cDNA (target) or human *TBP* DNA (endogenous reference). All data shown are the average of at least two independent experiments.

Loss of heterozygosity (LOH) analysis

LOH analysis of Ptch in murine tumor samples. Analysis of the *Ptch* locus in murine RMS was done by PCR and Southern blot hybridization. The primers 1, 2 and 3 (Figure 2, Table I) were used to amplify a ~650 bp fragment of the wild-type allele (primer 1 and primer 2) and a ~400 bp fragment of the targeted allele (primer 1 and primer 3) from DNA isolated from macrodissected tumor tissue. Loss of the 650 bp fragment in the presence of the 400 bp fragment was considered LOH at the *Ptch* locus. For Southern blot analysis, 15 µg of DNA was extracted from RMS and SM, digested with Xho I and transferred onto a nylon membrane. Preparation of the *Ptch* probe and hybridization were as described previously (13). Loss of the wt-allele (16 kb band in Figure 1b) was considered LOH at the *Ptch* locus.

LOH analysis of PTCH in human tumor samples. Genomic DNA was isolated from microdissected non-neoplastic and neoplastic tissue derived from 10 BCCs. LOH analysis at the *PTCH* locus was performed by amplification and sequencing of exon 12 and exon 23 of the human *PTCH* gene, which hold polymorphic sites at positions 1641, 1665 and 1686 in exon 12 and at position 3944 in exon 23. The primers used for the study were primer pair 12F/12R and 23F/23.R2 for amplification of exon 12 and exon 23, respectively (Table II). Loss of the polymorphic sites in neoplastic tissue in comparison to non-neoplastic tissue was considered LOH at the *PTCH* locus.

Four highly polymorphic microsatellite markers (D9S1820, D9S196, D9S287 and D9S1786) on human chromosome 9 in the vicinity of the

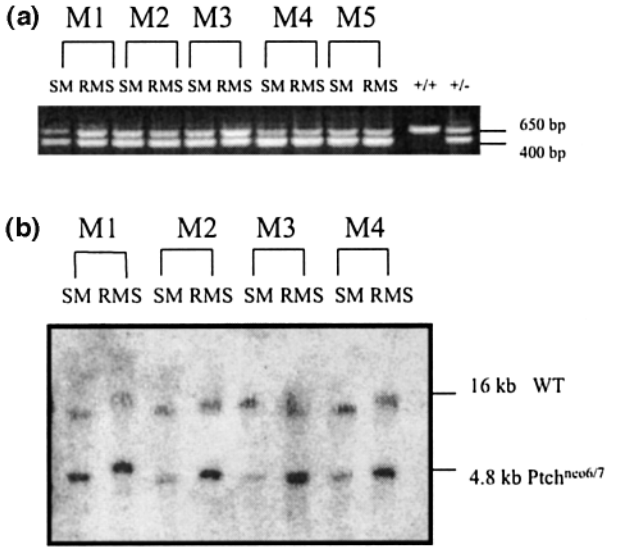

Fig. 1. Screen for loss of heterozygosity in RMS of *Ptch*$^{neo67/+}$ mice. DNA isolated from macrodissected RMS and normal skeletal muscle (SM) from heterozygous *Ptch*$^{neo67/+}$ mice (M1–M5) was analyzed by (**a**) a PCR based assay and (**b**) Southern hybridization. None of the RMS showed loss of the wild-type (WT) *Ptch* allele.

