Frequent p53 Mutations in Head and Neck Cancer

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ABSTRACT

Squamous cell carcinomas of the head and neck (SCCHN) are associated strongly with the use of tobacco and alcohol, but little is known about the molecular pathogenesis of these tumors. In the present study, we analyzed SCCHN for mutations in the tumor suppressor gene p53 by immunocytochemistry and complementary DNA sequencing. Overexpression of p53 protein was detected in 13 (100%) of 13 SCCHN cell lines and in tumor cells cultured directly from 10 (77%) of 13 patients with SCCHN. Direct evidence for p53 mutations was obtained by sequencing p53 complementary DNA from eight SCCHN cell lines and two xenografts. The genetic alterations included seven missense mutations resulting in single amino acid substitutions, a mutation encoding a stop codon, one 10-base pair deletion, and one 2-base pair addition. All seven missense mutations were G to T transversions, five of which were clustered at codons 245 and 248. A similar high frequency of G to T transversions predominates in lung cancer, another tobacco-related disease. Mutation of the p53 gene is the most common genetic alteration detected in SCCHN and implicates this gene locus as a critical site of specific damage by mutagenic carcinogens in tobacco, one of the important risk factors in the etiology of this disease.

INTRODUCTION

SCCHN4 is the sixth most frequent cancer in the world; an estimated 378,500 new cases were diagnosed in 1980, the latest date for which world-wide incidence data are available (1). In the United States, an estimated 44,000 new cases were detected and nearly 12,000 deaths were reported last year (2). The overall 5-year survival rate for patients with head and neck cancer is among the lowest of the major cancers and has not changed during the past two decades. Progress in understanding the etiology and pathogenesis of head and neck tumors has been slowed by a lack of molecular studies investigating genetic alterations that underlie the development and progression of these cancers. Two of the strongest risk factors for SCCHN are use of tobacco and consumption of alcohol. Tobacco smoking is also a major risk factor in human lung and esophageal cancer. Recent evidence has demonstrated that mutations in the tumor suppressor gene p53 are common in these cancers (3–6). Tumor suppressor genes function as negative regulators of cellular proliferation. Their inactivation, either by deletion or mutation, results in loss of function and unrestrained cellular growth (7). The p53 gene is frequently lost or mutated in several kinds of tumors, including colon, lung, breast, brain, ovary, and esophagus (reviewed in Ref. 8), implicating p53 as an important tumor suppressor gene. Given the common epidemiological risk factors associated with cancers of the aerodigestive tract, we suspected that p53 mutations might be an important genetic alteration in head and neck cancers. In the present study, we used both immunocytochemistry and cDNA sequencing to assess p53 gene mutations in cell lines derived from SCCHN, cells cultured as primary explants from tumor tissue, and SCCHN xenografts in nude mice. The results demonstrate a high frequency of p53 mutations in SCCHN with preferential G to T transversions clustered at codons 245 and 248.

MATERIALS AND METHODS

Cell Lines and Tumor Samples. Squamous carcinoma cell lines derived from the larynx (UMSCC10A, UMSCC10B, UMSCC16), tongue (UMSCC19), epiglottis (UMSCC23), and facial skin (UMSCC63) were provided by T. E. Carey. A SCC of the hypopharynx (FaDu), three tongue carcinoma lines (SCC4, SCC15, SCC25), a SCC of the vulva (A431), and a SCC of the cervix (SiHa) were obtained from the American Type Culture Collection. SCC lines derived from an alveolar ridge (EVSCC1), a tonsil (EVSCC3), and a floor of the mouth (EVSCC4) tumor were established in culture by us from nude mouse xenografts. Cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum or under conditions recommended by the suppliers. Tissue specimens from patients undergoing surgery for head and neck cancer and from head and neck tumors propagated as xenografts in nude mice (9) were used to initiate primary cell cultures. Primary tumor, adjacent normal tissue, and metastases (when present) were dissociated by collagenase treatment as described previously (10), seeded into flasks precoated with type I collagen (Collagen Corp., Palo Alto, CA), and grown in serum-free PC-1 medium (Endotronics, Minneapolis, MN). Tumors were staged by the tumor-node-metastasis (TNM) staging system for classification of head and neck cancers: extent of primary tumor (T), status of regional lymph nodes (N), and absence or presence of metastatic spread (M). Regional lymph node classification was based on size (N1, N2, N3), number (single [a] or multiple [b]), and location (homolateral [N1, N2] or bilateral [N3]). TNM categories are grouped into Stages I to IV that determine treatment and prognosis.

