p53 Mutations in Nonmelanoma Skin Cancer of the Head and Neck: Molecular Evidence for Field Cancerization

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ABSTRACT

Multiple and distinct p53 mutations were detected by DNA sequence analysis in tumor and adjacent nonmalignant skin samples from eight patients with nonmelanoma skin cancer of the head and neck, providing unambiguous evidence for field cancerization. The mutations consisted of C→T transitions at dipyrimidine sequences (30% of all single base substitutions), T→C transitions (47%), and G→T transversions (12%), suggesting that other carcinogens may act along with UV radiation in the development of nonmelanoma skin cancer. Patient interviews revealed that, in addition to substantial exposure to solar UV radiation, most had a history of smoking and were exposed to carcinogens from industrial or agricultural sources. These data show that extensive molecular epidemiological investigations are necessary to elucidate risk factors associated with the disease in localities where patients often report substantial exposure to environmental carcinogens.

INTRODUCTION

The 500,000 new cases of BCC4 and 100,000–150,000 new cases of SCC documented each year in the United States account for more than one-third of all cancers reported in the nation (1–5). One of the features of these cutaneous neoplasms is that several tumors may present simultaneously or consecutively (4, 5). It has been estimated that between 38 and 50% of NMSC patients develop a second primary tumor within 5 years of initial treatment (6–8). Management of multiple primary neoplasms, along with their adjacent premalignant lesions, requires extended follow-up, repeated surgical intervention, and, at times, postoperative radiotherapy (9, 10). Therefore, although the absolute number of yearly deaths caused by NMSC is relatively low (11), a substantial degree of morbidity and high medical costs are associated with treatment of the disease (12).

The hypothesis of field cancerization has been invoked to explain the occurrence of multiple primary neoplasms (13). According to this theory, entire regions of tissues or organs become genomically unstable and predisposed to aberrant growth as a result of prolonged exposure to carcinogens. The dysregulation of proliferation and differentiation in the “condemned” field is often associated with numerous premalignant lesions and a high incidence of multiple primary tumors. There is no previous report of a molecular investigation of the field origin of NMSC. However, fields of hyperplastic or neoplastic epidermis are readily discerned by microscopic examination (14), and cytogenetic studies support the concept of field cancerization in the skin (15). Recently, DNA sequence analysis of exons 5–8 of the p53 tumor suppressor gene in multiple primary tumors of the upper aerodigestive tract has provided a molecular method for detecting field cancerization (16). Whereas recurrences and metastases may harbor mutations that are present in the primary tumor (17), multiple primary neoplasms often arise from similar but independent mutational events (16). In addition, distinct types of base changes in p53 have been associated with exposure to specific carcinogens, providing molecular markers of the etiological factors involved in the process of carcinogenesis (18). Examples of such “signature” mutations include C→T transitions at dipyrimidine sequences and CC→TT double base transitions in DNA damaged by UV, bisulfite, or oxygen free radicals (19–21), and G→T transversions characteristic of exposure to carcinogens from tobacco smoke (22) or to hydrogen radicals generated, for example, by γ-radiation (23).

On average, 59 cases of BCC and 39 cases of SCC of the head and neck are treated each year at M. D. Anderson Cancer Center, of which a substantial number present with or develop multiple primary tumors. Many of the tumors also exhibit an unusually aggressive phenotype (12). We have, therefore, analyzed mutations in the p53 gene to test the presence of field effects in tumor-bearing areas of head and neck skin and to determine whether UV radiation (alone or in concert with other carcinogens) is involved in the induction of these mutations.

PATIENTS AND METHODS

Patients and Samples. Primary BCC or SCC and adjacent nonmalignant skin were excised from eight consenting patients at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center. The tumors were classified as clinically aggressive when they exceeded 2 cm in diameter; invaded muscle, bone, cartilage, or nerve; or metastasized to lymph nodes. Care was taken to prevent contamination between the tumor and nonmalignant skin samples, which were promptly snap-frozen. Frozen sections were performed on all samples and stained with hematoxylin and eosin for histological diagnosis. Peripheral blood lymphocytes were also available from two of the patients. Laboratory specimens were coded to maintain patient confidentiality and to prevent any bias before final analysis. The patients incurred no additional risks from this study, which was conducted after approval by the University of Texas M. D. Anderson Institutional Review Board.

