Significantly High Levels of Ultraviolet-specific Mutations in the Smoothened Gene in Basal Cell Carcinomas from DNA Repair-deficient Xeroderma Pigmentosus Patients

Sophie Couvé-Privat, Bakar Boudajjar, Marie Françoise Avril, Alain Sarasin, and Leela Daya-Grosjean

Abstract

The Sonic hedgehog (SHH) pathway is implicated in the etiology of the most common human cancer in Caucasians, the basal cell carcinoma (BCC). Mutations in the receptor of SHH, the patched gene, have been characterized in sporadic BCCs as well as those from patients with the rare genetic syndromes nevoid BCC and xeroderma pigmentosum (XP). To elucidate the role of UV in the deregulation of the SHH pathway, we analyzed for alterations of smoothened, a transmembrane signaling component regulated by patched, in BCCs and squamous cell carcinomas from UV hypersensitive XP patients. We find UV-specific smoothened mutations in 30% of XP BCCs, three times higher than those in sporadic Caucasian BCCs, confirming the high rate of UV-induced mutations in DNA repair-deficient XP patients. No alteration was found in XP squamous cell carcinomas, indicating the involvement of smoothened specifically in the development of BCC.

Introduction

The hedgehog signaling pathway directs crucial functions during embryonic development of both vertebrates and invertebrates (1). The family of hedgehog proteins includes the secreted protein SHH, first characterized in sporadic BCCs as well as those from patients with the rare genetic syndromes nevoid BCC and xeroderma pigmentosum (XP). The Sonic hedgehog (SHH) pathway is implicated in the etiology of the most common human cancer in Caucasians, the basal cell carcinoma (BCC). Mutations in the receptor of SHH, the patched gene, have been characterized in sporadic BCCs as well as those from patients with the rare genetic syndromes nevoid BCC and xeroderma pigmentosum (XP). To elucidate the role of UV in the deregulation of the SHH pathway, we analyzed for alterations of smoothened, a transmembrane signaling component regulated by patched, in BCCs and squamous cell carcinomas from UV hypersensitive XP patients. We find UV-specific smoothened mutations in 30% of XP BCCs, three times higher than those in sporadic Caucasian BCCs, confirming the high rate of UV-induced mutations in DNA repair-deficient XP patients. No alteration was found in XP squamous cell carcinomas, indicating the involvement of smoothened specifically in the development of BCC.

Materials and Methods

Clinical Samples. Fourteen SCCs and 30 BCCs of 28 XP patients were obtained mainly from the Centre Hospitalo-Universitaire de Bab El Oued (Alger, Algeria) or the Institut Gustave Roussy (Villejuif, France); the remaining samples were from different hospital sources in France and North Africa. Patients have been assigned a code number 1 to 28.

SSCP and Direct Sequence Analysis. Isolation of DNA, SSCP, and sequence analysis was carried out as described previously (4). The DNA was amplified using 13 pairs of primers described by Xie et al. (6), except for the exon 9 reverse primer, which we chose to give better amplification (5'-GGCAGGAGGGCTGGCTG-3').

Results and Discussion

In this study, we have analyzed for the involvement of the smoothened gene in the genesis of BCC by examining skin tumors from XP patients. Both BCCs and SCCs were screened for alterations in all 12 exons of the SMO gene by SSCP, followed by sequencing of bands showing altered mobility (Fig. 1). The absence of microdissection of our samples has forced us to use an established scanning method for detection of mutations. The SSCP shows a high sensitivity for detection of point mutation as well as small insertions and deletions, even in samples contaminated by stromal cells. The use of two polyacrylamide gel conditions (with or without glycerol) increases the SSCP sensitivity (8). No SMO gene mutations were found in the 14 XP SCCs analyzed in the present study whereas among 30 XP BCCs we found eight...
tumors from five patients presenting nine (30%) mutations, eight of which have not been described previously (Table 1). Seven of these alterations (23%) are missense mutations, one is a silent mutation in exon 1, and a base substitution located in intron 7 at the junction to exon 6 was also detected (Table 1). In two previous studies of 98 sporadic Caucasian BCCs only relatively low (9%) levels of SMO mutations, a postulated proto-oncogene, were observed, among which less than 30% are UV-specific alterations (4, 5). As shown in Table 1, the majority of the XP SMO mutations (89%) are CC→TT (six of nine) tandem mutations or C→T (two of nine) transitions occurring at bipyrimidine sequences, which are targets for UV-induced DNA lesions. This is exactly what is expected in the NER-deficient cells from XP patients where hypermutability by UV irradiation is correlated with their high predisposition to cutaneous cancers. Previous studies from our laboratory have already shown that XP tumors have significantly higher levels of UV-induced modifications in both oncogenes (ras, myc) and tumor suppressor genes (p53, patched) than in the same types of tumors from the normal population. Significantly, 67% of the SMO gene mutations exhibit the UV signature, the tandem CC→TT transitions that were not detected in previous studies of the SMO gene but found at elevated levels in the p53 and patched genes characterized in XP skin cancers (4, 9).

