Variation of \textit{p53} Mutational Spectra between Carcinoma of the Upper and Lower Respiratory Tract$^1$

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\textbf{ABSTRACT}

Mutations of the \textit{p53} tumor suppressor gene are the most common genetic alterations associated with human cancer. Tumor-associated \textit{p53} mutations often show characteristic tissue-specific profiles which may infer environmentally induced mutational mechanisms. The \textit{p53} mutational frequency and spectrum were determined for 95 carcinomas of the upper and lower respiratory tract (32 lung and 63 upper respiratory tract). Mutations were identified at a frequency of 30\% in upper respiratory tract (URT) tumors and 31\% in lung tumors. All 29 identified mutations were single-base substitutions. Comparison of the frequency of specific base substitutions between lung and URT showed a striking difference. Transitions occurred at a frequency of 68\% in URT, but only 30\% in lung. Mutations involving G:C$\rightarrow$A:T transitions, which are commonly reported in gastric and esophageal tumors, were the most frequently identified alteration in URT (11/19). Mutations involving G:C$\rightarrow$T:A transversions, which were relatively common in lung tumors (3/10) and are representative of tobacco smoke-induced mutations were rare in URT tumors (1/19). Interestingly, G:C$\rightarrow$A:T mutations at CpG sites, which are characteristic of endogenous processes, were observed frequently in URT tumors (9/19) but only rarely in lung tumors (1/10), suggesting that both endogenous and exogenous factors are responsible for the observed differences in mutational spectra between the upper and lower respiratory systems.

\textbf{INTRODUCTION}

Two common cancers with poor prognosis, high mortality, and clearly defined and overlapping environmental exposure risk factors are SCCHN$^3$ and lung cancer. SCCHN is the sixth most frequent cancer worldwide (1). In the United States, approximately 11,000 individuals die annually from SCCHN (2). There has been little improvement in the prognosis for SCCHN over the past 30 years, and it remains relatively poor, with an overall 5-year survival rate of 54\% (2, 3). Lung cancer is the leading form of cancer diagnosed in the United States with an overall incidence rate of 55.2/100,000 population (4). Cigarette smoking is widely accepted as the major risk factor for the development of lung cancer, with 80\% of lung cancer incidence attributed to exposure to tobacco smoke (5, 6). SCCHN shares tobacco smoking as a common major risk factor with lung cancer, although exposure to smokeless tobacco and alcohol are additional significant risk factors for SCCHN (7, 8).

Mutations of the \textit{p53} tumor suppressor gene are the most common genetic alteration associated with human cancer. Current evidence suggests that the wild-type \textit{p53} protein is essential for normal cell growth regulation and that its alteration or inactivation is associated with the development of cancer (9). A major function of the \textit{p53} gene is believed to be as a cell cycle check point gene (10). The \textit{p53} gene is induced by DNA damage with a resultant transient cell cycle arrest at the G$_1$-S interface (11–13). Cells lacking wild-type \textit{p53} do not display this DNA damage-induced cell cycle arrest (14, 15). The \textit{p53} gene has recently been shown to act as a transactivator of a cell cycle-associated protein that directly interacts with cyclin-dependent kinases involved in G$_1$ arrest (16, 17).

Many different point, deletion, and insertion mutations have been described which can inactivate \textit{p53}-mediated tumor suppression (18, 19). The analysis of tumor DNA has revealed that \textit{p53} mutations are usually missense mutations which lead to amino acid substitutions in the protein, and are primarily found in one of four evolutionarily conserved regions of nucleotide sequence located in exons 5–8 (20, 21). Mutations of the \textit{p53} tumor suppressor gene are common in both lung cancer (22–26) and in SCCHN (27–30).

The multistep process of carcinogenesis requires an accumulation of multiple genetic alterations in order for normal cells to progress to cancer. The role of mutations in the \textit{p53} tumor suppressor gene in human cancer has been well established. DNA damage may be induced by a number of factors including endogenous metabolites and exogenous chemical and physical carcinogetic compounds. It is known that various carcinogens can induce specific DNA base changes (31, 32). Tissue-specific mutational spectra may be indicative of the mutation-inducing

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\footnote{3}The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; URT, upper respiratory tract; LRT, lower respiratory tract.
carcinogenic agents and may reflect underlying mutational mechanisms. Therefore, the mutational spectra of the p53 gene for lung cancer and for head and neck cancer may provide information on the comparability of the underlying causes for these cancers. In the present study, the p53 mutational spectra (exons 5–8) were determined and compared for tumors of the URT and LRT.