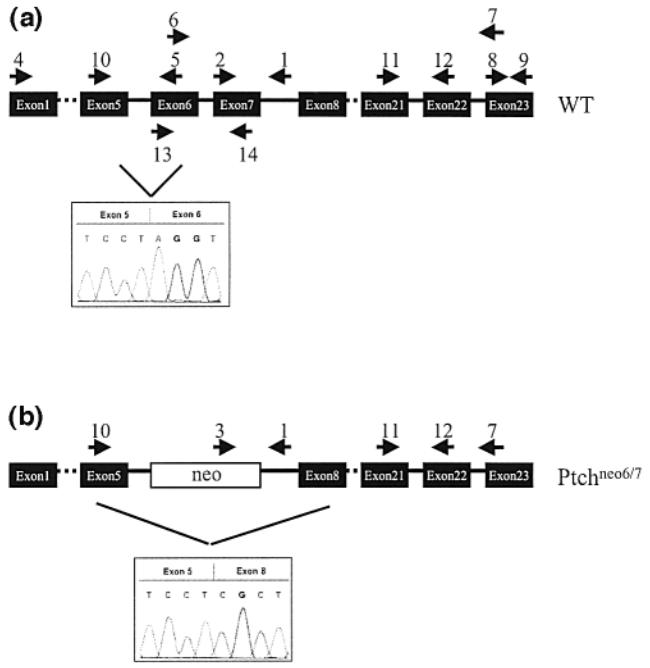

Fig. 2. Splicing of the *neo*-cassette in *Ptch*$^{neo67/+}$ mice. (**a**) Murine wild-type (WT) *Ptch* locus, sequence profile of the wild-type transcript and location of the primers designed for amplification of the wild-type allele (indicated by arrows). (**b**) Mutant (*Ptch*$^{neo67/+}$) *Ptch* locus and sequence profile of the resulting transcript. The *neo*-cassette of the targeted, 129Sv-derived *Ptch*$^{neo67/+}$ allele is excised and exon 5 is spliced together in frame with exon 8 of the *Ptch* gene. The locations of the primers used for amplification of the mutant transcript are marked by arrows. The numbering of exon 21, exon 22 and exon 23 of the murine *Ptch* transcripts are derived from the homologous exons of the human *PTCH* transcript.

PTCH gene were used to confirm the obtained data. The primer sequences of the microsatellite markers were obtained from the Genome Database (http://www.genome.wi.mit.edu). PCR amplification of genomic DNA was performed under standard conditions in a 15 µl reaction mixture with 1.5 mM $MgCl_2$ PCR Buffer, 1.25 mM dNTPs, 20 pmol of each primer (forward primers were fluorescently labeled) and 1.5 U Taq Polymerase. Amplification efficiency was determined by resolving 2 µl of the reaction products on an agarose gel. Equal amounts of the PCR products from normal and tumor DNA were subsequently analyzed using an automated ABI 377 sequencer and the ABI Prism Gene Scan software (Applied Biosystems).

Nucleotide sequencing

All sequencing reactions were performed using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Sequences were analyzed using the Sequencher software (Gene Codes Corp., Ann Arbor, MI).

Sequence analysis of Ptch in murine tumor samples

Primer pairs 4/5 and 6/7 were used to amplify 87% of the wild-type *Ptch* transcript from cDNA isolated from 5 RMS of heterozygous *Ptch*$^{neo67/+}$ mice (Figure 2a, Table I). For analysis of 11% of the remaining 3′-end of the wild-type transcript, genomic DNA was amplified from the same tumors using the primer pair 8/9 (Figure 2a, Table I). The *Ptch* transcript derived from the targeted allele in tumor tissue was amplified with primer pair 7/10 (Figure 2b, Table I).

Nucleotide polymorphisms detected in the wild-type and the mutant transcripts were confirmed on tail DNA isolated from 5 wild-type CD-1 mice (purchased from Charles River) and 1 wild-type 129Sv mouse using the primer pairs 11/12, 15/16, 17/18, 19/20, 21/22 and 8/9 (Table I) for amplification and sequencing.

To quantify the expression of the *Ptch* transcripts in tumor tissue a fragment amplified with primer pair 11/12 from cDNA of RMS was subcloned into the pCRII cloning vector (Invitrogen BV, Groningen, Netherlands). We have excluded by sequencing the existence of polymorphisms between CD1 and 129 mouse strains within regions binding PCR primers to avoid biased amplification of one allele. Ninety subclones were sequenced from both sides using the T7 and Sp6 primers. Inserts originating from either the wild-type or the mutant transcript were distinguished by nucleotide polymorphism at positions 3438 and 3498 of the murine *Ptch* gene.

Analysis of PTCH in human tumor samples

Genomic DNA isolated from microdissected non-neoplastic and neoplastic tissue derived from 10 BCCs was amplified and sequenced with primer pair 12F/12R and 23F/23.R2 (Table II). To determine allelic expression of *PTCH* in tumor tissue, cDNA isolated from neoplastic tissue was amplified and sequenced with the intron-spanning primer pairs Ex12F/Ex12R or Ex23F/Ex23R (Table II) that detect the polymorphic sites in exon 12 or exon 23, respectively. To quantify the level of expression of each *PTCH* allele, the resulting PCR fragments were cloned into the pCRII cloning vector (Invitrogen BV) and the subclones were sequenced using the T7 primer.