Immunocytochemical Analysis. Cells from fresh tissue specimens or tissue culture cells were plated onto glass coverslips, fixed in 95% ethanol at room temperature for 10 min, washed with phosphate-buffered saline, and incubated at room temperature for 30 min with monoclonal antibody PAb 1801 or PAb 240 (Oncogene Science, Uniondale, NY). PAb 1801 recognizes an epitope on the NH2-terminal domain of both mutant and wild-type human p53. PAB 240 recognizes an epitope between amino acids 156 and 355 of mutant p53. Isootype-matched normal mouse IgG was used as a negative control antibody. Primary antibody binding was detected by using the avidin-biotin-complex immunoperoxidase system (Vector Laboratories, Burlingame, CA), following manufacturer's recommendations.

RNA Extraction. Total cellular RNA was prepared from tissue culture cells or tumor tissue by the guanidinium isothiocyanate/phenol/chloroform extraction method (11).

PCR Amplification and Sequencing of p53. One µg of total RNA was reverse transcribed using oligodeoxythymidylate and Moloney-murine leukemia virus reverse transcriptase (BRL, Bethesda, MD). Following inactivation, one-tenth (2 µl) of the reaction was amplified by PCR with Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) and primers P53CL1 (5'-CTCGGTGTTCTCTGACGACCC-3') and P53CL2 (5'-CTGTTGGTGCCTCAGGACGACCC-3') which encompass the entire coding domain of the p53 gene, using an Erycop temperature cycler (Ercropic, San Diego, CA). The 1200-base pair product was

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4 The abbreviations used are: SCCHN, squamous cell carcinoma of head and neck; SCC, squamous cell carcinoma; cDNA, complementary DNA; PCR, polymerase chain reaction.
purified by ammonium acetate precipitation, and reamplified by asymmetric PCR (12, 13), using primers P53S1 (5′-CAGACTGCCTTC-GGGTGTCAC-3′) and P53AS1 (5′-GGAGGCTGTCACTGGGAAAC-3′) at a 0.01:1 ratio. The method of asymmetric PCR and direct sequencing without a cloning step was specifically chosen to reduce the possibility of identifying false positives introduced by the PCR amplification method itself. The predominantly single-strand product was purified by using Centricon 100 columns (Amicon, Beverly, MA), and sequenced by the dideoxy chain termination method with the use of a Sequenase 2.0 kit (US Biochemicals, Cleveland, OH). Samples were reamplified and resequenced for confirmation of mutations. The following internal sequence primers were used:

for positive strand sequence,

P53S1 5′-CAGACTGCCTTCGGGGTGTCAC-3′; P53SQ15 5′-TTTGATGCTGTCCCCGGACG-3′; P53SQ1 5′-CCTGTATCTTCTGTCCTTTCCAGC-3′; P53SQ5 5′-CCTCCCCCTGCCTCAACAAG-3′; P53SQ9 5′-GGCCATCTACAAGCAGTCC-3′; P53SQ7 5′-GTTGCTGCTAGCTTACCAAC-3′; P53SQ16 5′-CAGAGGAAGAAGAATCTGCACG-3′; P53SQ17 5′-CAGATCCGTGGCGTGAGC-3′;

for negative strand sequence:

P53SQ3 5′-CTAGGCGGCTCATAGGGCAC-3′; P53SQ6 5′-CATCGCTATCTGAGCAGCG-3′; P53SQ8 5′-CTCGG1TAGTGCTCCCTGG-3′; P53SQ10 5′-CTGGAATCTTCATGCTGG-3′;

The sequencing reaction mixes were separated by electrophoresis on 6% polyacrylamide gels.

RESULTS AND DISCUSSION

Clinicopathological features of the patients with SCCHN are shown in Tables 1 and 2. Patient age at tumor diagnosis ranged from 19 to 85 years. Nearly all patients had a history of chronic smoking and alcohol use. Three exceptions were patient EVSCC1, 55 years old, patient EVSCC7PX, 19 years old, and patient EVSCC17PX, 34 years old; all had no previous history of tobacco or alcohol use. We evaluated p53 protein expression in 13 cell lines derived from SCCHN by immunocytochemical staining with anti-p53 monoclonal antibodies PAB 1801 and PAB 240. Point mutations in the p53 gene result in an increased stability of the mutant protein which can be detected by immunocytochemistry (14). This approach has been used successfully for the screening for p53 mutations in breast, colorectal, and lung cancer (4, 15, 16).

Table 1 shows that all 13 of the SCCHN cell lines were positive for increased p53 expression using the two antibodies. In 11 of the cell lines, p53 immunostaining with PAB 1801 and PAB 240 was concordant, with positive cells demonstrating strong nuclear staining (Fig. 1, a and d). Two of the cell lines (SCC25, EVSSC1), did not react with PAB 1801, but stained positive with PAB 240, which recognizes a different epitope on mutant p53. Staining with PAB 240 was less intense and, in the case of EVSSC1, localized to the cytoplasm (Fig. 1c). p53 antibodies were nonreactive with SiHa cells (Fig. 1b), shown previously to contain wild-type p53 (17).

