Human Molecular Genetics, 2001, Vol. 10, No. 7 757–762

The hedgehog pathway and basal cell carcinomas

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Received 18 January 2001 ; Accepted 26 January 2001

Developmental pathways first elucidated by genetic studies in the fruit fly, *Drosophila melanogaster*, are conserved in vertebrates, and disruption of these pathways has been associated with various human congenital anomalies. Many developmental genes continue to play an important role in regulation of cell growth and differentiation after embryogenesis, and mutations in some of these genes can result in cancer. Basal cell carcinoma (BCC) of the skin is the most common type of cancer in humans. Although most BCCs are sporadic, in rare cases, individuals have a hereditary disease, Gorlin syndrome, that predisposes to multiple skin tumors as well as a variety of birth defects. Mutations in the human homolog of a *Drosophila* gene, *patched*, underlie Gorlin syndrome. Genetic studies in *Drosophila* show that *patched* is part of the hedgehog signaling pathway, important in determining embryonic patterning and cell fate in multiple structures of the developing embryo. Human *patched* is mutated in sporadic as well as hereditary BCCs, and inactivation of this gene is probably a necessary if not sufficient step for tumor formation. Delineation of the biochemical pathway in which *patched* functions may lead to rational medical therapy for skin cancer and possibly other tumors.

INTRODUCTION

Hedgehog is a secreted molecule that influences the differentiation of a variety of tissues during development. Hedgehog, its receptor, 'patched', and many downstream members of the hedgehog signal transduction pathway were originally discovered by developmental biologists studying embryogenesis in *Drosophila melanogaster* (1). *Drosophila* hedgehog works in concert with other molecules to lay down the basic framework of the embryo, determining anterior–posterior relationships ('segment polarity') in developing structures (2). Segment polarity defects in *Drosophila* lead to loss of anterior–posterior orientation and may be manifested as mirror-image duplication of structures that should have distinct anterior and posterior sides and fusion of paired structures where one is normally anterior and the other posterior.

Vertebrate homologs of many segment polarity genes have been identified, and in most cases a single gene in *Drosophila* corresponds to a family of related homologs in vertebrates. Mouse models and human disease states have shown that disruption of hedgehog signaling in developing vertebrate embryos can lead to defects analogous to segment polarity abnormalities in *Drosophila* (reviewed in 3). Many developmental genes continue to function in regulation of cell growth and differentiation after embryogenesis, and mutations of some members of the hedgehog pathway are associated with human cancer as well as birth defects. For example, germline mutations of *patched* cause Gorlin syndrome, an autosomal dominant disorder characterized by multiple skin cancers, other tumors and congenital anomalies of the brain, bones and teeth (4). *Patched* and other human segment polarity genes play key roles in a variety of sporadic tumor types as well as hereditary tumors.

Biochemistry of the hedgehog pathway

Hedgehog encodes a 45 kDa protein that undergoes autocatalytic cleavage and modification to give a 20 kDa active N-terminal fragment covalently bound to cholesterol (5). The role of cholesterol in hedgehog signaling is not known, but it may be important in limiting diffusion of the hedgehog molecule and the spatial distribution of its effects. Three vertebrate homologs of the *Drosophila hedgehog* gene have been identified including *sonic hedgehog* (*SHH*), *desert hedgehog* (*DHH*) and *Indian hedgehog* (*IHH*). *SHH* is the most broadly expressed member of this family and is probably responsible for the major effects on development of the brain, spinal cord, axial skeleton and limbs (6). *IHH* has been implicated in regulation of cartilage differentiation in the growth of long bones (7–9), and *DHH* exerts its effect mainly in the developing germline and in Schwann cells of the peripheral nervous system (10).

