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Indian hedgehog (IHH) and PTHrP normally participate as well as each control subject provided a signed consent form as approved by the institution's Research Ethics Board.

growth plate chondrocytes, thereby allowing orderly longitudinal bone growth [15, 21]. PTHrP is unable to downregulate IHH expression in enchondroma and chondrosarcoma organ cultures suggesting constitutive hedgehog signaling activation is present in a broad range of cartilaginous tumors [2]. IHH is a secreted glycoprotein that activates a transmembrane receptor complex formed by PATCHED-ONE (PTCH-1) and SMOOTHENED (SMO). When binding hedgehog, PTCH-1-mediated repression of SMO is relieved, thereby causing intracellular signal transduction, GLI activation, and upregulation of target genes, including PTCH-1 and GLI-1 from the hedgehog pathway itself [7, 24].

Mutations of the hedgehog receptor gene PTCH-1 and SMO cause activation of hedgehog signaling and are reported in a variety of tumor types [22, 34, 40, 41]. For example, PTCH mutations were detected in as many as 67% of sporadic basal cell skin carcinomas [23]. Because PTHrP is an uncommon mutation, found only in enchondromatosis, other events must be responsible for activating hedgehog signaling in the majority of cartilage tumors [27, 29].

Therefore, we asked whether mutations in PTCH-1 or SMO occur in cartilage tumors and whether they are somatic or germline alterations.

## Materials and Methods

We evaluated 46 sporadic cartilaginous tumors from surgically treated patients. Tumors consisted of 40 chondrosarcomas of histologic Grade I to IV, four chondroblastomas, and one synovial chondromatosis. Tumor specimens were obtained at the time of operative biopsy or definitive resection and were chosen by a pathologist with the aid of frozen histologic analysis to ensure the presence of viable tumor without normal tissue contamination. Tumor samples were collected immediately after surgery, snap-frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Genomic DNA was extracted using conventional techniques (Qiagen, Mississauga, Ontario, Canada). As controls, genomic DNA was also isolated from peripheral blood leukocytes of 127 healthy individuals without a history of sarcoma or a bone or cartilage tumor. Population-based control subjects were identified from Ontario, Canada, by calling a random list of residential telephone numbers. The control subjects completed health questionnaires and provided blood samples. Each

Mutational analysis of PTCH-1 (exons 2-23) and SMO (exons 2-11) was performed at the genomic level by single-strand conformation polymorphism analysis (SSCP) using PCR primers binding to intronic sequences flanking each exon [9]. All PCR primer sequences were used as previously published [5, 41, 44]. Placenta genomic DNA was used as a control. Fifty nanograms of tumor DNA was added to a 25- $\mu\text{L}$  reaction containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.8 to 2.6 mM  $\text{MgCl}_2$ , 0.4 mM of dNTPs, 6 pmol of each primer pair, 1 mCi  $^{32}\text{P}$ -dATP (10 mCi/ $\mu\text{L}$ ; Amersham, Piscataway, NJ), and two units of AmpliTaq (Perkin Elmer, Norwalk, CT). Each PCR product was screened for mutations by SSCP-dATP-incorporated PCR products were heat-denatured and electrophoresed on 8.5% native polyacrylamide gels containing 10% glycerol. Each fragment was evaluated under two different conditions with variation in temperature between 4 and 67°C. Sequence alterations detected as electrophoretic mobility shifts on SSCP gels were confirmed and characterized independently by direct manual sequencing using the same SSCP primers (ThermoSequenase cycle sequencing kit; Amersham Life Science, Arlington Heights, IL).

For a putative mutation identified, DNA from peripheral blood cells from the same patient as well as from uninformed control subjects was analyzed for the presence of the mutant sequence. PCR was performed with the same primer pair used for SSCP and sequencing (eg, for the site of the newly identified SMOR168H variant: forward primer 5'-AGTGAGGAGGGCCTTCA-3' reverse primer 5'-CAGAGAGCCTGGACCTTGTG-3'). Five nanograms of DNA from the peripheral blood of each patient or non-tumor control subject was added to a reaction buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM  $\text{MgCl}_2$ , 0.4 mM of dNTPs, 6 pmol of each primer, and two units of AmpliTaq. After denaturation at 94°C for 2 minutes, amplification was carried out over 37 cycles at 94°C for 15 seconds, at 56°C for 15 seconds, and at 72°C for 20 seconds. Electrophoresis of the amplified products was performed on 2% agarose gels after digestion with the HhaI stored at  $-70^{\circ}\text{C}$ . Genomic DNA was extracted using conventional techniques (Qiagen, Mississauga, Ontario, Canada). As controls, genomic DNA was also isolated to identify whether the SMOR168H variant was present. The gel was stained with ethidium bromide (0.2 mg/L) for 10 minutes and the digested fragments were visualized under ultraviolet illumination. Sizes of HhaI digested or undigested fragments were determined by comparison with a known size marker (100-basepair ladder) to facilitate identification.