DNA Amplification by PCR. Exons 4–9 of the p53 tumor suppressor gene (from tumor, nonmalignant skin, and available lymphocytes) were amplified under stringent conditions by PCR (24). Four sets of previously described primers amplifying exons 4, 5 through 6, 7, and 8 through 9 were used (25). In brief, for 10-μl reaction volumes, mixtures containing 50 ng genomic DNA, 0.2 μM 5’ primer, and 0.2 μM 3’ primer were incubated in Gene Amp reaction buffer (Perkin Elmer Cetus, Norwalk, CT) at 94°C for 2 min. After the addition of 0.7 μmol of each of the four deoxyribonucleotide triphosphates and 0.125 units of Taq polymerase (Perkin Elmer Cetus), the samples were amplified using consecutive annealing temperatures of 65°C for 2 cycles, 60°C for 3 cycles, and 55°C for 25 cycles.

Nucleotide Sequence Analysis. Amplified segments of p53 were cloned into the T-A Cloning vector pCR II (Invitrogen Corp., San Diego, CA) and transformed into INVaF competent Escherichia coli (Invitrogen). For each of the four segments of p53 amplified from each sample, plasmid preparations were made from nine individual colonies of transformed E. coli, and the inserts

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6 The abbreviations used are: BCC, basal cell carcinoma; NMSC, nonmelanoma skin cancer; SCC, squamous cell carcinoma; SSCP, single-strand conformation polymorphism.
were sequenced in both directions from M13 and T7 priming sites by using the Sequenase Version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH).

Evaluation of Carcinogen Exposure. Detailed information on suspected risk factors for skin cancer, including demographics, skin type, history of sunburns, occupational and recreational sun exposure, previous history of cancer, family history of cancer, tobacco usage, and exposure to other known carcinogens, was ascertained by conducting standardized telephone interviews with the patients. Because patient 5 was deceased when the survey was conducted, her daughter served as a proxy for the interview. Medical records of all eight patients were reviewed to obtain information on chemotherapy or radiotherapy undergone before surgical resection of any of the tissue samples because such treatments may influence the number and spectrum of mutations.

RESULTS

p53 Mutations in Tumor and Adjacent Nonmalignant Skin. To gather molecular evidence of field cancerization of the head and neck skin, we analyzed mutations in the p53 tumor suppressor gene in samples of histologically proven tumor and adjacent nonmalignant skin from the eight patients treated for NMSC of the head and neck. Representative photomicrographs of SCC and BCC (from patients 1 and 6, respectively), along with adjacent nonmalignant areas, are presented in Fig. 1. With the exception of tumor tissue from patient 4, all tissue samples harbored p53 mutations, as determined by DNA sequence analysis of cloned PCR products (Table 1). Although multiple mutations were present in both tumor tissues and adjacent nonmalignant skin samples, the mutations were always distinct from one another (Table 1; Fig. 2). To further confirm the absence of germ-line transmission of p53 mutations (26), we examined the available lymphocyte samples from two patients (patients 1 and 6). None of the mutations present in the corresponding tumor or adjacent tissue samples were identified in the lymphocytes.