The hedgehog signal is received and transduced at the membrane via a receptor complex consisting of patched and smoothened (Fig. 1). Patched, the component that specifically binds hedgehog, is a 1500 amino acid glycoprotein with 12 membrane-spanning domains (11,12) and two large extracellular loops that are required for hedgehog binding (13,14). There are several patched homologs in humans. *Patched1* (*PTCH*) is probably the major receptor molecule for all three forms of human hedgehog, and mutations in this gene are associated with a wide variety of birth defects (4,15). *Patched2* (*PTCH2*) is a close homolog of *PTCH*. Its normal function is not known, although it is mutated in rare medulloblastomas

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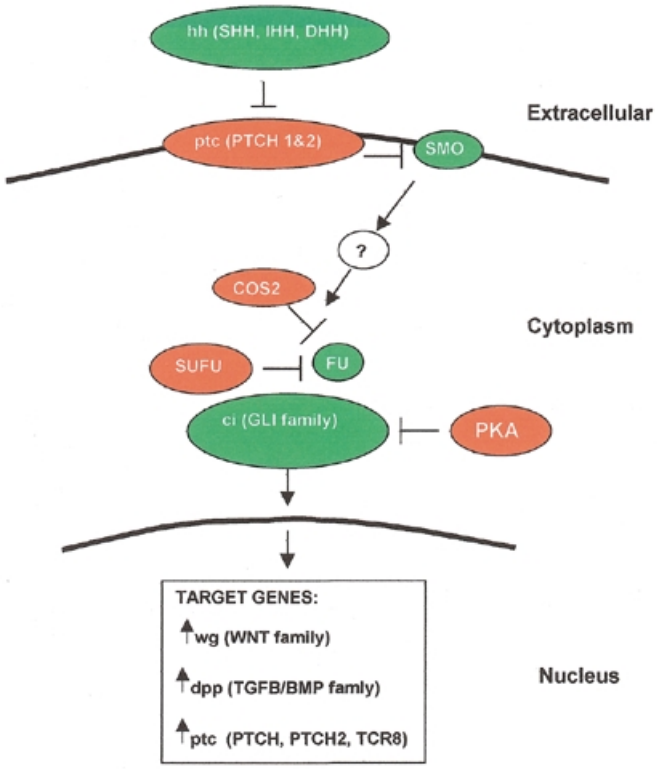

Figure 1. Elements of the hedgehog pathway conserved from *Drosophila* to vertebrates (human gene families in parentheses). Those members of the pathway that are activated in the presence of hedgehog are shown in green, and inhibitory members are shown in red. Hedgehog binds to patched releasing smoothened to transduce a signal. A downstream complex composed of fused, suppressor of fused, costal 2 and ci dissociates, and an active form of ci translocates to the nucleus where it switches on transcription of the target genes, *wingless*, *decapentaplegic* and *patched*. PKA, probably regulated by a parallel pathway, can inhibit activation ci.

and basal cell carcinomas (BCCs) (16,17). *TRC8* has homology to the region of *PTCH* encoding the membrane-spanning domains and second extracellular loop. It was found by cloning a translocation breakpoint in a renal cell carcinoma family and is mutated in some sporadic renal cell tumors (18). The transmembrane domains of patched show an intriguing homology to the 'cholesterol sensing' motifs of the Niemann–Pick disease protein (NPC1) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (19,20). The functional significance of this homology is not clear since there is no evidence that patched participates in cholesterol homeostasis, but this motif may have a broader role in intracellular trafficking of receptors and their ligands. In fact, hedgehog binding causes endocytosis of the hedgehog-patched complex and a decrease in the total amount of patched protein in the cell, presumably due to lysosomal degradation (21,22).

