High Frequency of Ultraviolet Mutations at the INK4a-ARF Locus in Squamous Cell Carcinomas from Psoralen-Plus-Ultraviolet-A-Treated Psoriasis Patients

Heidemarie Kreimer-Erlacher, Hannes Seidl, Barbara Bäck, Lorenzo Cerroni, Helmut Kerl, and Peter Wolf
Department of Dermatology, Karl-Franzens University, Graz, Austria

Squamous cell carcinomas in psoralen-plus-ultraviolet A (PUVA) treated patients frequently exhibit p53 tumor suppressor genes and Ha-ras protooncogenes that are mutated at dipyrimidine sites and carry the ultraviolet fingerprint (i.e., C-to-T or CC-to- TT transitions). To further broaden the knowledge of genetic mutations in PUVA-associated skin cancer, we used DNA sequencing analysis to study the mutational spectrum of the INK4a-ARF locus in 26 squamous cell carcinomas from 11 long-term PUVA-treated psoriasis patients and classified the mutations by origin (ultraviolet, ultraviolet and/or PUVA, or other). Nineteen INK4a-ARF missense/nonsense mutations were found in exons 1α, 1β, and 2 in 11 of 26 squamous cell carcinomas (42%) from seven of 11 patients (64%). Eleven mutations (58%) were of the ultraviolet type; three (16%) were of the ultraviolet and/or PUVA type (i.e., C-to-T transitions at dipyrimidine sites opposite a 3′TpG sequence, a potential psoralen binding site); and five (26%) were of other type. Interestingly, 10 of 11 patients (91%) showed intron polymorphism C500G at the 3′ untranslated region of exon 3. These data indicate that (i) INK4a-ARF mutations frequently occur in PUVA-associated squamous cell carcinomas; (ii) ultraviolet B radiation is the major cause of these mutations; and (iii) PUVA itself may play no direct role in development of most INK4a-ARF mutations. Key words: carcinogenesis/Ha-ras/mutagenesis/p53/skin cancer. J Invest Dermatol 120:676–682, 2003

Psoriasis patients who undergo long-term treatment with psoralen plus ultraviolet A (PUVA) and are exposed to high cumulative dosages of UVA are at increased risk for skin cancer, particularly squamous cell carcinoma (SCC) (for review see Stern et al., 1998). The exact causes and mechanisms of PUVA-associated skin carcinogenesis are unclear, however. PUVA is not only a tumor initiator (Griffin, 1959; Pathak et al., 1989; Kripke et al., 1982; Ananthaswamy, 1985; Yang et al., 1994; Gunther et al., 1995) and potential tumor promoter by virtue of its inflammatory properties, but also an immunosuppressor (Strauss et al., 1980; Kripke et al., 1983). It may thus permit the growth of skin cancers induced by itself or by other known or suspected carcinogenic agents and treatments (e.g., therapeutic and/or environmental UV radiation, ionizing radiation, methotretaxate (MTX), topical tar, and/or arsenic) (Maier et al., 1996).