Apart from one tandem triple base alteration (CCC→TTT in the morphologically normal skin from patient 2) and one tandem double base alteration (GT→AA in the tumor from patient 3), all of the mutations were single base changes. Of the 45 different codons altered in the 8 patients, as many as 19 (42%) were synonymous substitutions that did not alter the corresponding amino acids (Table 1), and in only two instances were previously defined skin cancer hotspots affected (codons 177 and 258). Fifteen (35%) of the 43 single base substitutions were C→T transitions, of which 13 (30%) were located at adjacent pyrimidine sequences (Table 2). There was no noticeable strand bias in the mutations, except for in those of patient 3. The mutations at dipyrimidine sequences were always on the nontranscribed strand for this patient. There were no C→T mutations at CpG sequences. The remaining single base substitutions consisted of 20 (47%) T→C transitions, 5 (12%) G→T transversions, 2 (5%) T→A transversions, and 1 (2%) T→G transversion (Tables 1 and 2). Except for the T→G transversion, which was present in an adjacent morphologically normal skin sample from patient 7, all types of nucleotide substitutions were detected in both tumor and adjacent tissues.

Patient Information. Table 3 presents the demographic, histological, and carcinogen exposure profiles of the eight patients with NMSC of the head and neck. The patients included four Caucasian men, two Caucasian women, one Hispanic man, and one Hispanic woman. The mean age of the group was 57 (range, 37–74). With the exception of patients 2 and 7, all patients were current smokers and had a history of smoking 20–40 cigarettes daily from their teenage or early adult years. Although patient 2 was a relatively light smoker, he also chewed tobacco. The mothers of patients 3 and 8 had skin cancer. The mother of patient 5 had breast cancer and the father had lung cancer. Patient 6 had a previous tongue tumor and had undergone radiotherapy in 1983. The degree of sun exposure during the last 20 years ranged from moderate to extensive, with patients 1, 2, 5, and 8 reporting severe blistering sunburns before age 16. Most patients were also exposed to other carcinogenic agents including liquid and vaporized products at oil wells, solvents, such as those used in carpentry, and chemicals used in agriculture.

DISCUSSION

Field Cancerization. Field cancerization has been investigated previously in the aerodigestive tract and is postulated to develop from prolonged exposure to carcinogens (13, 16). Numerous mutations accrue throughout the field or area sharing the environmental exposure, and some of the initiated cells progress to multiple primary neoplasms. The importance of determining the extent of field cancerization in a given anatomic site lies in the fact that initiated fields require substantially different strategies for prevention and intervention than do isolated tumors, which may be treated by surgery alone. As the risk of developing cancer in the entire exposed field remains high for several years, it is important to have extended follow-up periods and to attempt to block or reverse the progression from "condemned" field to multiple primary tumors.
Although metastases and recurrences often display mutations that are also harbored by the primary tumor, recent studies have demonstrated that multiple neoplasms arising in cancerized fields bear discordant p53 mutations (16, 17). Alterations in p53 are the genetic lesions observed most frequently in human cancers (18), and detailed characterization of these alterations in various types of tumors makes the gene an ideal candidate for molecular assessment of field cancerization (16). In addition, unlike colorectal cancers, which develop p53 mutations as one of the late events of tumor progression (27), NMSCs harbor p53 mutations during the early stages, before the onset of invasive properties (28-30). In our study of NMSC of the head and neck, we have extended the examination of p53 mutations to areas adjacent to the tumors (which are subjected essentially to the same environmental influences as the tumors). Our analysis supports strongly the notion of field cancerization in the head and neck skin because tumors and adjacent areas of nonmalignant skin were found to contain distinct p53 mutations (Table 1). In another recent investigation, five patients with more than one actinic keratosis occurring on sun-damaged areas of the arm were also found to harbor distinct p53 mutations (31). A previous report of elevated and immunohistochemically detectable levels of the p53 gene product (often associated with p53 mutations; Ref. 32) in keratinocytes surrounding BCCs of the head and neck also complements our results (28). In another study, 17 of 23 normal skin samples from exposed sites of Australian skin cancer patients were found to contain p53 mutations, whereas only 1 of 20 samples taken from the unexposed buttocks harbored the mu-