Finally, our analysis has allowed us to identify two new smoothened gene polymorphisms that do not modify the peptide sequence of the SMO protein (Table 2). A heterozygous G to A transition at nucleotide 852 was identified in the BCC and normal skin of patient 22 and was also found in one SCC of patient 17 but could not be confirmed because the normal skin of patient 17 was unavailable. Sequence analysis of four tumors presenting exon 6 SSCP variant bands allowed us to define the

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**Table 1. Mutational screening results for the SMO gene**

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Group</th>
<th>Tumor</th>
<th>Location</th>
<th>SMO mutation</th>
<th>TS/NTS</th>
<th>PTCH status</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>XPC</td>
<td>BCC1</td>
<td>Cheek</td>
<td>CC203TT</td>
<td>Ala68Val</td>
<td>NTS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BCC2</td>
<td>Nose</td>
<td>CC1046TT</td>
<td>Thr349Ile</td>
<td>NTS</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>BCC3</td>
<td>Forehead</td>
<td>CC1452TT</td>
<td>Arg485Trp</td>
<td>NTS</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>BCC</td>
<td>Unknown</td>
<td>CC2154TT</td>
<td>Leu515Phe</td>
<td>NTS</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>BCC1</td>
<td>Chin</td>
<td>C2344TT</td>
<td>Ala652Val</td>
<td>NTS</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>BCC</td>
<td>Arm</td>
<td>C2344TT</td>
<td>Cys-Leu</td>
<td>NTS</td>
</tr>
</tbody>
</table>

a The mutations are indicated in capitals on the coding strand, written 5’ to 3’, as described by Xie et al. (6).
b Location of the dipyrimidine lesion on the transcribed strand (TS) or the nontranscribed strand (NTS) of the SMO gene.
c PTCH mutations were described by Bodak et al. (4). ND, not determined; wt, wild type.

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**Table 2. Newly identified SMO gene polymorphisms**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Polymorphism</th>
<th>Heterozygous patient</th>
<th>Homozygous patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>aGt852aAt</td>
<td>Gly284</td>
<td>17, 22</td>
</tr>
<tr>
<td>6</td>
<td>gCa1164gGa</td>
<td>Gly388</td>
<td>4, 21, 27</td>
</tr>
</tbody>
</table>

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**Fig. 1.** A, SSCP analysis of amplified exon 9 DNA from XP patient 26. Lane 1, unexposed healthy skin; Lane 2, BCC. The presence of shifted bands (Lane 2, arrowhead) exclusively in the BCC sample indicates a modified sequence. B, direct sequence analysis of amplified exon 9 DNA from patient 26 (A, Lane 2). The G→T transversion at nucleotide 1604 is indicated.

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**Fig. 2.** Schematic representation of the location of mutations on the SMO gene. The SMO protein with the seven transmembrane domains is presented. The numbers refer to mutated codons with corresponding exons in parentheses. Black arrowheads denote point mutation in BCC from XP patients, and gray arrowheads denote point mutation in sporadic BCC (6, 7).
second polymorphism in our samples, nucleotide change C1164G, in codon 388.