Using the tools of molecular epidemiology (Perera, 1997), it may now be possible to determine the exact etiology of different types of tumors, including PUVA-associated skin cancers, by linking mutations at target genes (i.e., tumor suppressor and oncogenes) to their causative agents. Indeed, it is already known that particular carcinogens often cause “fingerprint” mutations in a tumor suppressor gene and/or oncogene. For instance, aflatoxin B1 induces a specific mutation (G:C to T:A transversion) at codon 249 of the p53 gene in hepatocellular carcinoma (Nataraj et al., 1995). UV-associated murine and human skin cancers exhibit so-called UV “fingerprint” mutations (i.e., C-to-T and CC-to- TT transitions at dipyrimidine DNA sequences) (Brash et al., 1991; Campbell et al., 1992; Kress et al., 1992; Rady et al., 1992; Kanjlal et al., 1993; Mole et al., 1993; Ziegler et al., 1993; Kubo et al., 1994; Nakazawa et al., 1994; Nelson et al., 1994; Ren et al., 1996). The application of psoralen and subsequent activation with UVA light that occurs during PUVA therapy leads to the formation of DNA photoproducts, which may in turn lead to skin cancer formation. In cell culture studies, psoralen photochemically and preferentially binds to repeated 5′TpA sequences, which makes such sequences primary targets for PUVA mutagenesis. Monofunctional adducts, which result from C4-cyclo addition of a psoralen molecule to the 5′,6′ double bond of pyrimidine bases (preferentially thymine) in DNA, can react photochemically with thymine, cytosine, or adenine in complementary DNA strands, thus resulting in cross-links. One important recent study demonstrated that PUVA-induced murine skin cancers contained p53 mutations at DNA cross-linking sites (Nataraj et al., 1996), with most of the mutations occurring at or near 5′ TA or 5′ TAT sequences. 5′ TA or 5′ TAT sequences are potential sites of psoralen–DNA binding quite different from those found in UV-induced skin cancers. Repeated AT sequences are particular hotspots for the photochemical reaction of 8-methoxypsoralen with DNA, and the reactivity of repeated AT sequences seems to increase in the following order: AT < TA < TAT < ATAT < ATATA (Sage and Moustacchi, 1987; Boyer et al., 1988; Sage and Bredberg, 1991). PUVA-associated base substitutions and mutations may also occur, however, at 5′TpG sites (Chiou and Yang, 1995; Gunther et al., 1994; Gunther et al., 1993; Moles et al., 1993; Ziegler et al., 1993; Kubo et al., 1994; Nakazawa et al., 1994; Nelson et al., 1994; Ren et al., 1996).
et al., 1995; Nataraj et al., 1997) and 5′TpA sites (Gunther et al., 1995). Recently, we and others performed molecular epidemiologic sequence analyses on the p53 tumor suppressor gene (Nataraj et al., 1997; Wang et al., 1997; Seidl et al., 2001) and Ha-ras protooncogene (Kreimer-Erlacher et al., 2001) in PUVA-associated nonmelanoma skin cancers and surprisingly found a high percentage of UV fingerprint mutations (i.e., C-to-T and CC-to-TT transitions at di- pyrimidine sites) in both. This in turn suggested that the damage caused by UVB exposure may be the most significant direct factor, after psoralen–DNA photoproducts, in the formation of skin cancers in PUVA-treated patients. Interestingly, potential PUVA-associated mutations were more often found at 5′TpG sequences than at 5′TpA sequences (Nataraj et al., 1997; Kreimer-Erlacher et al., 2001). Nevertheless, the lack of p53 and Ha-ras mutations in up to 46% of these human tumors and the unexpectedly high percentage of UV fingerprint mutations suggested that other genes besides p53 and Ha-ras (the most frequently mutated genes in human cancer) may be involved in PUVA carcinogenesis.

One candidate gene is INK4a-ARF. This locus is localized on the human chromosome 9p21 and encodes the alternative reading frame proteins p16INK4a (exons 1A, 2, and 3) and p14ARF (exons 1B, 2, and 3), both of which are involved in the negative control of cell proliferation and are known to function as tumor suppressor genes besides p53 and Ha-ras (Peris et al., 1999, cited therein) and more recently in nonmelanoma skin cancers. For instance, Soufr et al. found INK4a-ARF mutations in 10% (two of 20) of SCCs from the general population (Soufr et al., 1999) and in 29% (eight of 28) of nonmelanoma skin cancers (including basal cell carcinomas and SCCs) from patients with the genetic DNA repair disease xerodermia pigmentosum (XP) (Soufr et al., 2000). They also found significant positive associations between the frequency of p53 mutations on the one hand and p16INK4a and p14ARF mutations on the other. Therefore, in this study, we used tools of molecular epidemiology [i.e., polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP) and DNA sequencing analysis] to establish a mutation spectrum for the INK4a-ARF locus in 26 SCCs from 11 PUVA-treated psoriasis patients.

### MATERIALS AND METHODS

#### Patients and tumors

A total of 26 SCCs from 11 PUVA-treated psoriasis patients were examined for INK4a-ARF mutations. All 11 patients had been previously treated with PUVA and/or had presented for diagnosis and treatment of skin cancer at the Department of Dermatology of the University of Graz, Austria. The patients’ demographics, tumor characteristics, and PUVA treatment data are shown in Table I.

#### DNA extraction

For DNA extraction, paraffin-embedded specimens were macerated and/or microdissected (i) to eliminate nontumor tissue from tumor samples and (ii) to obtain normal tissue from each sample for control PCR-SSCP and DNA sequencing studies. Seven to 10 μm tissue sections on Superfrost Plus slides (Menzel, Braunschweig, Germany) were deparañized with xylene for 10 min and ethanol for 10 min, air dried, and dampened in deionized water. Tissue was scraped off the slides, suspended in 50–100 μl of a solution containing 0.1 M Tris–HCl (pH 8.0), 0.5 μg per μl proteinase K, incubated at 55°C overnight, boiled for 10 min, and finally stored at −20°C until analysis.

#### PCR-SSCP analysis

Oligonucleotide primers of INK4a-ARF (exons 1A, 1B, 2, and 3) (Fargnoli et al., 1998) were used in PCRs. Four microliters of DNA lysate was used as template in a 50 μl PCR containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2 (for exons 1A, 2, and 3), 0.25 mM of each dNTP, 0.1 μM of DNA, and 3 μM of each primer.