Smoothened is a 115 kDa protein with structural similarity to serpentine, G-protein coupled receptors (23,24). There is one known human smoothened gene (*SMO*), although it is homologous to members of the 'frizzled' (WNT receptor) family. In the absence of hedgehog, smoothened and patched form an inactive complex. When hedgehog binds to patched, the complex is altered and smoothened is then free to transduce the signal. There are conflicting data on the nature of the interaction between patched and smoothened and the effects of hedgehog binding on this complex. Immunoprecipitation suggests that hedgehog binding does not physically release smoothened from patched and that the release of inhibition reflects a modification or conformational change in smoothened (14). Analysis of subcellular localization, however, shows that hedgehog binding leads to loss of patched and an increase in smoothened on the cell membrane (21). The loss of patched has been shown to affect phosphorylation of smoothened and the stability of the smoothened protein (21,25). The interaction between patched and smoothened is not stochastic, suggesting that patched regulates modification of smoothened but does not complex with smoothened on a one-to-one basis. Smoothened has a long extracellular N-terminal domain, which in other members of the serpentine receptor family would bind a peptide ligand, but there is no evidence for a smoothened ligand. Likewise, smoothened is presumed to transduce the hedgehog signal to downstream members of the pathway through a G-protein, but no such interacting protein has been identified in *Drosophila* or vertebrates.

Based on epistatic interactions in *Drosophila*, fused, suppressor of fused, costal 2 and cubitus interruptus (ci) lie in the pathway downstream from smoothened (reviewed in 26). The ultimate member of the pathway in *Drosophila* is ci, a 155 kDa zinc finger transcription factor homologous to the GLI family in vertebrates. In cells not exposed to hedgehog, ci forms a tetrameric complex with costal-2, fused and suppressor of fused at the microtubules. In this form, ci can be cleaved to a 75 kDa N-terminal fragment that retains the zinc finger domain and can translocate to the nucleus and repress downstream target genes (27). In the presence of hedgehog, the complex dissociates and full-length ci is thought to mature into a short-lived

transcriptional activator which translocates to the nucleus and transcriptionally activates target genes. Within the tetrameric complex, costal 2 and suppressor of fused inhibit the activation of ci and are negative regulators of the pathway (27–29). Fused is believed to be activated by hedgehog signal leading to the release of active ci. PKA independently inhibits the activity of the hedgehog pathway and is believed to act directly on ci, probably contributing to its degradation (30).

Targets of the *Drosophila* hedgehog pathway include *wingless* (WNT family in vertebrates), *decapentaplegic* (*TGF beta* family in vertebrates) and *PTCH* itself. The wingless and decapentaplegic proteins may be the main mediators of hedgehog effect both by autocrine effects in the cells responding to the hedgehog signal and paracrine effects on surrounding tissues. The upregulation of PTCH expression results in the presentation of large amounts of PTCH protein at the cell membrane, which sequesters hedgehog and limits its spread beyond the cells in which it is produced (31).

THE HEDGEHOG PATHWAY AND HEREDITARY CANCER PREDISPOSITION

BCCs of the skin represent at least one-third of all cancers diagnosed in the US each year, and the cumulative lifetime risk for developing this type of tumor is approximately one in six (32). A minority (0.5%) of BCCs cases are attributable to Gorlin syndrome, also known as the nevoid BCC syndrome and basal cell nevus syndrome (33). This autosomal dominant disorder is characterized by multiple BCCs, medulloblastomas and ovarian fibromas and less frequently fibrosarcomas, meningiomas, rhabdomyosarcomas and cardiac fibromas. In addition to benign and malignant tumors, malformations are a striking component of Gorlin syndrome. The syndrome is associated with pits of the palms and soles, keratocysts of the jaw and other dental malformations, cleft palate, characteristic coarse facies, strabismus, dysgenesis of the corpus callosum, calcification of the falx cerebri, spina bifida occulta and other spine anomalies, bifid ribs and other rib anomalies, polydactyly, ectopic calcification, mesenteric cysts, macrocephaly and generalized overgrowth (33–35). Although not all patients with this syndrome are tall, some patients may reach gigantic proportions and exhibit features reminiscent of acromegaly.