Results

No evidence for LOH at the Ptch locus in RMS of heterozygous Ptch$^{neo67/+}$ *mice*

To investigate whether the wild-type *Ptch* allele was deleted in RMS of heterozygous *Ptch*$^{neo67/+}$ mice, PCR amplification was performed on both SM and RMS tissue of 5 *Ptch* mutant mice. The primers 1, 2 and 3 (Figure 2) allow the discrimination between the wild-type and the mutant *Ptch* allele (13). We found that both the wild-type and the targeted *Ptch* allele were present in all RMS and SM examined (Figure 1a). This result was confirmed by Southern blot hybridization of DNA isolated from the respective tissues. Both the 16 kb wild-type *Ptch* and the 4.8 kb *Ptch*^{neo67} fragment were detected in tumor tissue (Figure 1b). Altogether the data suggested that there was no LOH at the *Ptch* locus in tumor tissue.

No evidence for mutations in the wild-type Ptch allele in RMS of heterozygous Ptch$^{neo67/+}$ *mice*

To determine whether the wild-type *Ptch* allele was mutationally inactivated in the 5 tumors of *Ptch*$^{neo67/+}$ mice, we sequenced 87% of *Ptch* wild-type cDNA amplified from the RMS using primer pairs 4/5 and 6/7. A further 11% was amplified from genomic DNA of these tumors using primers 8 and 9 (Figure 2a). No mutations have been detected in the 98% of the wild-type *Ptch* coding region in any of the five tumors examined. Although 26 bp of the C-terminal end of the wild-type *Ptch* allele has not been sequenced in RMS the

Table III. Polymorphisms in genomic *Ptch* sequences from mouse strains CD-1 and 129Sv. The *Ptch* allele derived from strain 129Sv is overexpressed in *Ptch* cDNA isolated from RMS of $Ptch^{neo67/+}$ mice

Position	Primer pair for amplification	CD-1	129Sv	Amino acid change	Allele in RMS
3018 A–G	15/16	A or G	A	Gln (A)–Gln (G)	A
3180 T–C	17/18	T or C	C	Ile (T)–Ile (C)	C
3318 A–G[a]	19/20	G	G	Glu (A)–Glu (G)	G
3438 C–T	11/12	C	T	Thr (C)–Thr (T)	T
3498 G–A	11/12	G	A	Pro (G)–Pro (A)	A
3561 T–C	21/22	T or C	C	Pro (T)–Pro (C)	C
3800 C–A	8/9	C or A	A	Thr (C)–Asn (A)	A
4016 C–T[b]	8/9	C	T	Thr (C)–Met (T)	T

[a]The nucleic acid discrepancy at nucleotide 3318 between the mouse *Ptch* sequence registered in GenBank (Acc. No. U46155) and the *Ptch* sequence obtained from 129Sv and CD-1 was originally described in Wetmore *et al.* (16).
[b]This polymorphism was described in Wetmore *et al.* (16) at position 4015 instead of 4016.

data suggest that the wild-type allele does not harbor mutations in the protein-coding region.

Expression of the mutant allele in tissues of heterozygous $Ptch^{neo67/+}$ mice

Amplification of cDNA from RMS of the same 5 heterozygous $Ptch^{neo67/+}$ mice with primer pair 7/10 (Figure 2) uniformly revealed an aberrant *Ptch* transcript, in which the neo-cassette of the targeted 129Sv-derived $Ptch^{neo67/+}$ allele was excised and exon 5 was spliced together with exon 8 of the murine *Ptch* gene (Figure 2b).

Overxpression of the mutant Ptch allele in RMS of heterozygous $Ptch^{neo67/+}$ mice

In heterozygous $Ptch^{neo67/+}$ mice the mutant $Ptch^{neo67/+}$ allele is derived from 129Sv ES cells. Heterozygous $Ptch^{neo67/+}$ mice crossed onto the CD-1 background contain the targeted 129Sv-derived $Ptch^{neo67/+}$ allele as well as a wild-type CD-1-derived *Ptch* allele. When comparing the nucleotide sequence of the *Ptch* open reading frame derived from the mutant and the wild-type *Ptch* cDNA, several sequence discrepancies between the two transcripts were detected. Since the mutant transcript was derived from the 129Sv mouse strain and the wild-type transcript was derived from the CD-1 mouse strain, tail DNA isolated from 129Sv and CD-1 mice was examined. Our data showed that the variations represented 7 strain-specific polymorphisms. Six of these polymorphisms have been described previously for mouse strains C57BL/6 and 129Sv (16). Four of the polymorphisms showed allelic variance within the CD-1 genome and two of them resulted in amino acid exchanges (Table III). The polymorphisms were used to investigate expression of the *Ptch* alleles in normal and RMS tissue.