### Table I. Demographics of patients and PUVA treatment characteristics

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Phototype</th>
<th>Age at first PUVA treatment (y)</th>
<th>Total number of PUVA treatments</th>
<th>Cumulative UVA dose (J per cm²)</th>
<th>Non-PUVA risk factors</th>
<th>Total SCCs</th>
<th>SCC of study</th>
<th>Location of SCC</th>
<th>SCC appearance after first PUVA treatment (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M</td>
<td>III</td>
<td>47</td>
<td>1249</td>
<td>7488</td>
<td>N.A.</td>
<td>16 SCC</td>
<td>A1</td>
<td>Lower leg</td>
<td>34</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>III</td>
<td>37</td>
<td>1144</td>
<td>7996</td>
<td>UVB, arsenic, tar</td>
<td>25 SCC</td>
<td>B1</td>
<td>Lower arm</td>
<td>179</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>II</td>
<td>54</td>
<td>419</td>
<td>3056</td>
<td>UVB, MTX, tar</td>
<td>27 SCC</td>
<td>C1</td>
<td>Lower leg</td>
<td>93</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>II</td>
<td>48</td>
<td>445</td>
<td>2778</td>
<td>UVB, tar, X-rays (scalp)</td>
<td>17 SCC</td>
<td>D1</td>
<td>Temple</td>
<td>152</td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>II</td>
<td>43</td>
<td>82</td>
<td>222</td>
<td>UVB, MTX, tar</td>
<td>8 SCC</td>
<td>E1</td>
<td>Lower leg</td>
<td>51</td>
</tr>
<tr>
<td>F</td>
<td>M</td>
<td>III</td>
<td>47</td>
<td>813</td>
<td>4352</td>
<td>Tar, arsenic</td>
<td>9 SCC</td>
<td>F1</td>
<td>Lower arm</td>
<td>103</td>
</tr>
<tr>
<td>G</td>
<td>F</td>
<td>II</td>
<td>37</td>
<td>622</td>
<td>2732</td>
<td>UVB, tar, X-rays</td>
<td>5 SCC</td>
<td>G1</td>
<td>Hand</td>
<td>241</td>
</tr>
<tr>
<td>H</td>
<td>F</td>
<td>III</td>
<td>58</td>
<td>1288</td>
<td>6987</td>
<td>UVB</td>
<td>16 SCC</td>
<td>H1</td>
<td>Lower leg</td>
<td>181</td>
</tr>
<tr>
<td>I</td>
<td>F</td>
<td>III</td>
<td>59</td>
<td>332</td>
<td>1960</td>
<td>Tar</td>
<td>2 SCC</td>
<td>I1</td>
<td>Lower leg</td>
<td>247</td>
</tr>
<tr>
<td>J</td>
<td>M</td>
<td>II</td>
<td>44</td>
<td>146</td>
<td>728</td>
<td>Tar, X-rays (scalp), arsenic</td>
<td>80 SCC</td>
<td>J1</td>
<td>Scalp</td>
<td>None</td>
</tr>
<tr>
<td>K</td>
<td>F</td>
<td>III</td>
<td>34</td>
<td>996</td>
<td>5096</td>
<td>UVB, X-rays, tar</td>
<td>4 SCC</td>
<td>K1</td>
<td>Upper leg</td>
<td>251</td>
</tr>
</tbody>
</table>

1F: female; M: male.

3Skin phototype according to Fitzpatrick classification.
upstream and downstream primers (64 pM for exon 1, 120 pM for exon 1β, 30 pM for exon 2 part a, 40 pM for exons 2 part b and 3), 2.5 U of AmpliTaq Gold Polymerase (Perkin Elmer, Vienna, Austria), and 2.5 μl dimethylsulfoxide (exons 1α, 1β, and 2). Reaction mixtures were subjected to 40 cycles of amplification in a Perkin Elmer Thermocycler Model 2700 (Perkin Elmer, Foster City, CA). Each cycle consisted of denaturation at 94°C for 45 s, annealing at 57°C (exon 2 part b), 58°C (exons 1α and 1β), or 60°C (exons 2 part a and 3) for 30 s, and polymerization at 72°C for 30 s.