Since the syndrome was delineated in the late 1950s and '60s (36,37), numerous laboratory investigations have been undertaken to identify the underlying molecular basis. The prominence of developmental defects makes this syndrome unusual among autosomal dominant cancer predisposition syndromes. Nevertheless, Gorlin syndrome shares with other disorders the multiplicity, random distribution and early age of onset for neoplasms. Statistical analysis of the distribution of BCCs in affected individuals suggested that tumors in the syndrome arise through a two-hit mechanism (38) and that the underlying defect might be mutation in a tumor suppressor gene (39). This theory was strongly supported by the mapping of the Gorlin syndrome gene to chromosome 9q22–31 and the demonstration that the exact same region was deleted in a high percentage of BCCs and other tumors related to the disorder (40).

Positional cloning identified the human homolog of *Drosophila patched* as a candidate gene (4,41). Vertebrate patched was known to function in the developing neural tube, pharyngeal pouches, somites and limb buds (42). Many of the clinical features of Gorlin syndrome, including abnormalities of the brain, craniofacial structures, ribs, vertebrae and limbs correlate well with the apparent role of *patched* in the development of these structures, making *patched* a good candidate gene for this syndrome. Furthermore bifid ribs and polydactyly were 'segment polarity-like' features that one might expect in a syndrome caused by disruption of the hedgehog pathway. Screening of the *patched* coding region revealed a wide spectrum of mutations in Gorlin syndrome patients, with the majority predicted to result in premature protein truncation (43,44). Mutations are spread throughout the entire gene with no apparent clustering. The extensive phenotypic variability does not correlate with the nature or location of mutations in *patched*. Different kindreds with identical mutations differ dramatically in the extent of clinical features, suggesting that genetic background or environmental factors may have an important role in modifying the spectrum of both developmental and neoplastic traits.

SPORADIC BCCs

In addition to germline mutations in Gorlin syndrome individuals, *patched* mutations occur frequently in sporadic BCCs and in BCCs associated with xeroderma pigmentosa (45–48). Minute BCCs are as likely as large tumors to have mutations, and all histological subtypes, whether primary or recurrent, have a high frequency of loss of patched.

The mutational spectrum of *patched* in sporadic BCCs suggests that environmental factors other than ultraviolet B (UVB), the predominant carcinogenic component of sunlight, may play a role in tumorigenesis. UVB typically causes formation of photodimers that result in G·C to A·T transitions opposite dipyrimidine sites (49). Mutations in p53, which occur in ~50% of BCCs, are almost always UVB related (50) and mutations in the ras family of proto-oncogenes are often of the type caused by UVB (51). However, <50% of the *patched* mutations in sporadic BCCs have the typical UVB signature (45). In contrast to sporadic tumors, those in patients with xeroderma pigmentosa have a high rate of UVB-signature mutations (47,48). These data suggest that individuals with defects in repair of UV damage develop tumors through UVB-induced mutagenesis but that other carcinogens, possibly UVA or cosmic rays, play a more important role in the etiology of sporadic BCCs.

Given that activation of SMO, like inactivation of PTCH, upregulates transcription of hedgehog target genes, it is not surprising that activating mutations in the *SMO* gene have been detected in a proportion of sporadic BCCs (52). One common mutation (Trp535Leu) in the seventh transmembrane domain of Smo has been detected in almost all BCCs lacking PTCH mutations. In contrast to wild-type smoothened, the mutant form has been shown to result in constitutive smoothened signaling in an *in vitro* focus forming assay and BCC-like tumors in transgenic mice expressing this mutant gene under the control of an epidermis promoter.

Since hedgehog itself is primarily responsible for activation of this pathway, it is feasible that it may also be mutated in associated tumors, a premise supported by the finding that transgenic mice overexpressing *Sonic hedgehog* develop BCC-like skin lesions (53). Accordingly, a single recurrent mutation

in *Sonic hedgehog* was initially reported in a range of tumor types including BCC, but failure of other workers to detect this mutation suggests that it is extremely rare.

Taken together, inactivation of *PTCH* or oncogenic activation of *SMO* occurs in almost all BCCs, suggesting that dysregulation of hedgehog signaling is a requirement for BCC formation. The term gatekeeper was coined by Kinzler and Vogelstein (54) to describe genes that must be inactivated or activated to give rise to a particular type of tumor. Although it has previously been suggested that *patched* is the gatekeeper gene for BCC formation (55), it may be more accurate to regard the receptor complex consisting of patched and smoothened as the BCC gatekeeper.