RMS of heterozygous $Ptch^{neo67/+}$ mice express elevated levels of *Ptch* RNA (13). To investigate whether the wild-type or the mutant transcript contribute to *Ptch* overexpression in these tumors, both the primer pair 11/12 and the primer pair 8/9 were used to amplify *Ptch* from RMS and corresponding normal muscle tissue. These primer pairs detect the polymorphisms at positions 3438, 3498 and 4016 (Table III). Equivalent levels of both the wild-type and the mutant allele were amplified from genomic DNA isolated from 5 macrodissected RMS (Figure 3a). However, when cDNA of the same tumors was amplified using the same primer pair, only the 129Sv *Ptch* sequence (mutant allele) was detected (Figure 3a). On the contrary, amplification of cDNA derived from the corresponding normal skeletal muscle (SM) or from other non-cancerous

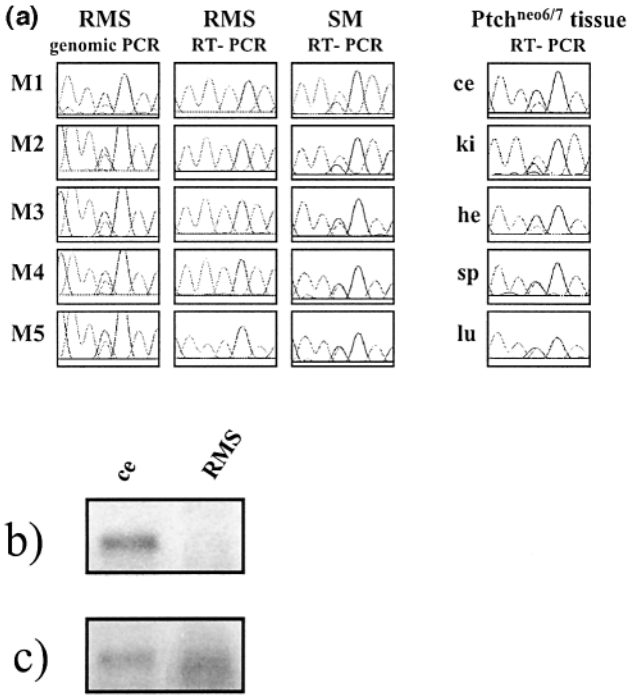

Fig. 3. Overexpression of the mutant *Ptch* allele in RMS of $Ptch^{neo67/+}$ mice. (**a**) Genomic DNA and cDNA of macrodissected RMS and normal skeletal muscle (SM) of heterozygous $Ptch^{neo67/+}$ mice (M1–M5) were analyzed by sequencing of the CD-1-derived (wild-type) and 129Sv-derived (mutant) polymorphism at position 4016 of the murine *Ptch* transcript (accession no. U46155). Genomic DNA extracted from RMS revealed both the CD-1-derived and 129Sv-derived polymorphism, demonstrating retention of both *Ptch* alleles in tumor tissue. However, cDNA extracted from the same tumors expressed only the 129Sv-derived allele. This expression was tumor-specific since cDNA isolated from SM of the same animals expressed both polymorphisms, as did cDNA extracted from other non-cancerous $Ptch^{neo67/+}$ tissues (ce, cerebellum; ki, kidney; he, heart; sp, spleen; lu, lung). (**b,c**) Northern blot analysis of total RNA prepared from RMS and control tissues isolated from a heterozygous $Ptch^{neo67/+}$ mouse. Hybridizations were performed with a probe corresponding to exon 6 and 7 of the murine *Ptch* cDNA, which is deleted in the mutant *Ptch* allele (b) and with a probe corresponding to exons 22 and 23 of murine *Ptch* cDNA (c) that detected a strong signal in RNA isolated from RMS tissue.

tissues of $Ptch^{neo67/+}$ mice showed expression of both *Ptch* alleles (Figure 3a). These data were confirmed by northern blot analysis using two different probes (Figure 3b,c). The probe used in Figure 3b corresponds to exons 6 and 7, which are deleted in the mutated allele. The probe used in Figure 3c