Results

We observed no PTCH-1 mutations in the 46 cartilage tumors examined. SSCP identified five band shifts in the PTCH-1 gene; however, subsequent manual sequencing revealed each of these was the result of a polymorphism. Two band-shifted fragments were the result of two new single nucleotide polymorphisms (SNPs) in exons 14 and 18 of PTCH-1 in two tumors. One new SNP was a 2199 A → G (A to G switch at nucleotide 2199) of exon 14, which was the third position of codon 773. Three additional tumors all carried the same 314 T → C change, which is a previously described SNP in exon 18 of the PTCH-1 gene [17].

SSCP revealed two shifts in SMO representing mutations in two of 46 (4%) cases. The same heterozygous mutation at nucleotide 783 (A nucleotide substitution resulting in an arginine to histidine amino acid change at codon 168 (R168H) in the N-terminus extracellular domain of the SMO protein was detected in two of 46 unrelated patients with cartilaginous tumors (Fig.1). One of these patients had a dedifferentiated chondrosarcoma and the other synovial chondromatosis. The frequency of SMO mutations in chondrosarcoma was one in 40 (2.5%).

In the patient with synovial chondromatosis, the SMO R168H substitution may represent a germline variant rather than a true germline mutation. Of the two patients carrying the SMO mutation, DNA from peripheral blood leukocytes was only available for testing from the patient with synovial chondromatosis. SSCP and sequencing revealed the same SMO R168H substitution, suggesting that in this patient, the mutant SMO allele was carried in the germline. To further investigate the germline status of this alteration, we devised a strategy based on PCR and restriction enzyme digestion for analysis of normal DNA from healthy control individuals. The restriction enzyme HhaI can digest the wild-type SMO fragment leading to a 213-basepair (bp) and 70-bp fragment, although usually only the larger of these two normal fragments (ie, 213 bp) can be visualized after gel electrophoresis (Fig. 2, Lane 2). In comparison, HhaI cannot digest the PCR-amplified product carrying the R168H mutation, leading to a 283-bp fragment being visualized on the agarose gel. Analysis of DNA from these two cartilaginous tumors and blood from the patient with synovial chondromatosis by

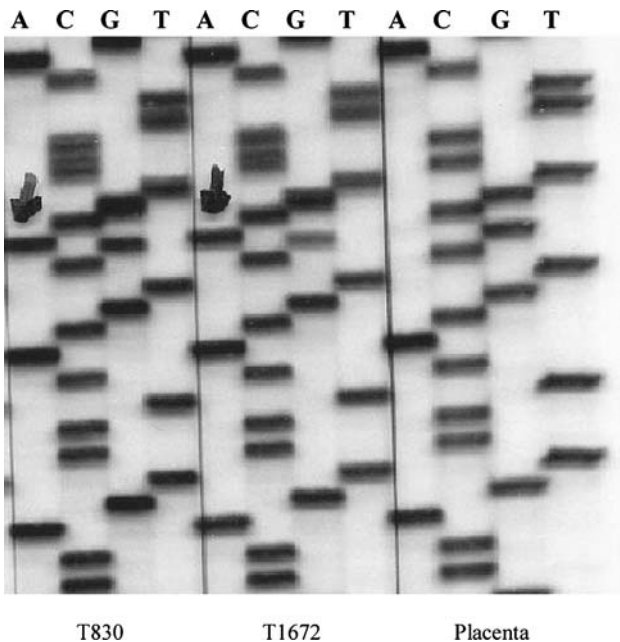


Fig. 1 The same SMO R168H variant is present in two separate normal placenta control shows one 213-bp band resulting from HhaI subfragment cleavage. Lanes 3 and 4: blood and tumor samples, respectively, from the patient with synovial chondromatosis (T830) and dedifferentiated chondrosarcoma (T1672). Lanes 3 to 5 demonstrate the presence of a 213-bp band as well as a 283-bp band indicating the same heterozygous DNA alteration at nucleotide 783.