PATHOGENESIS OF HEDGEHOG-INDUCED NEOPLASIA

The mechanism by which activation of the hedgehog pathway leads to carcinogenesis is not entirely clear. Hedgehog signaling has been shown to oppose cell cycle arrest and increase the replicative capacity of cultured epithelial cells (56). Neither the known members of the pathway nor its downstream target genes are direct regulators of the cell cycle or cell senescence, but the human homologs of many of these genes are known to function either as oncogenes or as tumor suppressor genes. For example, the human GLI1 gene has previously been shown to act as an oncogene in brain tumors including medulloblastomas (57). Furthermore, mouse models over expressing GLI1 or GLI2 in epidermis develop skin tumors that resemble BCCs (58,59). These data support a role of the GLI genes in mediating the carcinogenic effect of hedgehog pathway activation.

Switching on target genes that encode secreted proteins may contribute to neoplasia through autocrine activity. WNT1, a vertebrate homolog of *Drosophila* wingless, is known to cause mammary tumors in mice when activated (60). Switching on the WNT pathway in humans results in a variety of tumors including medulloblastomas (APC mutations in Turcot syndrome) (61) and skin tumors (62). Members of the TGF beta superfamily, homologous to *Drosophila decapentaplegic*, have complex roles in cell growth and differentiation. The mad (mothers against dpp) gene product is a component of the signal transduction pathway of dpp in *Drosophila*, and two human homologs of mad, DPC4 and MADR2, have been shown to act as tumor suppressors (63–65). It is plausible that dysregulation of the TGF beta family contributes to carcinogenesis through autocrine activity.

Secreted proteins may also play a contributory role in carcinogenesis through paracrine activity. BCCs are unusual among malignancies because they almost never metastasize. The failure of cells to separate from the primary tumor and grow in another tissue environment may reflect a need for interaction with a 'conditioned' stroma. Transplantation of human BCCs to nude mice provides some evidence that surrounding stroma is necessary for the maintenance of the malignant state (66,67). Paracrine activity of hedgehog target genes in neoplasia has not been explored extensively. However, members of the TGF beta family have been shown to play a role in enhancing growth conditions for BCCs by altering surrounding tissue (68).

RATIONAL MEDICAL THERAPY FOR BCCs

With the assumption that BCCs require activation of the hedgehog pathway not only for initiation but also for maintaining the malignant state, agents that modulate the activity of the pathway might be used to treat these tumors. Switching on inhibitory members of the pathway (e.g. SUFU) or switching off stimulatory members downstream from PTCH (e.g. SMO) would be expected to suppress the growth of these tumors or cause their regression.

One promising compound of the latter type was discovered through epidemiological investigations of malformed sheep (69). If Gorlin syndrome is thought of as the result of excessive activation of the hedgehog pathway, then its molecular flip side is holoprosencephaly—a disease caused by inactivating mutations of *Sonic hedgehog* (70). Like many other birth defects, holoprosencephaly can be caused by either intrinsic genetic defects or the effects of environmental substances on genetically normal embryos. Pregnant sheep grazing on a common lily plant were noted to have a high rate of holoprosencephaly in their offspring. The relevant chemical from the lily was dubbed 'cyclopamine'. As holoprosencephaly can be caused by either mutations in *Sonic hedgehog* or exposure to cyclopamine, it was suggested that cyclopamine acts by repressing the hedgehog pathway. Several studies (71–73) showed that cyclopamine reverses activation of the pathway downstream of patched and upstream of GLI. In fact, the evidence points to smoothened as the site of cyclopamine's action. Together with the finding that adult sheep do not suffer ill effects of cyclopamine, these results support the possibility that this agent can be used as a treatment for BCCs and any other tumor type associated with mutations in patched or smoothened.

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