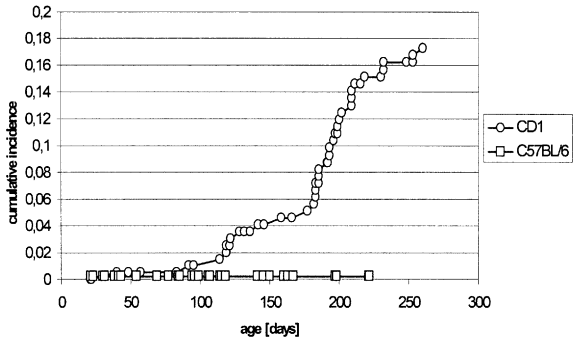

Fig. 4. Incidence of RMS in heterozygous *Ptch*$^{neo67/+}$ mice. Heterozygous *Ptch*$^{neo67/+}$ mice on a mixed 129Sv × C57BL/6 background were bred to wild-type CD-1 or C57BL/6 mice for 10 generation. The F10 progenies were scored for the presence of RMS.

corresponds to exons 22 and 23. On the contrary to a control tissue expressing Ptch (cerebellum), RMS contained no wild-type (i.e. exon 6 and 7-containing) transcripts.

Altogether the data suggested an imbalance of allelic expression of *Ptch* and furthermore showed that the mutant *Ptch* allele is tumor-specifically overexpressed in RMS of *Ptch*$^{neo67/+}$ mice.

To quantify the expression level of the mutant transcript in RMS, one of the fragments derived from amplification of *Ptch* cDNA isolated from RMS using the intron-spanning primer pair 11/12 was subcloned in the pCRII vector and 89 subclones were sequenced from both sides using the T7 and Sp6 primers. Inserts originating from wild-type or mutant transcripts were distinguished by the nucleotide polymorphisms at position 3438 and 3498 as described above. Eighty-seven subclones were derived from the mutant 129Sv allele whereas two subclones were derived from the wild-type CD-1 *Ptch* allele. Thus, the mutant transcript was 48-fold overexpressed in RMS of *Ptch*$^{neo67/+}$ *mice.*

Frequency of RMS of Ptch$^{neo67/+}$ *mice is modified by the genetic background*

At 225 days of age, 15% of heterozygous *Ptch*$^{neo67/+}$ mice on CD-1 background had developed soft tissue tumors histologically confirmed as RMS (Figure 4). Within this period no RMS were detected in animals maintained on the C57BL/6 background. This indicates that the frequency of RMS in heterozygous *Ptch*$^{neo67/+}$ mice is strongly affected by genetic background.

LOH analysis at the PTCH locus in human BCCs

The source of normal DNA of all patients was normal skin microdissected from frozen BCC sections. The polymorphic sites in exon 12 and exon 23 of *PTCH* were informative in seven of 10 patients (Table IV). LOH was detected in tumor tissue of BCCs 1, 2 and 3 whereas tumor tissues of BCCs 4–7 did not exhibit LOH at the *PTCH* locus. Confirmation of the status of the *PTCH* locus was performed by microsatellite analysis using four markers in the vicinity of the *PTCH* gene. In tumors 1, 2 and 3 LOH was detected at least two of the markers flanking the *PTCH* gene. Tumors 4, 5 and 7 showed no LOH at these markers and tumor 6 was not informative (data not shown).

Human BCCs without LOH at the PTCH locus exhibit monoallelic or biallelic overexpression of PTCH

It has been reported that, in contrary to normal skin, essentially all BCCs express high levels of *PTCH* mRNA (7,17). As

Table IV. LOH analysis of the *PTCH* locus and expression of *PTCH* in human BCC employing highly polymorphic sites in exon 12 and exon 24 of *PTCH*

Sample	LOH *PTCH*	Overexpression of *PTCH* expression	Monoallelic
BCC 1	LOH	yes	yes
BCC 2	LOH	yes	yes
BCC 3	LOH	yes	yes
BCC 4	no LOH	yes	no
BCC 5	no LOH	yes	no
BCC 6	no LOH	yes	no
BCC 7	no LOH	yes	yes (27-fold)
BCC 8	NI	ND	ND
BCC 9	NI	ND	ND
BCC 10	NI	ND	ND

NI: not informative.
ND: not determined.