Table 1  
*p53 Mutations in tumor and adjacent tissues of eight NMSC patients*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Codon</th>
<th>Sequence change</th>
<th>Amino acid change</th>
<th>Exon</th>
<th>Codon</th>
<th>Sequence change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>90</td>
<td>TCC → TTC</td>
<td>Ser → Phe</td>
<td>6</td>
<td>187</td>
<td>GGT → GCC</td>
<td>Gly → Gly</td>
</tr>
<tr>
<td>4</td>
<td>111</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>143</td>
<td>GGT → GCC</td>
<td>Gly → Gly</td>
</tr>
<tr>
<td>5</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>202</td>
<td>GGT → AGT</td>
<td>Gly → Ser</td>
</tr>
<tr>
<td>5</td>
<td>141</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>326</td>
<td>GAA → GGA</td>
<td>Gly → Gly</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>177</td>
<td>GCC → TTT</td>
<td>Gly → Gly</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>102</td>
<td>ACC → GCC</td>
<td>Thr → Ala</td>
</tr>
<tr>
<td>6</td>
<td>205</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>143</td>
<td>GGT → GCC</td>
<td>Val → Ala</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>111</td>
<td>GGC → AGC</td>
<td>Gly → Gly</td>
<td>4</td>
<td>65</td>
<td>AGA → AGG</td>
<td>Arg → Arg</td>
</tr>
<tr>
<td>5</td>
<td>145</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>63</td>
<td>GCT → GCC</td>
<td>Ala → Ala</td>
</tr>
<tr>
<td>5</td>
<td>156</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>52</td>
<td>CCA → CCT</td>
<td>Pro → Ser</td>
</tr>
<tr>
<td>6</td>
<td>215</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>67</td>
<td>CCA → CCT</td>
<td>Pro → Pro</td>
</tr>
<tr>
<td>7</td>
<td>258</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>119</td>
<td>GCC → ACC</td>
<td>Ala → Ala</td>
</tr>
<tr>
<td>9</td>
<td>325</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>213</td>
<td>CAG → CGG</td>
<td>Arg → Arg</td>
</tr>
</tbody>
</table>

*Synonymous substitution.*

Fig. 2. Multiple discordant mutations detected in the tumor (A) and morphologically normal adjacent tissue (B) from patient 1. Numbers below the sequences indicate affected codons, the sequences of which are presented on the right. Coding strand sequences run from top to bottom, and arrows point to the altered bases. Note that an intron is located in the genomic sequence between the second and third bases of codon 187.
p53 mutations in seven cases (88%) with no apparent distinction between basal and basosquamous carcinomas of the head and neck revealed multiple missense mutations (33). Biopsies of unexposed skin from the buttocks or the upper inner arms of 15 French (33) and 10 American (34) control subjects were also found to lack p53 mutations.

There are also reports of p53 mutations in carcinogen-exposed normal tissue from other organs. For example, samples from nonmalignant human livers that were naturally exposed to varying levels of aflatoxin B1 have been reported to contain specific p53 mutations at frequencies that parallel the degree of exposure to the toxin (35). Therefore, the alterations in p53 probably depend on the nature, duration, and intensity of carcinogen exposure. In fact, it has been suggested that measurement of mutations may serve as a relevant indicator of carcinogen exposure and provide information about the risk of developing subsequent cancers (33).

**p53 Mutations in BCC and SCC.** Using the rapid screening procedures of direct sequence analysis and, in some cases, SSCP analysis (36), a number of laboratories have reported that between 15 and 69% of SCCs (19, 25, 29, 34, 37-40) and 12 and 56% of BCCs (38-40) exhibit p53 mutations. Our examination of cloned segments of p53 from eight NMSC (three basal, three squamous, and two basosquamous carcinomas) of the head and neck revealed multiple p53 mutations in seven cases (88%) with no apparent distinction between basal and basosquamous carcinomas of the head and neck revealed multiple p53 mutations in seven cases (88%) with no apparent distinction based on tumor histology. In six tumors (75%), these mutations led to at least two distinct amino acid replacements/sample. Such coding changes occurred mostly in the site-specific DNA-binding core domain (41) or within the minimal unit required for tetramerization (42).

