

Ultraviolet Radiation Mutagenesis of Hedgehog Pathway Genes in Basal Cell Carcinomas

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The identification of mutations in Hedgehog (HH) pathway genes in some basal cell carcinomas (BCC) and the detection of HH pathway dysregulation in almost all BCC confirms the importance of this developmental regulatory pathway in human BCC tumorigenesis. Moreover, the occurrence of UVB signature mutations in key HH pathway genes in BCC provides the first genetic evidence that UV radiation (UVR) may be the principal mutagen involved in BCC tumorigenesis. We review herein current advances in the understanding of the role

of the HH pathway in BCC tumorigenesis including transgenic and knock-out animal models of HH pathway dysregulation. Furthermore, we summarize abnormalities in other tumor suppressors and oncogenes including *ras* and *p53* and evidence for interactions between these regulatory genes and the HH pathway. Key words: basal cell carcinoma/Hedgehog pathway/PATCHED/p53 and ultraviolet radiation. Journal of Investigative Dermatology Symposium Proceedings 4:41-45, 1999

Although ultraviolet radiation (UVR) is considered the primary mutagen involved in basal cell carcinoma (BCC) tumorigenesis, there is a paucity of experimental data to confirm this association. Until recently, research on the pathogenesis of BCC has been hampered by the lack of animal models and by a limited understanding of the genetic basis of the cancer. The identification of mutations in Hedgehog (HH) pathway genes including *PATCHED* (*PTC*), *SONIC HEDGEHOG* (*SHH*), and *SMOOTHENED* (*SMO*) has expanded our understanding of the genetic basis of BCC and facilitated the development of *in vitro* and *in vivo* models of BCC. In light of these recent advances, we will review epidemiologic and genetic evidence linking UVR to BCC tumorigenesis and *PTC* mutagenesis and the potentially collaborative role of mutations in other oncogenes and tumor suppressor genes in BCC tumorigenesis.

EPIDEMIOLOGIC EVIDENCE

Basal cell carcinoma is the most common human cancer, affecting an estimated 750 000 Americans per year (Miller and Weinstock, 1994). BCC incidence is rising – 3% annually in two sampled US geographic regions between 1971 and 1978 (Fears and Scotto, 1982), and doubling in a 10-y period in Stockholm, Sweden (Wallberg and Skog, 1991). This increasing incidence is commonly attributed to lifestyle changes favoring sun exposure in spite of efforts to educate the public on the dangers of UV exposure. It is estimated that 28% of Caucasians

born after 1994 will develop a BCC in their lifetime (Miller and Weinstock, 1994).

A wealth of epidemiologic data establishes the importance of UV radiation exposure to BCC tumorigenesis. For example, these tumors occur on sites commonly exposed to sunlight – primarily the head and neck (approximately 66%) (Giles *et al*, 1988) – they occur with greater frequency in those with fair skin, an inability to tan, and a tendency to freckle or sunburn (Kricger *et al*, 1991), and they occur rarely in African-Americans (Scott *et al*, 1983). There is also a direct correlation between increasing BCC incidence and increasing equatorial proximity (Kricger *et al*, 1991) and thus higher solar irradiance. This correlation is further confirmed by studies finding a lower incidence of BCC in migrants from high latitude countries (low levels of childhood UV exposure) to Australia (low latitude) than in native-born Australians (Giles *et al*, 1988).

MOLECULAR PATHOGENESIS

In 1996, two groups simultaneously reported the identification of mutations in the *PTC* gene in sporadic BCC and in patients with basal cell nevus syndrome (BCNS) (Hahn *et al*, 1996b; Johnson *et al*, 1996). BCNS is an autosomal dominant disorder characterized by the development of multiple BCC, central nervous system (CNS) tumors (medulloblastomas and meningiomas), jaw keratocysts, and skeletal abnormalities (Gorlin, 1987). BCNS-associated BCC and jaw cysts retain the mutant germ-line *PTC* allele but lose the wild-type (WT) allele by loss of large chromosomal fragments at 9q (Bonifas *et al*, 1994; Levant *et al*, 1996). *PTC* gene mutations and loss of the remaining WT allele have also been identified in sporadic medulloblastomas and meningiomas (11%), suggesting a common genetic basis for the sporadic and syndrome-associated cancers (Raffel *et al*, 1997; Vorechovsky *et al*, 1997; Wolter *et al*, 1997; Xie *et al*, 1997). In accordance with Knudson's two hit hypothesis, BCNS patients – born with only one functional *PTC* allele – are susceptible to developing these rare CNS tumors and to developing BCC in greater numbers (tens to hundreds), and at a younger average age than most sporadic cases.