Before the first cycle, the PCR tubes were incubated for 12 min at 94°C; after the last cycle, for 7 min at 72°C. Tubes without template DNA were included in each PCR run to control for potential contamination of PCRs. For SSCP analysis, PCR products were purified by gel electrophoresis on 3% metaphor agarose (FMC Bioproducts, Rockland, ME) and subsequent gel extraction (Qiagen, Hilden, Germany). Samples were then applied to a commercially available GenGel Excel 12.5/24 gel (Pharmacia Biotech, Uppsala, Sweden) and run for 1 h 45 min at 5°C (exon 2 part a and b), 10°C (exons 1α and 1β), or 20°C (exon 1α). The running conditions were as follows: voltage, 600 V; current, 25 mA; power, 15 W. Finally, the gels were stained using a DNA silver staining kit (Pharmacia Biotech). PCR-SSCP was performed at least twice per tumor sample.

Direct sequencing In brief, 60 ng of each tumor's DNA was amplified with 3.2 pM of primer for the different exons (Fargnoli et al, 1998) (PE Applied Biosystems, Weiterstadt, Germany). The amplified products were then precipitated and analyzed on an ABI Prism 310 system (Perkin Elmer). Applied Biosystems, Weiterstadt, Germany). The amplified products were then precipitated and analyzed on an ABI Prism 310 system (Perkin Elmer). AmpliTaq Gold Polymerase (Perkin Elmer, Vienna, Austria), and 2.5 μl dimethylsulfoxide (exons 1α, 1β, and 2). Reaction mixtures were subjected to 40 cycles of amplification in a Perkin Elmer Thermocycler Model 2700 (Perkin Elmer, Foster City, CA). Each cycle consisted of denaturation at 94°C for 45 s, annealing at 57°C (exon 2 part b), 58°C (exons 1α and 1β), or 60°C (exons 2 part a and 3) for 30 s, and polymerization at 72°C for 30 s.

Before the first cycle, the PCR tubes were incubated for 12 min at 94°C; after the last cycle, for 7 min at 72°C. Tubes without template DNA were included in each PCR run to control for potential contamination of PCRs. For SSCP analysis, PCR products were purified by gel electrophoresis on 3% metaphor agarose (FMC Bioproducts, Rockland, ME) and subsequent gel extraction (Qiagen, Hilden, Germany). Samples were then applied to a commercially available GenGel Excel 12.5/24 gel (Pharmacia Biotech, Uppsala, Sweden) and run for 1 h 45 min at 5°C (exon 2 part a and b), 10°C (exons 1α and 1β), or 20°C (exon 1α). The running conditions were as follows: voltage, 600 V; current, 25 mA; power, 15 W. Finally, the gels were stained using a DNA silver staining kit (Pharmacia Biotech). PCR-SSCP was performed at least twice per tumor sample.

Statistical analysis For statistical analysis, we used Fisher's exact test or the χ2 test in the StatView® statistical analysis program version 5.01 (SAS Institute, Cary, NC). p ≥ 0.05 was considered to indicate a statistically significant difference.

RESULTS

High frequency of UV fingerprint mutations at the INK4a-ARF locus DNA sequencing analysis revealed 19 INK4a-ARF missense/nonsense mutations in 11 of 26 SCCs (42%) from seven of 11 patients (64%) (Table II). Mutations were detected in exons 1α, 1β, and 2 of the INK4a-ARF locus. Three of these 19 mutations (16%) affected the amino acid sequence of both p16INK4A and p14ARF; 10 mutations (53%) affected only p16INK4A; and six (32%) affected only p14ARF. Most of the mutations (17 of 19, 89%) were single-nucleotide substitutions, except for a two-nucleotide substitution in one case and a 1 bp deletion in another. Eleven of the 19 missense/nonsense mutations (58%) were of the UV type; three (16%) were of the UV and/or PUVA type (i.e., C-to-T transitions at dipyrimidine sites opposite a 5'TpG sequence, a potential psoralen binding site); and five (26%) were of other type (including one deletion at nucleotide position 205 of exon 2) (Fig 1). Two tumors taken from the lower leg of patient C (C1 and C2) had the same C-to-T mutation on codon 48 at a site containing multiple (n = 3) pyrimidines. Although tumor C2 was excised more than 12 y after tumor C1, the fact that the same mutation type was seen in both cases suggests that tumor C2 may have developed from the same precursor cell(s) that gave rise to tumor C1 or from tumor tissue that remained after excision of tumor C1. Importantly, similar to p53 mutations in PUVA-associated SCC (Naraj et al, 1997) and basal cell carcinoma (Seidl et al, 2001), all tumors analyzed in this study contained both wild-type and shifted SSCP bands. Shifted SSCP bands were often of lower intensity than wild-type bands, however (data not shown), suggesting that (i) only one allele was mutated and the other was wild-type and/or (ii) only a portion of tumor cells harbored mutation(s). No somatic mutations were found in any sample of normal tumor-adjacent skin of this study, excluding the presence of not only