Among the seven smoothened mutations, six mutations are located in the extramembrane domains of the protein (Fig. 2). Thus, the codon 68 mutation found here changes the conserved amino acid alanine to valine, effecting the amino-terminal extracellular domain of SMO, and may alter its postulated binding affinity to PTCH (10). Two other mutations are situated in the third large extramembrane loop, resulting in arginine 485 to tryptophan and leucine 515 to phenylalanine amino acid changes. The two mutations closely situated in the large extracellular loop indicate it to be a major functional domain of the SMO protein, and this could be the binding site for a putative ligand that may be important in modulating SMO signaling. Three mutations have been found in the intracellular domain of the SMO protein. One of these effects codon 349 located in the second intracellular loop, resulting in a threonine to isoleucine amino acid change. Two other mutations are closely located in the cytoplasmic tail of the SMO protein, which gives rise to an alanine to valine change at codon 652 and a proline to phenylalanine change at codon 755 (Fig. 2). Indeed, it should be noted that the cytoplasmic tail and the intracellular loops of GPCRs are critical domains for interaction with G proteins, a step essential in signal transduction (11). By homology, it is clear that the three modifications located in the cytoplasmic domain of the SMO protein, a putative GPCR, must alter dramatically SMO protein function. Modifications in this region of the protein may also be important in affecting receptor desensitization or sequestration, as described previously for GPCRs. Further homology with the GPCRs indicates that the seventh transmembrane domain and the third intracellular loop of the SMO protein are also required in activation of downstream signaling. In our study, we have found one mutation situated in the seventh transmembrane domain of the SMO protein, a G to T transversion that modifies the conserved tryptophan residue among all SMO proteins into leucine at codon 535 (Fig. 2). In the majority of GPCRs, the codon 535 corresponds to a conserved tyrosine residue, the function of which is to keep these receptors in a latent state by the formation of a polar pocket. Disruption of this site by mutation of SMO would result in constitutive SHH signaling that could be important in BCC development. The significance of the alterations at this location is manifested by the fact that the G→T transversion at codon 535 has also been detected in the three other studies analyzing for SMO modifications in sporadic BCCs (6, 7, 12). Interestingly, the study by Lam et al. (12), analyzing only for modifications in codons 535 and 562 in sporadic BCCs of an Asian population, found a high frequency (21%) of the 1604 G→T transversion. It is clear that this site constitutes a hot spot for SMO protein modification. Also, it is well established that the G→T substitution can result from replication of DNA containing 8-oxoguanine, a base modification that can be caused by reactive oxygen species known to be produced by both UV radiation and arsenic compounds (13). The latter are known to be significant because they are often part of the dietary consumption in Asian populations and could explain the differences in frequencies of this mutations observed in BCCs between the Caucasian and Asian populations.

We have already shown the presence of high levels of patched gene mutations in BCCs from XP patients, and it should be noted that three of six of the tumors bearing SMO missense mutations also carry a mutated patched gene, which is unrelated to any differences in histology of the BCCs (Table 1). The tumor suppressor gene PTCH is considered to be the gatekeeper gene for BCCs, suggesting it is modified at an early stage of skin carcinogenesis (14). Alterations of SMO, a proto-oncogene, could occur at any stage of the carcinogenic process and give a selective advantage in BCC formation (12). Hence, mutations of PTCH and SMO in the same cell could favor clonal expansion resulting in BCC. However, it cannot be ruled out that the tumors are made up of different clonal populations, each carrying different mutated genes. Our results and those from other studies provide sound evidence that both XP and sporadic BCCs can carry alterations of several genes, including those involved in the SHH signaling pathway (4, 7). Thus, to date, the loss-of-function of tumor suppressor genes like patched or the gain-of-function of proto-oncogenes such as SMO are often caused by UV-induced lesions and have been shown to contribute to BCC tumorigenesis by uncontrolled activation of the SHH signaling cascade (15).

Our study was carried out in tumors from XP patients that belong either to one of the seven complementation groups associated with NER deficiency (XP group A to XP group G) or to the XP variant group (16). Although we could not analyze the complementation groups of the XP patients studied here, we know that the majority of them being of North African origin belong to the XPC group, which represents between 50% and 70% of this population. Because the XPCs are deficient in global genomic repair but fully proficient in transcription-coupled repair, all mutations found in genes in XPC skin cancers, such as p53 or PTCH, are due to replication of unrepaird lesions on the nontranscribed strands of DNA (5, 9). By analyzing the mutations in Table 1, we can see that 100% of the C to T or CC to TT transitions are due to unrepaird lesions targeted at bipyrmidine sequences on the nontranscribed strand of the SMO gene. Moreover, we have already shown that the tandem CC to TT is specifically found much more frequently in XPC patients (17). Therefore, we can deduce that most of the XP patients with SMO mutations in BCCs analyzed here must, indeed, belong to the XPC group.

Our study showing the presence of relatively high levels of SMO proto-oncogene mutations in BCCs from UV-sensitive skin cancer-prone XP patients has confirmed its importance in BCC development. Interestingly, we and others have not been able to show mutations of patched or smoothened in XP or sporadic SCCs, indicating that the SHH pathway is probably not directly implicated in their formation (4, 18). The growing amount of data becoming available from biochemical studies as well as from animal models should enable us to better understand the differences in development of the two major nonmelanoma skin cancers, BCCs and SCCs. In conclusion, our data demonstrating high levels of UV-specific SMO gene alterations have also substantiated the interest of the XP model in epidemiological and molecular studies that offer a clear advantage in analyzing the importance of particular genes involved in skin carcinogenesis.

References
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