The most common gross genetic alteration found in sporadic BCC (68%) is loss of heterozygosity (LOH) at the region of the *PTC* locus

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Abbreviations: *APRT*, adenine phosphoribosyltransferase; BCNS, basal cell nevus syndrome; CNS, central nervous system; CYO, cytochrome p450; HH, Hedgehog; *HPRT*, hypoxanthine phosphoribosyltransferase; KO, knockout; LOH, loss of heterozygosity; NMSC, nonmelanoma skin cancer; *rasGAP*, *ras* GTPase activating protein; *Shh*, Sonic Hedgehog; *SHH*, *Sonic Hedgehog*; *SMO*, *SMOOTHENED*; *PTC*, *PATCHED*.

(9q22) (Gailani *et al.*, 1996a). LOH at 9q22 can be detected in small BCC (<1 cm diameter) and tumors of varying histology and invasiveness, but does not correlate with tumor aggressiveness (Gailani *et al.*, 1996a). These findings suggest that LOH at the region of the *PTC* locus may be an early event in BCC tumorigenesis. Interestingly, although UV exposure is an obvious risk factor for sporadic BCC tumorigenesis, investigators have found a poor correlation between LOH at 9q and the predicted level of solar irradiance at the tumor site (Gailani *et al.*, 1996a). In one study, BCC located on sites of high solar irradiance (the nose) had a lower incidence (56%) of 9q LOH than body sites with lower or no UV exposure (76%). Moreover, there was no statistically significant correlation between the presence of p53 UVB signature mutations (the selected surrogate marker of high solar irradiance) and LOH at 9q. As the authors stated, however, UVB radiation most commonly results in point mutations rather than gross chromosomal rearrangements or deletions, and therefore 9q LOH (as detected by loss of microsatellite markers in this study) is an insensitive measure of UVB-induced mutagenesis. Since the publication of this study, the identification of the *PTC* gene coding sequence has enabled the detection of these UV-induced point mutations.

PTC gene mutations have been identified in less than one third of screened BCC (37 of 137, 27%). Of those mutations that were identified in sporadic BCC, approximately 40% are the C→T or CC→TT transitions at dipyrimidine sites typical of UVB-induced DNA damage, implicating UVB in *PTC* mutagenesis (Hahn *et al.*, 1996b; Johnson *et al.*, 1996; Gailani *et al.*, 1996b; Uden *et al.*, 1996; Wolter *et al.*, 1997; Aszterbaum *et al.*, 1998; Reifenberger *et al.*, 1998). The incidence of these UV signature mutations in *PTC* in human BCC appears to be within the wide range observed in other mammalian genes in UV-irradiated cultured cells. For example, *in vitro* UV irradiation of cultured repair-proficient human fibroblasts resulted in hypoxanthine phosphoribosyltransferase (*HPRT*) gene mutations, of which 37% were the C→T (or alternatively represented by G-C→A-T) or CC→TT UV signature type (McGregor *et al.*, 1991), and irradiation studies of Chinese hamster ovary cells resulted in a 62% incidence of G-C→A-T transitions in the adenine phosphoribosyltransferase gene (*APRT*) (Drobetsky *et al.*, 1987). The incidence of UVB signature mutations in p53 in BCC and SCC occurring in Caucasians is 65% and 67%, respectively (Brash *et al.*, 1991; Pierceall *et al.*, 1991b; Rady *et al.*, 1992; Campbell *et al.*, 1993; Moles *et al.*, 1993; Ziegler *et al.*, 1993; Kubo *et al.*, 1994; van der Riet *et al.*, 1994; Gailani *et al.*, 1996b). Thus, the greater UVB signature mutation rate in p53 compared with *PTC* in non-melanoma skin cancers implies that UVB plays a greater role in p53 inactivation than in Ptc inactivation. Nevertheless, both are within the wide range of signature mutation incidence in *in vitro* model systems. The large variability in incidence of C→T and CC→TT substitutions in *in vitro* UV mutagenesis studies fails to provide an expected, baseline incidence rate of UV signature mutations in mammalian cells, thus making it difficult to conclude with statistical certainty that *PTC* gene mutagenesis is purely UV mediated.