Table II. Types of p16INK4a and p14ARF mutations found in SCCs of PUVA-treated patients

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Exon</th>
<th>Nucleotide and surrounding sequence</th>
<th>Base change</th>
<th>Strand with affected pyrimidine</th>
<th>Type of mutation</th>
<th>Codon p16INK4a</th>
<th>Effect on p16INK4a protein</th>
<th>Codon p14ARF</th>
<th>Effect on p14ARF protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>2</td>
<td>gcCag</td>
<td>C → T</td>
<td>NT</td>
<td>UV/PUVA</td>
<td>117</td>
<td>None</td>
<td>132</td>
<td>Gly → Ser</td>
</tr>
<tr>
<td>B1</td>
<td>2</td>
<td>acCCgt</td>
<td>CC → TT</td>
<td>T</td>
<td>UV</td>
<td>81</td>
<td>Pro → Leu</td>
<td>95, 96</td>
<td>None</td>
</tr>
<tr>
<td>B3</td>
<td>2</td>
<td>gcCgg</td>
<td>C → T</td>
<td>T</td>
<td>UV</td>
<td>114</td>
<td>Pro → Leu</td>
<td>128</td>
<td>None</td>
</tr>
<tr>
<td>B4</td>
<td>2</td>
<td>gcCgt</td>
<td>C → T</td>
<td>T</td>
<td>UV</td>
<td>114</td>
<td>None</td>
<td>129</td>
<td>Arg → Cys</td>
</tr>
<tr>
<td>Cl, C2</td>
<td>1α</td>
<td>gcCga</td>
<td>C → T</td>
<td>T</td>
<td>UV</td>
<td>38</td>
<td>Pro → Leu</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cl</td>
<td>1β</td>
<td>caCga</td>
<td>C → T</td>
<td>NT</td>
<td>Other</td>
<td>None</td>
<td>24</td>
<td>32</td>
<td>None</td>
</tr>
<tr>
<td>C1</td>
<td>2</td>
<td>ccCcg</td>
<td>C → T</td>
<td>NT</td>
<td>UV/PUVA</td>
<td>110</td>
<td>Trp → Stop</td>
<td>124</td>
<td>None</td>
</tr>
<tr>
<td>C1</td>
<td>2</td>
<td>acCcg</td>
<td>C → T</td>
<td>T</td>
<td>UV</td>
<td>79</td>
<td>None</td>
<td>94</td>
<td>Pro → Ser</td>
</tr>
<tr>
<td>D2</td>
<td>1α</td>
<td>acCcg</td>
<td>C → T</td>
<td>NT</td>
<td>UV</td>
<td>35</td>
<td>Gly → Arg</td>
<td>32</td>
<td>None</td>
</tr>
<tr>
<td>D4</td>
<td>1α</td>
<td>taCcg</td>
<td>C → T</td>
<td>NT</td>
<td>UV</td>
<td>25</td>
<td>Val → Ileu</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>E2</td>
<td>2</td>
<td>gcCgg</td>
<td>C → T</td>
<td>Other</td>
<td>None</td>
<td>None</td>
<td>82</td>
<td>Arg → Cys</td>
<td>None</td>
</tr>
<tr>
<td>E2</td>
<td>2</td>
<td>gcCgg</td>
<td>Del</td>
<td>N.A.</td>
<td>N.A.</td>
<td>68</td>
<td>Frameshift</td>
<td>82</td>
<td>Frameshift</td>
</tr>
<tr>
<td>F1</td>
<td>2</td>
<td>gcCga</td>
<td>C → T</td>
<td>NT</td>
<td>Other</td>
<td>None</td>
<td>110</td>
<td>125</td>
<td>Gly → Arg</td>
</tr>
<tr>
<td>F1</td>
<td>2</td>
<td>gcCgg</td>
<td>C → T</td>
<td>NT</td>
<td>Other</td>
<td>None</td>
<td>148</td>
<td>17</td>
<td>Pro → Ser</td>
</tr>
<tr>
<td>H1</td>
<td>1α</td>
<td>gcCga</td>
<td>C → T</td>
<td>NT</td>
<td>UV/PUVA</td>
<td>15</td>
<td>Trp → Stop</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>H1</td>
<td>1β</td>
<td>gcCga</td>
<td>C → T</td>
<td>NT</td>
<td>UV</td>
<td>22</td>
<td>Arg → Gln</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>H1</td>
<td>1β</td>
<td>gcCgt</td>
<td>C → T</td>
<td>T</td>
<td>UV</td>
<td>None</td>
<td>None</td>
<td>17</td>
<td>3 Pro → Ser</td>
</tr>
<tr>
<td>I1</td>
<td>1α</td>
<td>gcCgg</td>
<td>C → T</td>
<td>Other</td>
<td>None</td>
<td>34</td>
<td>Ala → Val</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*No missense mutations were found in tumors A1, A2, B2, B5, D1, D3, E2, G1, G2, H2, J1, J2.