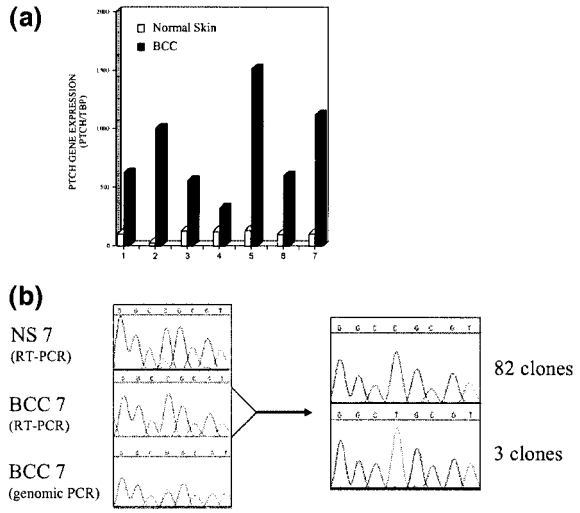

Fig. 5. Analysis of *PTCH* expression in human BCC. (**a**) Expression of *PTCH* in BCCs (black bars) and in normal epidermis (white bars) from seven different patients, analyzed by quantitative RT-PCR. (**b**) Allelic expression of *PTCH* in BCC 7. Chromatograms of DNA sequence analysis of an informative polymorphism in exon 12 of *PTCH* amplified from normal skin (NS) and tumor tissue (BCC) of case 7 are shown. Subcloning of the RT–PCR fragment isolated from tumor tissue revealed 27-fold overexpression of one *PTCH* allele.

shown in Figure 5a, all BCCs examined in this study displayed an up to 1500-fold increase of *PTCH* transcription in comparison to normal skin.

To determine which *Ptch* allele contributed to the elevated *PTCH* transcription, cDNA isolated from microdissected BCCs was amplified with the intron-spanning primer pairs Ex12F/Ex12R (polymorphic sites in exon 12) or Ex23F/Ex23R (polymorphic site in exon 23). The resulting fragments were sequenced using the same primers. As expected, the high levels of *PTCH* expression resulted from expression of one *PTCH* allele in those BCCs that exhibit LOH at the *PTCH* locus (Table IV). BCCs 4, 5 and 6 showed retention of heterozygosity at the *PTCH* locus and expressed PTCH biallelically (Table IV). Allelic expression imbalance of *PTCH* was detected in BCC 7. Although BCC 7 exhibited retention of heterozygosity at the *PTCH* locus, only one *PTCH* allele was expressed (Table IV, Figure 5b). To quantify the allelic expression level the PCR fragment amplified with Ex12F/Ex12R of this tumor was subcloned into the pCRII-cloning

vector and 85 subclones were sequenced using the T7 primer. A 27-fold overrepresentation of one allele was found (Figure 5b).

Altogether these results indicate that unlike in mice, most human tumors exhibit heterozygosity at the *PTCH* locus and express *PTCH* biallelically.

Discussion

PTCH has been proposed to act as a tumor suppressor gene in humans (18). According to Knudson's model, a tumor is the result of inactivation of the two alleles of the gene. Inactivation of the suppressor gene frequently occurs through point mutations of one allele and somatic loss of the second allele. The latter mechanism often involves loss of chromosomal material, which can be assayed by LOH (for review see ref. 19). However, the inactivation of both alleles has been formally shown only in a variable fraction of only some tumors associated with *PTCH* deficiency (20,21).

We have investigated the mutational status and the expression of *Ptch* in RMS in murine *Ptch* mutants. Here we show that the wild-type *Ptch* allele is retained and appears to be intact in RMS of heterozygous $Ptch^{neo67/+}$ animals. This conclusion is based on several lines of evidence. First of all, there is no evidence for the loss of the chromosomal segment harboring the wild-type allele of *Ptch*, as determined by LOH analysis. Secondly, mRNA transcripts derived from the wild-type allele can be detected in RMS and they carry no inactivating mutations within the protein-coding region. Interestingly, using an independent *Ptch* mutant mouse strain (14), Wetmore *et al.* and Zurawel *et al.* also found no evidence for the mutational inactivation of the wild-type allele in the other frequent tumor in murine *Ptch* mutants, medulloblastoma (16,22).