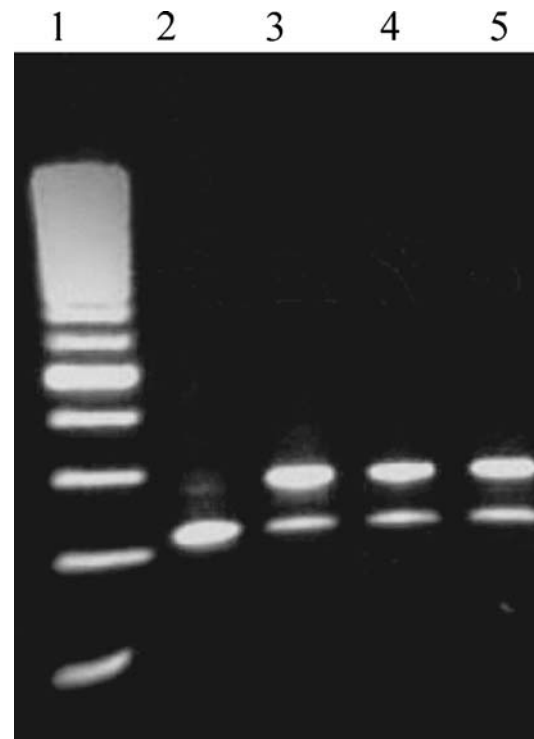


Fig. 2 SMO genotyping reveals the SMO R168H variant is present in the germline in one patient. Lane 1: 100-basepair (bp) marker. Lane 2: normal placenta control shows one 213-bp band resulting from HhaI subfragment cleavage. Lanes 3 and 4: blood and tumor samples, respectively, from the patient with synovial chondromatosis (T830) and dedifferentiated chondrosarcoma (T1672). Lanes 3 to 5 demonstrate the presence of a 213-bp band as well as a 283-bp band indicating the same heterozygous DNA alteration at nucleotide 783.

this HhaI assay reconfirmed the presence of the 783G amino acid change in all samples, indicative of SMO R168H substitution (Fig 2, Lanes 3&5). For each of these three samples, both a 213- and 283-bp fragment were also evident, indicating this alternation is heterozygous. Of the 127 healthy subjects, three (2.4%) carried this same alternation in their leukocyte DNA suggesting it represents a germline variant.

## Discussion

We identified PTCH-1 and SMO as plausible mutational targets in cartilaginous neoplasia based on three independent findings: (1) constitutive activation of hedgehog signaling is common in both benign and malignant cartilage tumors; (2) mutations of PTCH-1 and SMO have been identified in a variety of different cancers; and (3) inactivating mutations of PTCH-1, most commonly resulting from premature protein truncation, and constitutively activating missense SMO mutations both lead to overactivation of hedgehog signaling [17, 22, 32, 34, 38, 40, 41]. We searched for mutations of the hedgehog receptors and identified only three polymorphisms in the PTCH-1 gene and one relatively rare variant involving SMO. Although this novel SMO R168H heterozygous amino acid substitution was identified in unrelated tumors and was also present in the germline of at least one of these patients, it most likely represents a variant rather than a true mutation, because the same DNA change was also identified in 2.4% (three of 127) of healthy control subjects. The lack of somatic mutations in the cartilaginous tumors we examined suggests PTCH-1 and SMO alterations are uncommon causative factors underlying cartilaginous neoplasia.

A potential limitation of our study was the entire coding region of the two genes was not analyzed. The exon sequence of PTCH-1 was not assessed because numerous previous studies failed to identify mutations in this region of the gene [17, 33]. We were unable to successfully amplify SMO exons 1 and 12 as a result of the high GC content in these two exon sequences. However, SMO exon 1 contains untranslated sequences, making it an unlikely site for mutation [41]. In addition, no mutations have been described in human tumors in exons 1 or 12 of SMO gene. No mutations have been identified in the noncoding regions of PTCH-1, which cause human diseases [33]. Although such noncoding mutations could dysregulate PTCH-1 expression, our group and others have found PTCH-1 expressed in a variety of cartilage tumors, including chondrosarcoma, thereby ruling out the presence of inactivating alterations [25, 32, 37]. Therefore, expanding the analyses to include these additional regions

would be unlikely to substantially change the findings of this study. The majority of tumors evaluated in this study were chondrosarcomas, so the finding of infrequent hedgehog receptor mutations in cartilaginous malignancy types of cartilage neoplasia. In fact, the only sample of the benign condition synovial chondromatosis, which was examined, harbored the same SMO variant as a dedifferentiated chondrosarcoma. Although we did not investigate the functional aspects of the PTCH-1 polymorphisms or R168H SMO variant as part of this study, these alterations may still affect the hedgehog signaling pathway, which we previously found was overexpressed in a variety of cartilage neoplasia [9, 32, 37].