The sequence is shown in 5’ → 3’ for the strand (transcribed and nontranscribed) containing the pyrimidines. Mutated bases are written in capital letters. Dipyrimidines with UV-type mutations (C → T and CC → TT transitions) are underlined once; potential psoralen binding sites (5’TpA, 5’ApT, 5’TpG/5’CpA, 5’TpI/5’SapA sites) with mutations are underlined twice.

T, transcribed strand; NT, nontranscribed strand.

PCR-generated mutations but also mutations arising from PCR across residual carcinogen adducts. In the cases of two patients (patients E and K), however, all of their tumor and normal adjacent skin samples showed a C-to-T mutation at codon 148, a polymorphism that has been previously described (Fargnoli et al., 1998; Aitken et al., 1999; Holland et al., 1999; Goldstein et al., 2000). This C-to-T base change was also seen in tumor F1 but not in tumor F2 and in samples taken from normal skin adjacent to tumors F1 and F2 in the case of patient F, indicating that in tumor F1 the base change was in fact a somatic mutation. Interestingly, tumor samples from 10 of 11 patients (91%) showed the intron polymorphism C500G at the 3' untranslated region of exon 3, a mutation previously described in malignant melanomas from patients of Celtic ancestry (Fargnoli et al., 1998; Aitken et al., 1999).

Mutation frequency and spectrum comparison The mutation spectrum obtained in this study was compared with a mutation spectrum derived from pooled data from previous sequencing studies of the INK4a-ARF locus in SCCs from normal patients (Kubo et al., 1997; Soufri et al., 1999) and XP patients (Soufri et al., 2000). As shown in Fig. 2, there was no significant difference in percentage of mutation types (UV and/or PUVA, other) among the different groups of subjects [including SCCs of PUVA-treated and non-PUVA-treated non-XP patients, and SCCs of (presumably non-PUVA-treated) XP patients] although the number of patients was too small to allow any definite conclusions to be drawn. Most mutations at the INK4a-ARF locus in the SCCs from each group of patients were of the UV type. The total percentage of INK4a-ARF missense or nonsense UV fingerprint mutations at dipyrimidine sites in SCCs was 74% (14 of 19) in PUVA-treated patients [or 58% (11 of 19), when not counting the three C-to-T transitions at 5'TpG sequences], 80% (four of five) in patients from the general population, and 78% (seven of nine) in XP patients (Fig. 2). Potential PUVA-type mutations were only seen in SCCs from XP patients and PUVA-treated patients. As outlined above, however, in the case of PUVA-associated SCCs, the origin of these mutations could not be unambiguously traced back to PUVA or UV due to the DNA sequences at the sites of mutation (i.e., a dipyrimidine DNA sequence on one DNA strand and a 5'TpG sequence on the opposite strand). Interestingly, the total percentage of SCCs exhibiting mutations at the INK4a-ARF locus was 42% (11 of 26) in PUVA-treated patients, 12% (five of 41) in patients from the general population, and 33% (six of 18) in XP patients. The difference between the INK4a-ARF mutation percentage in SCCs from PUVA-treated patients and that from the general population was statistically significant (p = 0.0077, Fisher's exact test).

DISCUSSION

Our DNA sequencing analysis revealed 19 INK4a-ARF missense/nonsense mutations in 11 of 26 SCCs (42%) from seven of 11 patients (64%) (Table II). Most of these mutations had either the UV fingerprint (58%) or the UV and/or PUVA fingerprint (16%). Most (17 of 19, 89%) were single-nucleotide substitutions, except for a two-nucleotide substitution in one sample and a 1bp deletion in another. Interestingly, all UV type mutations were C-to-T transitions (and one tandem CC-to-TT transition) exclusively found at C-C sequences with the lack of neighboring T. This is unexpected because for random base mutations one would expect at least in some cases a T neighboring the C-C sequences at the mutated sites. The exact reason for the predominance of this particular mutation type is unknown at present. It does not seem
to be specific for PUVA-associated skin cancer, however, because C-to-T transitions at C-C sequences without neighboring T have been previously found to be more prevalent than other mutation types in skin cancer from the general population as well, for instance at the p53 gene (Brash et al., 1991; Ziegler et al., 1993). In our study, no mutation occurred at 5’Tpa sequences, the most likely sites for psoralen binding and thus potential markers for a PUVA effect.