Surprisingly, the overexpression of *Ptch* in RMS appears to be caused by an overabundance of transcripts derived almost exclusively from the mutant allele. This observation is specific for the tumor and it is unlikely caused by different stabilities of the wild-type and mutant *Ptch* alleles. Indeed, both transcripts are expressed at comparable levels in non-tumor tissues. An overexpression could be caused either by a silencing of the wild-type allele or by an increased transcription of the mutant one. In respect of the first possibility, the wild-type allele of *Ptch* could be switched off by a mechanism different from mutational inactivation. There is growing evidence that epigenetic silencing rather than mutation is a common mechanism for loss of function of one or sometimes both alleles of tumor suppressor genes. Examples include *VHL*, *MLH1*, *p16* and possibly *BRCA1* and the underlying mechanism involves allele-specific methylation of CpG islands in the promoter regions of these genes (19,23).

The strong under-representation of transcripts derived from the wild-type allele in the *Ptch* transcript pool in RMS is in agreement with such a mechanism. In addition, a selective silencing of the wild-type allele would explain the apparent disagreement between the *Ptch* mRNA expression status in RMS and the current model of its regulation. According to this model, Ptch represses its own transcription via a negative feedback loop. Conversely, reduced Ptch activity, such as that expected in the case of a mutational inactivation increases *Ptch* transcription (for review see ref. 24). This model is in agreement with the overexpression of *Ptch* transcripts observed in a variety of tumors associated with *Ptch* mutations. However, it does not explain the dramatic difference between the relative expression levels of wild-type and the mutated *Ptch* alleles. To explain this difference, one would have to postulate an additional mechanism, such as a selective silencing of the wild-type allele. To confirm or reject this hypothesis it will be necessary to provide evidence for functionally relevant and tumor-specific differences in the methylation status of both *Ptch* alleles in RMS. Furthermore, an analysis of the protein expression from either allele will have to be conducted. This should become possible following the development of the appropriate exon-specific antibodies. Alternatively, it is possible that the wild-type allele is expressed at 'normal' levels in RMS and that haploinsufficiency for *Ptch* is sufficient for the formation of this tumor. To verify this hypothesis it will be necessary to first identify the cell subpopulation from which RMS develop and to determine its *Ptch* expression level. If the expression of wild-type allele is indeed not repressed in RMS, this would mean that RMS formation requires an event at a gene locus different from *Ptch*. The strong dependency of the RMS prevalence on the genetic background is in agreement with a significant contribution of other gene loci to RMS formation. The experimental verification could be provided by a time-point defined and skeletal muscle-specific inactivation of the wild-type allele. This should be achievable by means of conditional knockout technology. Furthermore, it should be possible to identify loci responsible for the differences in the Ptch phenotypes on different genetic backgrounds by means of QTL analysis.

In either case these data suggest that *Ptch* may not act as a classic tumor suppressor gene in mice. In humans, the mode of action of PTCH may be different. In accordance with previous studies (7,25) and similarly to murine tumors, all BCCs examined showed high levels of *PTCH* expression. However, in contrast to RMS in mice some human BCCs exhibited LOH at the *PTCH* locus. Obviously, all *PTCH* transcripts in these BCCs were derived from the remaining *PTCH* allele. Approximately every second BCC showed retention of heterozygosity at the *PTCH* locus and most of these tumors expressed *PTCH* biallelically. Thus, unbalanced expression of *PTCH* appears not to be strictly maintained in all *PTCH*-associated tumors. Altogether, these results raise the possibility that tumorigenesis associated with *PTCH* mutations may vary depending on tissue or species context. Similar observations have been made with other tumor suppressor genes. For instance, loss of function of the cyclin-dependent kinase inhibitor *p16* may occur through deletion, point mutation, or promoter hypermethylation and interestingly, the frequency of each mechanism differs between tumor types (23). A proof of tissue- or species-specific differences in the inactivation (e.g. through silencing) of *PTCH* will have to include an analysis of human BCCs with mutations in only one allele of this gene. This task should become possible following identification of all *cis*-acting genetic elements, which control *PTCH* expression.

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References

1. Gailani,M.R., Bale S.J., Leffell,D.J., *et al.* (1992) Developmental defects in Gorlin syndrome related to a putative tumor suppressor gene on chromosome 9. *Cell*, **69**, 111–117.