Germline PTCH-1 alterations account for the majority of cases of Gorlin's syndrome (nevoid basal cell carcinoma syndrome), a hereditary disease in which patients have developmental anomalies as well as numerous cancers, including multiple basal cell carcinomas, medulloblastoma, rhabdomyosarcoma, and others [10, 34, 35, 38]. It was subsequently shown that in sporadic basal cell cancers, PTCH-1 is mutated in up to 67% of cases as SMO is altered in another 10% to 20% [6, 23, 41]. Other cancers that occur as part of Gorlin's syndrome also frequently harbor PTCH-1 or SMO mutations when they occur sporadically. Although chondrosarcoma is not part of this syndrome, overactive hedgehog signaling is a feature shared by all these tumors. However, SMO variant was present in only one of 40 (2.5%) chondrosarcomas and there were no PTCH-1 mutations.

Most other solid tumors exhibiting dysregulated hedgehog signaling, similar to cartilage neoplasia, failed to reveal the presence of mutations of hedgehog pathway members to account for disruption of normal IHH/PTHrP signaling [2, 11, 12, 31, 36, 42]. Therefore, a hedgehog ligand-dependent mechanism likely underlies development and maintenance of the majority of cartilage neoplasia as well as other more common malignancies arising from the breast, prostate, lung, pancreas, and gastrointestinal tract that are similarly not associated with Gorlin's syndrome. This pathway may also provide a novel therapeutic target in many of these malignancies as we recently demonstrated for chondrosarcoma [28, 32, 39].

Previous studies implicated the IHH/PTHrP pathway in cartilaginous tumorigenesis. We previously showed hedgehog signaling is constitutively active in enchondromas and chondrosarcomas, and the normal negative feedback loop controlling the IHH/PTHrP pathway is uncoupled leading to ongoing proliferation without differentiation control [8, 32]. Both IHH and PTHrP as well as EGF signaling are active in chondroblastomas [25]. Osteochondromas are frequently caused by mutations in EXT genes, which regulate the diffusion of hedgehog ligands

[3]. Furthermore, the level of expression of PTHrP correlates with the grade of malignancy in chondrosarcoma [20, 26].

It is intriguing that the patient with SMO germline variant had synovial chondromatosis, a benign soft tissue type of cartilage tumor. We previously observed mice deficient in Gli3, a type of suppressor protein of the hedgehog signaling pathway, were predisposed to develop synovial chondromatosis [9]. Furthermore, in human patient samples of synovial chondromatosis, we demonstrated evidence of overactive hedgehog signaling [43]. Although the SMOR168H alteration we identified in this study is likely an uncommon genetic variant, it may still have a direct effect on hedgehog signaling and play a role in cartilaginous neoplasia [7]. A recent study showed the hedgehog ligand causes SMO activation by inducing a conformational switch, which is essential for pathway activation [43]. This process is negatively regulated by arginine clusters in the SMO cytoplasmic tail through intramolecular electrostatic interactions and opposed by phosphorylation, which leads to an active conformational switch. Although the arginine SMO codon 168 is situated in the aminoterminal extracellular domain, it is highly conserved among the human, mouse, and drosophila proteins and is positioned adjacent to a cysteine residue, so the R168H change to histidine may influence protein structure, function, and hedgehog signaling [30]. Interestingly, the PTHR1 R150C mutation we identified in two unrelated patients with enchondromatosis was also present in the germline of one of the patients and was inherited from his father, who had no evidence of the disease. Although subsequent investigations suggested the R150C mutation is uncommon [27, 29], the functional evaluation of the R150C variant led to the concept of overactive hedgehog signaling being a critical event in the development of cartilaginous neoplasia [8].

Because activation of hedgehog signaling in chondrosarcoma is rarely caused by PTCH-1, SMO, or PTHR1 mutations, other key components that are downstream from IHH or interact with the IHH/PTHrP pathway should be considered candidates for harboring alterations that are involved in the development of cartilage tumors [32]. Cell proliferation genes that are transcriptional targets downstream of GLI signaling may be important for the oncogenic function of the hedgehog pathway and include CYCLIN D, CYCLIN E, and components of the epidermal growth factor pathway [4, 26]. TGF- $\beta$ , BMP, and the FGF pathways also interact with hedgehog signaling [10]. Overactive hedgehog-mediated signaling appears a critical event for the development of cartilage neoplasia. Searching for additional molecular events upregulating the hedgehog pathway may provide a better understanding of tumorigenesis as well as lead to novel targeted therapeutics.

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