When we compared our results with those of previous studies on the INK4a-ARF locus in SCCs from normal patients (Kubo et al., 1997; Soufr et al., 1999) and XP patients (Soufr et al., 2000), the mutation spectra were very similar (Fig 2). In those studies, most of the INK4a-ARF locus mutation ranges (58%–80%) had the UV fingerprint. Potential PUVA-type mutations, however, were only found in tumors from PUVA-treated patients and presumably non-PUVA-treated XP patients. Though unusual, the occurrence of PUVA-type mutations in the XP patients may be explained by exposure to naturally occurring psoralens or by chance.

In comparing our mutational findings in the INK4a-ARF locus with those in p53 and Ha-ras genes (Fig 3), we observed that potential PUVA-type mutations occur more frequently in the p53 and Ha-ras genes than at the INK4a-ARF locus. This observation may be explained at least in part by the higher number of psoralen binding sites found in the former versus the latter. Indeed, the percentage of potential psoralen binding sites (i.e., 5’Atp/5’Tpa, 5’Tpa/5’Tpa sequences) per total DNA length is 32.3% (290/897) at p53 exons 4-9 and 30.4% (173/570) at Ha-ras exons 1-4, but only 19.4% (128/660) at INK4a-ARF exons 1-3 (INK4a-ARF locus versus Ha-ras or p53, p<0.0001, χ² test). In contrast, the relatively high percentage of UV fingerprint mutations at the INK4a-ARF locus cannot easily be explained by the number of dipyrimidines at this locus versus the other genes. Indeed, the percentage of dipyrimidine sites is 20.6% (136/660) at the INK4a-ARF locus exons 1–3 and 19.8% (113/570) at Ha-ras exons 1–4, but 29.3% (263/897) at p53 exons 4–9 (INK4a-ARF locus or Ha-ras versus p53, p<0.0001, χ² test).

Three of the 19 INK4a-ARF mutations (16%) affected the coding region of both p16INK4a and p14ARF. Four (53%) affected only that of p16INK4a, and six (32%) affected only that of p14ARF. Five mutations (26%) were found at mutational sites previously reported from malignant melanoma. Of those, two (Trp15Stop (tumor D4), Arg22Cln (tumor H1), and Ala34Val (tumor I)) affecting p16INK4a; Gly132Ser (tumor B1), Val24Met (tumor C1), Pro94Ser (tumor C1), Arg82Cys (tumor E2), and Pro17Ser (tumor H1) affecting p14ARF, and Trp110Stop (tumor F1) affecting both p16INK4a and p14ARF) have not been reported previously and their effect on protein function is unknown.

In addition, two of our 11 patients with SCCs (18%) (E and K) exhibited a previously described polymorphism, i.e., a C-to-T base change at codon 148 that results in an amino acid change (Ala148Thr) in the p16INK4a protein (Fargnoli et al., 1998; Aitken et al., 1999; Holland et al., 1999; Walker et al., 1999; Hashemi et al., 2000; Tsao et al., 2000). This mutation has no functional effect, however (Walker et al., 1999; Hashemi et al., 2000; Tsao et al., 2000). The prevalence of this polymorphism in our study was consistent with previous studies, in which it ranged from 11.5% (5 of 131) (Holland et al., 1999) to 22% (four of 28) (Fargnoli et al., 1998). Interestingly, 10 of 11 patients (91%) exhibited the known intron polymorphism C500G at the 3’ untranslated region of exon 3 (Fargnoli et al., 1998; Aitken et al., 1999). In an Australian study, Aitken et al. (1999) found that the prevalence of the C500G polymorphism in patients from families with a history of melanoma increased linearly from 11.2% in the control group to 30.4% in the high-risk familial melanoma group. It was suggested that this association probably reflected the Celtic ancestry of the patients in the high-risk group. In similar studies, MacKie et al. (1998) found the C500G polymorphism in only one of 16 melanoma families from Australia (6.3%), whereas Fargnoli et al. (1998) found it in seven of 10 melanoma families from Italy and Austria (70%). In any case, the very high frequency of C500G polymorphisms in PUVA-treated patients in our study suggests that it may be a marker of increased risk of PUVA-inducible SCC.