To verify that overexpression of p53 protein in SCCHN cell lines was due to a mutated p53 gene, we reverse transcribed RNA from 8 cell lines and 2 tumor xenografts, and amplified the cDNA by PCR. As a control we sequenced the cDNA from A431, an epidermoid carcinoma cell line, shown previously to contain a mutated p53 gene, CGT to CAT (Arg to His) at codon 273 (18). p53 gene mutations were detected in all 8 cell lines and both tumor xenografts (Fig. 2A). In each case analyzed, there was a direct correlation between abnormal p53 protein expression and gene mutation (Table 1). We confirmed a point mutation at codon 273 in A431 (data not shown). Six of the SCCHN cell lines and one xenograft harbored missense point mutations, all of which were G to T transversions. Five of the missense mutations resulting in amino acid substitutions were at codons 245 and 248, and one each at codons 242 and 176. UMSGAC10A and UMSGAC10B are a primary and recurrent laryngeal tumor, respectively, derived from the same patient. Both lines have the same p53 mutation at codon 245, indicating that the recurrent tumor was derived from the same clone. EVSSC1 had a nonsense mutation at codon 213 resulting in chain termination, and did not retain a wild-type allele (Fig. 2B). By immunostaining, p53 was specifically localized to the cytoplasm of EVSSC1 cells (Fig. 1c). This result is consistent

### Table 1 Clinicopathological features and alterations of p53 in SCCHN cell lines and tumor xenografts

<table>
<thead>
<tr>
<th>Source</th>
<th>Site</th>
<th>Age</th>
<th>Sex</th>
<th>Stage</th>
<th>Tobacco pack (yr)</th>
<th>p53 immunostaining**</th>
<th>p53 mutation</th>
<th>Codon</th>
<th>Mutation</th>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>248</td>
<td>CGG → CTG</td>
<td>Arg → Leu</td>
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**Tumor sites: hypopharynx (HPX), larynx (LX), tongue (TG), alveolar ridge (AR), tonsil (TL), floor of mouth (FOM), vulva (VUL), cervix (CX).

** Packs/day x years smoked.

** Staining is defined as (+) strongly positive nuclear staining in all cells; (±) nuclear staining in some cells, (−) no staining.

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Table 2 Clinicopathological features and p53 overexpression in primary explant cultures of SCCHN

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<td>M</td>
<td>IV</td>
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*a Tumor sites: tonsil (TL), hypopharynx (HPX), larynx (LG), tongue (TG), buccal cavity (BC).

b PX suffix designates primary tumor grown as nude mouse xenograft; N, P, M are normal tissue, primary tumor, or metastasis, respectively.

Fig. 1. Immunocytochemical staining of p53 in SCCHN. a, positive nuclear staining of FaDu cells; b, negative staining of SiHa cells; c, positive cytoplasmic staining of EVSCC1 cells; d, positive nuclear staining of UMSCC108 cells; e, positive nuclear staining of fresh tumor sample EVSCC5PX. Cells were reacted with PAb1801 in a, b, d, and e, and with PAb240 in e.

with the predicted chain termination and loss of nuclear localization signals in the carboxyl terminus of the p53 protein (19). UMSCC19 had a 10-base pair deletion that resulted in the deletion of codons 248 to 250 inclusive, and a frame shift involving codon 251. Tumor xenograft EVSCC4X had a 2-base pair (TT) addition at codon 289–290 resulting in a frame shift. Only the mutant allele was found in 6 of the SCCHN cell lines and the 2 xenografts, indicating that the p53 locus was hemizygous or homozygous in these cells. This finding implies that the wild-type allele is either transcriptionally inactive or has been lost by the tumor cells. Both mutant and wild-type p53 alleles were detected in UMSCC23 and UMSCC63, indicating that either the normal allele was retained in the tumor cells or the cell lines are a mixture of tumor and normal cells. The latter explanation is unlikely since immunostaining with p53 antibodies showed uniform nuclear staining of all UMSCC23 and UMSCC63 cells.

Detection of p53 mutations in SCCHN cell lines prompted us to investigate the prevalence of aberrant p53 in primary explant cultures of tumors either resected directly from the patient or propagated as xenografts in nude mice. We evaluated tumor tissue from 13 patients for increased p53 protein by immunocytochemical staining with PAb 1801 and PAb 240. In two cases, normal tissue adjacent to the tumor and, in four cases,
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REFERENCES

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