MATERIALS AND METHODS

The specimens were surgically resected, histologically confirmed tumors obtained from patients with non-small cell lung cancer or head and neck cancer who were treated at the University of Pittsburgh Medical Center in conjunction with the Pittsburgh Cancer Institute. Specimens for this study were selected without regard to clinical stage, patient prognosis, therapy regimen, metastasis, or primary origin of tumor site in the head and neck region, except that tumors of the thyroid, esophagus, and skin were not included. Cases were collected consecutively and selection criteria were based on availability of tissue. Patients with prior treatment with radiation or chemotherapy were not excluded from the study but comprised only a small portion of the study population (15/95). Demographic information was obtained by questionnaire at the time of tissue biopsy and was entered into a computer data base by the personnel of the Pittsburgh Cancer Institute Tissue and Serum Bank.

DNA was isolated from primary fresh-frozen tumor tissue by guanidine thiocyanate extraction (33) using the commercially available IsoQuick kit (MicroProbe, Garden Grove, CA). PCR amplification and sequencing of p53 exons 5 through 8 (including exon-intron boundaries) were performed using previously published PCR primers (34). All PCR reactions were carried out in 100 μl total volume on an automatic thermocycler (Perkin Elmer/Cetus 480 or 9600). The reaction conditions were: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% w/v gelatin, 0.5 μM each deoxynucleoside triphosphate, 0.3 μM each primer, and 1.25 units Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT). The PCR thermocycler parameters for all amplifications consisted of an initial denaturation at 95°C for 5 min followed by 28 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. To confirm correct amplification, the products were subjected to electrophoresis on 1.6% agarose minigels, visualized by staining with ethidium bromide, and photographed under UV light. Removal of primers and deoxynucleoside triphosphates as well as concentrations of the PCR products was done by the use of Microcon 100 microcentrators (Amicon, Beverly, MA). Direct sequencing of the double-stranded PCR product was performed by a modification of the standard dideoxynucleotide chain terminating method (35). PCR products were directly sequenced with fluorescent dye-labeled dideoxynucleotides using Taq polymerase and cycle sequencing. The sequencing reactions were performed using the Taq DyeDeoxy Terminator Cycle Sequencing kit (ABI, Foster City, CA). Sequencing products were purified of unincorporated dye-labeled dideoxynucleotides by processing through Centri-Sep spin columns (Princeton Separations, Princeton, NJ). Sequencing products were electrophoretically fractionated through 6% denaturing polyacrylamide gels (0.4-mm thick). Electrophoresis, band visualization, and sequence analysis were automatically performed on the Applied Biosystems 373A automatic sequencer. All mutations were confirmed by sequencing both DNA strands.

RESULTS

Ninety-five tumor specimens were directly sequenced for p53 mutations in exons 5–8. Mutations were identified in 29 of these samples, for an overall frequency of approximately 31%. All identified mutations were single-base substitutions. Four were nonsense mutations leading to premature stop codons, 1 was a splice site mutation, 4 were same-sense mutations with no change in amino acid, and 20 were missense mutations leading to amino acid substitutions in the protein (Table 1). There was a total of 16 base transitions (7 C→T, 7 G→A, and 2 A→G) and 13 base transversions (8 G→C, 3 G→T, 1 C→A, 1 C→G). The overall distribution of these mutations was relatively random across p53 exons 5–8, with 8 mutations identified in exon 8, 3 in exon 7, 10 in exon 6, 7 in exon 5, and 1 splice site mutation at the 3' end of intron 4. The most frequently mutated codons were codons 221, 273, and 299. The mutation at codon 273 was identified in three different URT tumor samples and was always a G→A transition at the second nucleotide position of the codon. This missense mutation should lead to an amino acid substitution of histidine for arginine in the protein. The mutation at codon 221 was identified in four different tumor samples (three URT and one lung) and was always the same G=C base substitution at the third nucleotide position of the codon in the three lung tumors. This mutation leads to an amino acid substitution of aspartic acid for glutamic acid in the protein. The remaining codon 221 mutation was a G→A base substitution at the third base position which leads to no amino acid substitution. The codon 299 base substitution does not lead to an amino acid substitution and is not located in one of the evolutionarily conserved domains of p53. This silent mutation was found in three different URT tumor specimens, was always a G→C base substitution at the third nucleotide position of codon 299, and could not be identified in DNA extracted from the nontumor tissue of patients. Most of the identified mutations appeared to be heterozygous, with both mutant and wild-type peaks present on sequencing chromatographs (Fig. 1). Tumor specimen sectioning was performed with careful pathological review but specimens were not microdissected. The true allelic status of these mutations (homozygous or heterozygous) is difficult to assess since normal tissue contamination of the gross tumor specimen is unavoidable.

The mutational data was stratified for comparison between the URT and LRT (Table 1). The frequency of identified mutations was nearly identical at 30% (19/63) for URT tumors and 31% (10/32) for lung cancer. The functional nature of the various types of mutations did not differ dramatically between URT and LRT tumors (Fig. 2). Missense mutations made up a significant majority of all identified mutations. Mutations leading to protein truncation (splice site and premature stop codons) comprised approximately 16 and 20% of mutations identified in URT and LRT tumors, respectively.