2. Gorlin,R.J. (1987) Nevoid basal-cell carcinoma syndrome. *Medicine (Baltimore)*, **66**, 98–113.
3. Johnson,R.L., Rothman,A.L., Xie J., *et al.* Quinn (1996) Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science*, **272**, 1668–1671.
4. Hahn,H., Wicking,C., Zaphiropoulous,P.G., *et al.* (1996) Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. *Cell*, **85**, 841–851.
5. Knudson,A.G., Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl Acad. Sci. USA*, **68**, 820–823.
6. Cavenee,W.K., Dryja T.P., Phillips,R.A., Benedict,W.F., Godbout,R., Gallie,B.L., Murphree A.L., Strong,L.C. and White,R.L. (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*, **305**, 779–784.
7. Gailani,M.R., Stahle-Backdahl,M., Leffell,D.J., *et al.* (1996) The role of the human homologue of *Drosophila* patched in sporadic basal cell carcinomas [see comments]. *Nat. Genet.*, **14**, 78–81.
8. Wicking,C., Shanley S., Smyth I., *et al.* (1997) Most germ-line mutations in the nevoid basal cell carcinoma syndrome lead to a premature termination of the PATCHED protein and no genotype-phenotype correlations are evident. *Am. J. Hum. Genet.*, **60**, 21–26.
9. Gailani,M.R. and Bale,A.E. (1997) Developmental genes and cancer: role of patched in basal cell carcinoma of the skin. *J. Natl Cancer Inst.*, **89**, 1103–1109.
10. Stone,D.M., Hynes,M., Armanini,M., *et al.* (1996) The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog [see comments]. *Nature*, **384**, 129–134.
11. Marigo,V., Davey,R.A., Zuo,Y., Cunningham,J.M. and Tabin,C.J. (1996) Biochemical evidence that patched is the Hedgehog receptor [see comments]. *Nature*, **384**, 176–179.
12. Toftgard,R. (2000) Hedgehog signalling in cancer. *Cell. Mol. Life Sci.*, **57**, 1720–1731.
13. Hahn,H., Wojnowski,L., Zimmer,A.M., Hall,J., Miller,G. and Zimmer,A. (1998) Rhabdomyosarcomas and radiation hypersensitivity in a mouse model of Gorlin syndrome. *Nature Med.*, **4**, 619–622.
14. Goodrich,L.V., Milenkovic,L., Higgins,K.M. and Scott,M.P. (1997) Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science*, **277**, 1109–1113.
15. Specht,K., Richter,T., Muller,U., Walch,A., Werner,M. and Hofler,H. (2001) Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am. J. Pathol.*, **158**, 419–429.
16. Wetmore,C., Eberhart,D.E. and Curran,T. (2000) The normal patched allele is expressed in medulloblastomas from mice with heterozygous germ-line mutation of patched. *Cancer Res.*, **60**, 2239–2246.
17. Kallassy,M., Toftgard,R., Ueda,M., Nakazawa,K., Vorechovsky,I., Yamasaki,H. and Nakazawa,H. (1997) Patched (ptch)-associated preferential expression of smoothened (smoh) in human basal cell carcinoma of the skin. *Cancer Res.*, **57**, 4731–4735.
18. Wicking,C. and Bale,A.E. (1997) Molecular basis of the nevoid basal cell carcinoma syndrome. *Curr. Opin. Pediatr.*, **9**, 630–635.
19. Ponder,B.A. (2001) Cancer genetics. *Nature*, **411**, 336–341.
20. Xie,J., Johnson R.L., Zhang X., *et al*. (1997) Mutations of the PATCHED gene in several types of sporadic extracutaneous tumors. *Cancer Res.*, **57**, 2369–2372.
21. Cohen,M.M., Jr. (1999) Nevoid basal cell carcinoma syndrome: molecular biology and new hypotheses. *Int. J. Oral Maxillofac. Surg.*, **28**, 216–223.
22. Zurawel,R.H., Allen,C., Wechsler-Reya,R., Scott,M.P. and Raffel,C. (2000) Evidence that haploinsufficiency of Ptch leads to medulloblastoma in mice. *Genes Chromosomes Cancer*, **28**, 77–81.
23. Baylin,S.B. and Herman,J.G. (2000) DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet.*, **16**, 168–174.
24. Freeman,M. (2000) Feedback control of intercellular signalling in development. *Nature*, **408**, 313–319.
25. Reifenberger,J., Wolter,M., Weber,R.G., Megahed,M., Ruzicka,T., Lichter,P. and Reifenberger,G. (1998) Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.*, **58**, 1798–1803.

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