Taken together, our results and those of previous studies on p53 and Ha-ras in PUVA-associated SCC suggest that mutations in the INK4a-ARF, p53, and Ha-ras genes occur frequently in these tumors, which in turn indirectly suggests that there might be functional cooperation among the proteins resulting from those mutations. In addition, the fact that all three of these genes show the UV fingerprint when mutated in PUVA-associated SCCs suggests that PUVA may somehow promote the mutagenic effects of UVB (possibly via immunosuppression or tumor promotion) and thus consequently promote carcinogenesis. The likely reasons for the frequent presence of UV fingerprint mutations in tumor suppressor genes and/or oncogenes of PUVA-associated SCCs have been previously discussed in detail (Nataraj et al., 1997; Kreimer-Erlacher et al., 2001; Seidl et al., 2001). Three of the most important appear to be (i) a history of therapeutic exposure to artificial UVB, (ii) habitual exposure to natural sunlight, and (iii) therapeutic exposure to natural sunlight that is beneficial in

---

**Figure 3.** Comparison of mutation spectra of human PUVA-associated SCC. Pooled data from the literature on p53 exons 4–9 (Nataraj et al., 1997; Wang et al., 1997) and Ha-ras exons 1–4 (Kreimer-Erlacher et al., 2000) are compared with data from this study on INK4a-ARF locus exons 1β, 1fβ, 2, and 3. Mutation types are defined as in Table II.
psoriasis (Nataraj et al., 1997). All three of these factors may, alone or in combination, produce UV fingerprint mutations. Importantly, most SCCs of this study harboring mutations were located on the lower legs and arms, representing also the most common sites of SCC occurrence in those PUVA-treated patients. Pсорiatic lesions particularly on the lower legs are common and often therapy resistant, needing additional therapeutic exposure to treatments such as UVB, PUVA, X-rays, and/or tar, which might be responsible alone or in combination for the appearance of mutations and tumors at those sites.

In the light of previous cell culture studies (Sage and Bredberg, 1991; Sage et al., 1993; Yang et al., 1994; Chioi and Yang, 1995; Gunther et al., 1995) and murine studies (Nataraj et al., 1996), it is difficult to understand the rarity and even absence of mutations at 5'TpA sequences in the INK4a-ARF, p53, and Ha-nas genes in PUVA-associated SCCs in our study, especially at 5'TpA sequences are the most likely psoralen binding sites and thus targets for PUVA carcinogenesis, at least based on cell culture (Sage and Bredberg, 1991; Sage et al., 1993; Yang et al., 1994; Chioi and Yang, 1995; Gunther et al., 1995) and murine studies (Nataraj et al., 1996). It has been a consistent finding, in human skin cancer however, that 5'TpG sites, but not 5'TpA or 5'TpT, are the most frequent sites of potential PUVA type mutations in all genes examined so far. Potential reasons for the occurrence of sizable portions of mutations at 5'TpG sites in humans have been previously discussed (Nataraj et al., 1997; Wang et al., 1997; Kreimer-Erlacher et al., 2001; Seidl et al., 2001). Nataraj et al (1997) suggested that the processing of psoralen adducts in vivo in humans might produce different mutation spectra than in rodent or cultured cells. Some have speculated that, in most in vitro mutagenesis studies, the doses of PUVA are too high (and thus clinically irrelevant) and result in mutation spectra that differ from those in human cancer (Nataraj et al., 1997). This hypothesis is supported by the finding that, when murine fibroblasts containing supF DNA were treated with low doses of PUVA, only 19% of the resulting mutations (eight of 42) occurred at 5'TpA sites versus 29% (12 of 42) at 5'TpG sites (Gunther et al., 1995). Interestingly, four of the 19 INK4a-ARF mutations (21%) we observed in this study occurred at CpG sites and resulted in a C-to-T transition. One possible cause of this mutation is DNA methylation and deamination (Vogel and Kopun, 1977; Cooper and Krawczak, 1993; Laird and Jaenisch, 1996) and many studies in eukaryotes (reviewed in Cooper and Krawczak, 1993) have shown that the CpG dinucleotide is specifically associated with frequent C-to-T transitions.

In summary, our results demonstrate that (i) mutations at the INK4a-ARF locus occur frequently in PUVA-associated SCC, (ii) UVB radiation seems to be the major cause of these mutations, and (iii) PUVA itself may play no direct role in the development of most mutations at the INK4a-ARF locus. Taken together with findings of previous studies on p53 and Ha-nas, these results indirectly suggest that PUVA's primary contribution to carcinogenesis in PUVA-treated patients may be its potential for nonmutational effects, such as tumor promotion and/or immunosuppression, that in turn promote tumor growth.

REFERENCES
Gunther EJ, Yeasky TM, Gasparro FP, Glazer PM. Mutagenesis by 8-methoxypsoralen and 5-methylangelicin photoadducts in mouse fibroblasts: mutations at cross-linkable sites induced by monoadducts as well as cross-links. Cancer Res 53:1283–1288, 1993


Sage E, Moustacchi E: Sequence context effects on 8-methoxypsoralen photobinding to defined DNA fragments. *Biochemistry* 26:3307–3314, 1987