The *hh* and *ptc* genes were first identified as important regulators of *Drosophila* larval segment polarity (anterior-posterior axis) and imaginal disc pattern development. In the developing fly and developing mouse, *ptc* is expressed in target tissues of hedgehog (Hh), a diffusible protein. The *ptc* protein is an integral membrane protein predicted to have 12 transmembrane domains. This model posits that Ptc protein inhibits HH target gene expression through its interaction with the Smoothed (Smo) protein, predicted to be a seven transmembrane protein resembling a G protein-coupled receptor. According to this model, Ptc inhibition of Smo is relieved upon binding of Sonic Hedgehog (Shh) to Ptc (Chen and Struhl, 1996; Marigo *et al.*, 1996) or following mutational inactivation of Ptc. Smo signaling may activate transcription of HH targets including *PTC* through activation of the putative transcription factor, *Gli* (Fig 1). Therefore, mutational inactivation of *PTC* and consequent loss of Ptc protein function results in increased Ptc expression and the accumulation of high levels of *PTC* transcript – high levels of *PTC* mRNA correspond with low levels of Ptc protein function.

Whereas only 37% of BCC have identifiable *PTC* gene mutations, *in situ* hybridization studies detect high levels of *PTC* message in

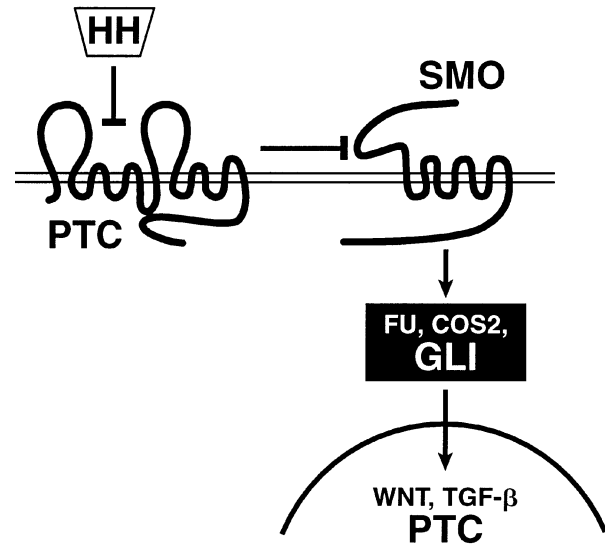


Figure 1. Hedgehog signaling pathway proteins are involved in BCC tumorigenesis. Hh is a soluble protein, which binds and suppresses Ptc, a 12 transmembrane domain protein with two large extracellular loops. If not bound to Hh, Ptc acts as a repressor of another transmembrane protein, Smoothed (Smo). If Ptc is mutationally inactivated or Smo is mutationally activated, Smo acts constitutively to activate downstream pathway targets including transcription factor Gli. Gli acts intranuclearly to activate transcription of *PTC*.

essentially all BCC, in contrast to the absence of message in normal epidermal keratinocytes (Uden *et al.*, 1997). In one recent report, 24 of 25 BCC studied demonstrated a significant increase in *PTC* expression relative to normal skin (Reifenberger *et al.*, 1998), and all BCC having *PTC* upregulation also had upregulation of other HH pathway components including *SMO* and *Gli* (Dahmane *et al.*, 1997; Wolter *et al.*, 1997; Green *et al.*, 1998; Xie *et al.*, 1998). Of the putative transcription factor *Gli* family genes (*Gli1*, *Gli2*, and *Gli3*), *Gli1* is the most highly expressed in human BCC. *Gli1* RNA levels were reported to be increased in 46 of 47 BCC examined, with the highest levels being found in tumor nodules invading the dermis (Dahmane *et al.*, 1997). Of note, *Gli1* is not expressed in interfollicular human basal cells but is expressed during the growing phase of follicular development.