This high incidence rate of p53 mutations in skin cancers is in accordance with the reports from Shea et al. (28), who detected the overexpression of a mutant p53 protein in 30 of 36 BCCs (83%) of the head and neck. Our previous studies on UV-induced murine skin cancers also corroborate the high rate of p53 mutations, because all skin tumors examined were found to harbor p53 mutations (43).

The discordance among published reports regarding the incidence rates of p53 mutations in NMSC (19, 25, 29, 34, 37-40) may be a result of differences in sensitivity of the procedures used for screening mutations, as well as genetic and environmental factors. The method of direct sequencing reveals the predominant alleles present in the entire sample and, therefore, is affected by the presence of wild-type sequences (29) and intratumor heterogeneity of mutations (43). Although the relatively rapid procedure of SSCP analysis may reveal the presence of multiple heterogeneous mutations, many mutations remain undetected by this method (43). Moreover, unless individual bands from SSCP gels are reamplified and sequenced individually, it is not possible to correlate them with specific mutations. Another reason for differences may be that, although most studies examine only the functionally important domains within exons 5-8 of the p53 gene (41, 44), others also include flanking sequences. In this study, 47% of the affected codons were located in exon 4 (Table 1). The clinical significance of the occurrence of a substantial number of exon 4 mutations in human NMSC is not known as yet. Because our study was designed to examine mutations induced by field effects, and not only those that predominate in the tumors as a result of clonal expansion (plasmid preparations were not pooled before sequencing), a relatively large number of silent mutations were detected (Table 1). The incidence of p53 mutations may also depend on the clinical phenotype of the tumors. A relatively large number of patients with advanced BCC or SCC are treated at M. D. Anderson Cancer Center, and this may have contributed to the number of p53 mutations found in this study.

### Table 2: Nature of mutations identified in the p53 tumor suppressor gene

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor tissue mutations (n)</th>
<th>Adjacent tissue mutations (n)</th>
<th>Distinct mutationsa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C → T</td>
<td>T → C</td>
<td>G → T</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>

a Distinct mutations indicates whether mutations in the adjacent tissue were distinct from those identified in the tumor tissue from the same patient.

b NA, not applicable.

### Table 3: Characteristics of eight patients with NMSC of the head and neck

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Race b</th>
<th>Sexa</th>
<th>Complexion</th>
<th>Skin typec</th>
<th>Family history of cancer</th>
<th>Occupation</th>
<th>Tumor phenotypec</th>
<th>Tumor histology</th>
<th>Sun exposure (sunburns)</th>
<th>Cigarettes per day (years)</th>
<th>Other carcinogen exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>C</td>
<td>M</td>
<td>Fair</td>
<td>II</td>
<td>none</td>
<td>oil well drill operator</td>
<td>+</td>
<td>squamous</td>
<td>moderate (&gt;)5</td>
<td>20 (53)</td>
<td>agricultural chemicals and petrochemicals</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>C</td>
<td>M</td>
<td>Fair</td>
<td>IV</td>
<td>skin—mother</td>
<td>geologist</td>
<td>+</td>
<td>basosquamous</td>
<td>extensive (1)</td>
<td>1–2 (20)</td>
<td>agricultural chemicals and petrochemicals</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>C</td>
<td>M</td>
<td>Dark</td>
<td>IV</td>
<td>skin—mother</td>
<td>oil well serviceman</td>
<td>+</td>
<td>squamous</td>
<td>extensive (0)</td>
<td>40 (50)</td>
<td>agricultural chemicals and petrochemicals</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>C</td>
<td>F</td>
<td>Fair</td>
<td>III</td>
<td>none</td>
<td>teacher</td>
<td>+</td>
<td>basal</td>
<td>moderate (NR)</td>
<td>20–40 (20)</td>
<td>none</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>C</td>
<td>M</td>
<td>Medium</td>
<td>I</td>
<td>breast—mother</td>
<td>nurse</td>
<td>+</td>
<td>squamous</td>
<td>moderate (&gt;)5</td>
<td>40 (40)</td>
<td>agricultural chemicals</td>
</tr>
<tr>
<td>6</td>
<td>74</td>
<td>H</td>
<td>M</td>
<td>Fair</td>
<td>III</td>
<td>lung—father</td>
<td>none</td>
<td>+</td>
<td>squamous</td>
<td>extensive (0)</td>
<td>20 (53)</td>
<td>agricultural chemicals and radiotherapy</td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>H</td>
<td>F</td>
<td>Dark</td>
<td>III</td>
<td>skin—mother</td>
<td>farmer</td>
<td>+</td>
<td>squamous</td>
<td>extensive (0)</td>
<td>0</td>
<td>agricultural chemicals and solvents</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>C</td>
<td>M</td>
<td>Medium</td>
<td>IV</td>
<td>skin—mother</td>
<td>carpenter</td>
<td>+</td>
<td>squamous</td>
<td>extensive (&gt;)5</td>
<td>40 (50)</td>
<td>agricultural chemicals</td>
</tr>
</tbody>
</table>