Mutations in other HH pathway genes have been identified in sporadic BCC, implying that the principal trigger for tumorigenesis is not simply mutation of the *PTC* gene, but is more likely HH pathway dysregulation. Xie *et al.* (1998) described mutations in *SMO* in 6% (three of 47) of human BCC at codons 535 or 562, and Reifenberger *et al.* (1998) reported *SMO* mutations in 13% of BCC (four of 31) at codons 535 or 199. The codon 535 mutation results in an amino acid substitution in a highly conserved region in the seventh transmembrane domain. *SMO* mutations were identified only in one *SMO* allele in each tumor, with retention of the WT allele, pointing to the oncogenic potential of Smo. Co-precipitation studies indicated that the codon 535 and 562 mutations described by Xie *et al.* (1998) did not affect binding of Smo to pTC, although the fidelity of this attachment has not yet been elucidated. Half of those BCC with *SMO* mutations (two of four) also had mutations in *PTC* (Reifenberger *et al.*, 1998). BCC with *SMO* mutations all had upregulated expression of *PTC* mRNA and *Gli1* mRNA. Of note, one of the four *SMO* mutations Reifenberger described in BCC from UV-exposed areas was a C→T substitution; however, these limited data are insufficient to confirm that UVR is a significant *SMO* mutagen.

The oncogenic potential of mutant *SMO* was further confirmed by Xie *et al.* by *in vitro* assays using rat embryonic fibroblasts (REF52). Transfection of REF52 cells with adenovirus E1A and mutant (but not WT) *SMO* converted the REF52 cells to a malignant phenotype, i.e., foci formation over a confluent monolayer (Xie *et al.*, 1998).

Table I. Current *in vivo* models of basal cell carcinoma tumorigenesis

Species	Genetic alteration	Tumor type	Capable of reproduction	Reference
mouse	<i>Shh</i> transgenic	BCC	No	Oro <i>et al.</i> , 1997
mouse	<i>Smo</i> ^{mut} transgenic	BCC	No	Xie <i>et al.</i> , 1998
frog	<i>Gli 1</i> transfection	BCC	No	Dahmane <i>et al.</i> , 1997
mouse	<i>ptc</i> KO (+/-)	BCC and trichoblastoma	Yes	Aszterbaum <i>et al.</i> , manuscript in preparation

Moreover, in contrast to normal REF52 cells, cells from these foci were able to grow in soft agar. From these *in vitro* studies it appears that simple overexpression of *SMO* in mammalian cells is insufficient for malignant transformation and that mutant expression is required.

Mutations in *SHH* have also been identified in sporadic BCC. Oro *et al.* (1997) reported the identification of identical *SHH* mutations at codon 133 (His to Tyr) in a sporadic BCC (one of 43) and a medulloblastoma (one of 14). The predicted activating-type mutation is a C→T transition at the 3' cytosine of a dipyrimidine site and therefore possibly represents a UVB signature mutation; however, this limited data and the occurrence of the same mutation in a medulloblastoma, clearly not a UV-related cancer, warrants a cautious interpretation of these findings.

ANIMAL MODELS

Although in humans UV appears to be a causative factor in BCC tumorigenesis, it has proved difficult to induce BCC in experimental mice. Experimental mice commonly develop SCC in response to UV irradiation, but only rarely develop BCC. The most successful studies of BCC induction in mice have been by application of polyoxyethylene sorbitan monostearate and DMBA (Merenemies, 1959; Della Porta *et al.*, 1960). In contrast, rats are susceptible to BCC tumorigenesis by exposure to X-ray (Zackheim *et al.*, 1964) and topical carcinogens (Zackheim *et al.*, 1959). Surprisingly, these rat BCC models have not been widely used. Study of BCC tumorigenesis has also been hindered by the inability to achieve sustained tumor growth of human BCC transplanted onto SCID mice (n = 10; Aszterbaum, unpublished data), and by the difficulty of establishing BCC cell lines. Our increased understanding of the role of HH pathway dysregulation in BCC tumorigenesis, however, has enabled the development of animal models that facilitate study of BCC pathogenesis.

Oro *et al.* (1997) described a keratin 14 (K-14) promoter driven *SHH* transgenic mouse that expressed high levels not only of *SHH* RNA but also of *PTC* RNA in the epidermis, suggestive of target gene activation. K-14 *SHH* transgenic mice displayed several phenotypic findings similar to BCNS patients, including polydactyly, spina bifida, and multiple BCC-like epidermal proliferations throughout their skin surface early in skin development. Because K-14 *SHH* mice are not viable beyond the perinatal period, tumor progression was examined by transplantation of embryonic skin onto SCID mice; however, the BCC-like growths failed to enlarge further and displayed partial mature hair follicle differentiation. A second mouse model of BCC tumorigenesis was described by Xie *et al.* (1998) in which a keratin 5 (K-5) promoter drove expression of mutant (codon 535) *SMO* (*SMO*^{mut}) in the epidermis. These mice express high levels of mutant *SMO* in the basal layer of the epidermis and develop BCC-like cutaneous growths, establishing the role of *SMO* in BCC tumorigenesis. These mice, however, fail to produce viable offspring and are therefore impractical models for further study (Table I).

An amphibian model also links BCC tumorigenesis to HH pathway dysregulation. Dahmane *et al.* (1997) reported the overexpression of *Gli1* in frog embryo epidermis by injection of ectodermal cells with a *Gli1* construct. Transfected frog embryos demonstrated ectopic expression of *Gli1* and, importantly, activation of HH target genes. Moreover, frog embryos developed abnormal BCC-like tumors, suggesting that *Gli1* overexpression may be sufficient to result in BCC tumorigenesis. This amphibian model and the mouse models herein described provide powerful evidence that BCC pathogenesis can result from disruption of at least three important regulatory points in the HH pathway.

The *PTC* knockout (KO) mouse model, first described by Goodrich

et al. (1997), confirms the importance of *PTC* in mammalian development and BCC tumorigenesis. These *ptc* KO mice have a deletion of exons 1 and 2 and insertion of *lacZ* reporter gene at the deleted site. Although homozygous KO (*ptc*^{-/-}) mice die at embryonic day 8.5, the heterozygote survives to adulthood. *ptc*^{+/-} mice, like *PTC*^{+/-} patients, have a high incidence of developmental abnormalities and extracutaneous tumors. These mice, like mice with inactivation of exons 6 and 7 reported by Hahn *et al.* (1998), have varying combinations of medulloblastomas, rhabdomyosarcomas, polydactyly, and jaw cysts but have not been previously reported to develop BCC. We have examined the skin of these mice (through maturity), and find that, like *PTC*^{+/-} BCNS patients, *ptc*^{+/-} mice develop BCC and that these tumors demonstrate upregulation of *ptc* promoter activity. Although the skin of untreated exon 1 and 2 *ptc*^{+/-} mice at 3–17 mo of age appeared grossly normal, 30% (26 of 85) of biopsied mice had microscopically detectable basaloid cell proliferations (average cross-sectional area 0.002 mm²), some resembling superficial BCC and others resembling a related follicular germinative tumor known as trichoblastoma. Most of these basaloid cell proliferations appeared after 9 mo of age (25 of 26) (Aszterbaum *et al.*, in preparation). In contrast, none of 27 WT littermate controls had a skin tumor detectable in biopsied skin. These findings further emphasize the importance of *PTC* inactivation in BCC and BCC-like tumors. Furthermore, the *ptc* heterozygote KO mouse is the first and only model of murine BCC tumorigenesis in which mice survive to adulthood and produce viable offspring (Table I).