a C, Caucasian; H, Hispanic.
b M, Male; F, Female.
c Fitzpatrick skin type: Type I, severe burning (blistering) and no tanning; Type II, moderate burning and mild tanning; Type III, mild burning and moderate tanning; Type IV, rare burning, easy tanning.

d Tumor phenotype: +, aggressive; −, non-aggressive. The clinical phenotype of the tumor was characterized as aggressive if the tumor exceeded 2 cm in diameter; invaded muscle, cartilage, bone, or nerve; or if metastasized to regional lymph nodes.
e Occupational and recreational exposure to the sun during the last 20 years. Moderate, <20 h/week. Extensive, >20 h/week. Numbers of severe blistering sunburns before age 16 are presented in parentheses. NR, not reported.
Intrasample Heterogeneity of p53 Mutations. The multiple mutations found in our study by sequencing cloned segments, underscore the complex, heterogeneous nature of the somatic mutations in tissue samples (Table 1; Fig. 2). A high level of cytogenetic heterogeneity was reported previously in BCC and other epidermal skin tumors (13). It has been suggested that at least some BCC may have a multicellular origin and may appear to be polyclonal or monoclonal depending on the point in clonal evolution at which the tumors are examined (15). In a previous study involving UV-induced murine skin cancers, we have observed heterogeneity with respect to p53 mutations within individual samples (43). Such mutations may also arise as a result of continued exposure to environmental carcinogens. Our recent analysis of one of the heterogeneous murine tumors showed that the mutations are distributed within the tumor and are separable into distinct clonal lines.\(^5\) These studies, along with other reports of multiple p53 mutations in skin lesions (31), unequivocally confirm the existence of intratumor heterogeneity of p53 mutations.

The multiple heterogeneous mutations may be detected by isolation of individual mutant alleles from the PCR products by cloning into suitable vectors before sequencing of multiple clones (see “Materials and Methods”) but may be missed by direct sequencing of the PCR products.\(^5\) We believe that the mutations observed in this study are not artifacts of PCR amplification with Taq polymerase because: (a) relatively stringent conditions were maintained during PCR amplification; (b) no mutations were observed in the lymphocyte samples, which were examined in exactly the same manner; (c) twice as many mutations were detected in the tumor tissues compared with the nonmalignant tissues; (d) we have demonstrated previously the reproducibility of our assays in 11 samples by amplifying and sequencing each segment twice (43). The same set of mutations in the p53 gene was always associated with any given sample; and (e) we have separated clonal lines containing distinct p53 mutations from a tumor that was initially shown to contain multiple mutations by our assay (43).\(^5\)