MUTATIONS IN OTHER GENES

Oncogenes and tumor suppressor genes commonly implicated in extracutaneous tumorigenesis, including *ras* and p53, have also been implicated in BCC tumorigenesis. p53 is an important cell cycle inhibitor and a regulator of DNA repair and apoptosis of cells with damaged DNA. Almost half of all types of sporadic human tumors occurring in the general population have mutations of p53 (Beroud *et al.*, 1996). In normal appearing UV-exposed human epidermis, islands of keratinocytes immunostain for mutant p53 (Jonason *et al.*, 1996). These islands suggest that p53 mutant cells may be clonally expanded and may have a selective growth advantage over cells WT for p53. p53 mutations have been identified in 50% of BCC, and 65% of these are UV-type mutations (Brash *et al.*, 1991; Rady *et al.*, 1992; Campbell *et al.*, 1993; Moles *et al.*, 1993; Ziegler *et al.*, 1993; Kubo *et al.*, 1994; van der Riet *et al.*, 1994; Gailani *et al.*, 1996a). As mentioned previously, 9q (*PTC* locus) LOH in BCC does not correlate with p53 UV signature mutations; however, *in situ* detection of high levels of *PTC* mRNA in BCC was accompanied by positive p53 immunostaining in two-thirds of tumors (Unden *et al.*, 1997). These findings suggest a collaborative role for p53 in BCC tumorigenesis, but no currently available evidence confirms a direct interaction between p53 and HH pathway genes.

BCC tumor incidence and location has also been associated with polymorphisms in glutathione synthetase (GST) genes, which are important in detoxification of reactive oxygen species, and in cytochrome p450 (CYP) genes, which are important in metabolizing environmental carcinogens such as nitrosamines and polycyclic aromatic hydrocarbons. In patients who present with at least one BCC, those which are GSTT1 null or have polymorphisms in GSTM1 or CYP2D6 have a significantly decreased time before the appearance of a new BCC (Lear *et al.*, 1997). Therefore these genes may play an important role in BCC tumorigenesis.

The *ras* oncogenes, comprised of H-*ras*, N-*ras*, and K-*ras*, are mutant

in a variety of human cancers, including breast, bladder, and skin cancers. Single base substitutions have been found in H-*ras* or K-*ras* in 13% to 31% of screened BCC (van der Schroeff *et al*, 1990; Pierceall *et al*, 1991b). These activating mutations have been identified in BCC from sun-exposed sites (neck, face, and shoulder), and some are UV signature type mutations (Pierceall *et al*, 1991b). In patients with non-melanoma skin cancer, there is a high incidence (36% for patients with BCC and 90% for patients with SCC) of Ha-*ras* LOH in normal appearing skin (Ananthaswamy *et al*, 1989). The *ras* GTPase activating protein (*ras*GAP) gene, both a downregulator of *ras* and an effector of downstream *ras* signaling, has also been found to be mutant in human BCC (Friedman *et al*, 1993). These findings suggest a potential interaction between the *ras* signaling pathway and BCC and tumor promotion.

In vitro studies suggest that the *ras* gene may be involved in HH pathway regulation. Immortalized human keratinocytes containing p53 mutations, HaCaT cells, express markedly increased levels of *PTC* message when transfected with EJ-*ras*, a mutated c-Ha-*ras* gene. *SMO* is also overexpressed in HaCaT cell lines, although with less consistency, and *SMO* expression level in HaCaT cell lines correlates with doubling time – the highest level of *SMO* expression is associated with the shortest doubling time. Overall, EJ-*ras* transfection of HaCaT cells results in an almost 3-fold increase in *PTC* message and a 30% increase in *SMO* expression (Kallassy *et al*, 1997).

CONCLUSION

The molecular mechanisms by which UV radiation transforms normal basal keratinocytes to BCC cancer cells is not fully elucidated. This review has provided an overview of some molecular alterations detected in clinically apparent human BCC with particular emphasis on mutations in the HH pathway genes. Animal and *in vitro* models herein discussed establish the importance of HH pathway dysregulation in BCC tumorigenesis. Furthermore, the high frequency of UVB signature mutations in *PTC* indicates that UV radiation is important to HH pathway gene mutagenesis. Therefore, we conclude that existing genetic evidence supports current epidemiologic evidence that UVR is central to the pathogenesis of human BCC. Mutations in other tumor suppressor genes or oncogenes including p53 and *ras* may potentiate tumor progression via interaction with the HH pathway; however, future studies will attempt to replicate UV mediated tumorigenesis in animal models and further elucidate the molecular mechanisms of BCC tumorigenesis.

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