UV and Other Carcinogens in the Etiology of NMSCs. The nature of the mutations detected in this study provide important clues on the identity of local environmental mutagens that may contribute to the carcinogenic process (Table 2). Although three patients reported having at least one parent with a history of cancer, the mutations within exons 4–9 of p53 were heterogeneous and did not appear to be present in the germ line. The absence of germ-line mutations was confirmed in the lymphocyte samples available from two patients. In addition, no C—T transitions at CpG sites were identified, indicating that external mutagenic agents were responsible for the bulk of the mutations (45). With two exceptions, the identified mutations did not coincide with known skin cancer hotspots (37). The p53 mutations commonly reported in NMSC are the C—T and CC—TT transitions that are often associated with exposure to UV radiation (19, 25, 37, 40). This is also the case in experiments using animal models in which UV is the only carcinogen to which the animals are exposed (43, 46). In addition, mutations of this type predominate in NMSC from patients with xeroderma pigmentosum, a disease in which repair of UV-induced DNA damage is impaired (47, 48). This study, however, showed that only 30% of the single base substitutions were UV-related, whereas 47% were T—C transitions (Table 2). As may be expected in fields of cancerization, both types of transitions were present in the corresponding tumor and adjacent tissues. It is curious that 75% of the T—C transitions were also located at dipyrimidine sequences. On considering only those single nucleotide substitutions that led to amino acid replacements, 33% of the mutations in either tumor or adjacent tissue could have been induced by UV, whereas T—C transitions constituted 56 and 50% of the changes in tumor and adjacent tissues, respectively. In human cancers as a whole, T—C mutations constitute only 10% of all mutations reported in the p53 gene (49), and, at present, it is unclear whether these transitions represent any particular signature mutation. In a study of p53 mutations in breast cancer, however, T—C transitions were found to be present more often in a cohort of African-American women with a relatively high mortality rate (49).

Three of the patients (2, 3, and 8) also exhibited G—T mutations (Table 2), which are often associated with exposure to carcinogens found in cigarette smoke (22). These patients had no known exposure to γ-radiation (another possible etiological agent associated with G—T transversions; 23), but all had a long history of smoking (Table 3). A few G—T transversions were reported in previous studies on skin tumors, but the carcinogen exposures of the patients in those studies were not documented extensively (19, 25, 29, 38, 39). Although the G—T transversions detected in this study do not result in any change in the amino acid sequence of p53, it is possible that, in other cases, such transversions may result in amino acid replacements that lead to inactivation of tumor suppressive properties.

It is interesting to note that, of the eight patients whose samples were subjected to p53 sequence analysis, seven reported occupational exposure to liquid and vaporized products at oil wells, agricultural chemicals, or solvents such as those used in carpentry (Table 3). The tumors were classified as aggressive in five patients (Table 3), and studies are being initiated to investigate the relationship between occupational exposure to carcinogens and the unusually aggressive phenotype of the NMSCs that are encountered here.

Concluding Comments. The multifocal nature of NMSC of the head and neck is a major contributory factor to the high degree of morbidity associated with the disease. We present molecular evidence of field cancerization of the skin based on extensive sequence analysis of the p53 gene in tumor and adjacent nonmalignant skin from patients with NMSC of the head and neck. Because of the limited number of patients examined in this study, it is not possible to make any conclusions about the etiology of NMSC treated at our institute. However, the prevalence of T—C transitions is particularly interesting given a recent report on the high incidence of such transitions in a cohort of women with high mortality from breast cancer (49). It is clear that a number of carcinogens may act in concert with UV radiation in the induction of field cancerization of the head and neck skin. Extensive molecular epidemiological investigations are necessary to elucidate the specific risk factors associated with NMSC, especially in regions such as ours, in which the patients report substantial occupational exposure to carcinogens, and the tumors often display an unusually aggressive phenotype.

REFERENCES


\(^5\) S. Kanjiyal and H. N. Ananthaswamy, manuscript in preparation.

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p53 Mutations in Nonmelanoma Skin Cancer of the Head and Neck: Molecular Evidence for Field Cancerization

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