Differences in p53 mutational profiles were observed between URT and LRT tumors. Mutations identified in lung cancer were primarily transversions (70%), whereas the muta-
which were relatively common in lung tumors (3/10), were rare
base substitutions (coding strand). URT tumor mutations in-
(C) wild-type
history of alcohol consumption (Table 2).

between the type of mutation and gender, smoking history, or
variable analyses failed to show any statistical relationship
for possible associations between specific nucleotide substitu-
served when the mutational data were further stratified to look
in URT tumors (1/19). No significant associations were ob-
associated mutations included 3 C-T, 3 G-*T, and 4 G-C
difference in observed mutational spectra (Fig. 3). Lung cancer-
p53 (EDRN 4135). Sequence around
Fig. I p53 DNA sequence electrochromatogram of an URT tumor
(EDRN 4135). Sequence around p53 codon 196 demonstrating both
wild-type (C) and mutated (T) alleles.

Table 1 p53 Mutational Spectra for URT and LRT

<table>
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<th>Sample</th>
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<th>Codon</th>
<th>Mutationa</th>
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</table>

Total analyzed (n = 63)
Mutations (19/63)
Transitions (13/19) 3 G-*T; 4 C-T; 2 A-G
Transversions (6/19) 4 G-T; 1 C-A; 1 C-G

Total analyzed (n = 32)
Mutations (10/32)
Transitions (3/10) 3 C-T
Transversions (7/10) 3 G-T: 4 G-C

a Coding strand sequence.

DISCUSSION

The combined mutational data on URT and LRT tumors suggest that p53 mutations are relatively frequent in respiratory tract cancers, and that the nature of the mutations and their distribution are not unusual when compared with p53 mutations identified in other human cancers. However, comparison of the frequency of specific base substitutions between URT and LRT tumors showed a striking difference in p53 mutational profiles. Among tissues with mutations, transversion mutations occurred twice as often in lung tumors and transition mutations twice as often in URT tumors. This difference achieved a borderline level of statistical significance (P = 0.064). This difference could not be explained by differences in environmental expo-
An even more pronounced difference in the p53 mutational spectra is observed between the URT and LRT when only functionally relevant mutations are compared. Elimination of the codon 299 (G→C) same-sense mutation identified in three URT tumors results in a URT mutational profile which consists of 81% (13/16) base transitions, of which 85% (11/13) were G:C→A:T. A difference in mutational spectrum between head and neck squamous cell carcinoma samples and lung cancer has been previously reported (37). The p53 mutational profile of URT tumors derived from our data more closely fits that of previously reported p53 mutational spectra observed in gastrointestinal tract cancers (36, 38, 39). A recent, extensive compilation and comparison of 2567 reported p53 mutations in human cancers showed a similar, albeit not as pronounced, difference between the profiles of 897 lung cancer mutations and 524 head and neck tumor mutations (36). Approximately 1.5 times as many transversions were observed in lung tumors as in head and neck tumors with a preponderance of G:C→T:A lung mutations (40%) and a preponderance of G:C→A:T (31%) head and neck mutations (36). The reasons for the observed difference in mutational profiles between the URT and LRT is uncertain, but may reflect differences in both exogenously induced tissue-specific mutational mechanisms and underlying endogenously induced mutation rate variation between the tissues of the upper and lower respiratory systems. The G:C→A:T mutations at CpG dinucleotides are due to frequent methylation of cytosine to 5-methylcytosine and subsequent spontaneous deamination to thymine, resulting in G:T mismatches which may not be repaired accurately (40). This mutational mechanism is an endogenous process for which no exogenous factors have yet been identified that alter the frequency of methylation, deamination, or the efficiency of repair leading to these CpG-related mutations (41). In our study, G:C→A:T mutations at CpG sites were observed in 47% of URT tumors but only in 10% of lung tumors. This suggests that endogenous biological factors may play a role in determining some of the observed differences in the p53 mutational spectra between the upper and lower respiratory systems. The reasons for differences in endogenous tissue-specific mutation rates for the p53 gene, as ascertained by CpG mutations, is unknown but has been well documented (reviewed in Ref. 41).

The higher frequency of G:C→T:A mutations in lung tumors is representative of the type of mutation known to be caused by polycyclic aromatic hydrocarbons, most notably benz(a)pyrene, that are present in tobacco smoke. The upper aerodigestive and LRTs share tobacco smoke as a significant common exogenous mutagenic risk factor. However, our data suggest that either different tobacco-related mutagens or different mutational mechanisms are involved in p53 mutation in URT and LRT cancers. Tobacco smoke consists of many potentially mutagenic substances, some of which have been well characterized, such as N-nitrosamines and polycyclic aromatic hydrocarbons, but tobacco smoke also consists of many other compounds which have not been well defined and whose mutagenic potentials are unknown. The concentration and duration of exposure to the various compounds in tobacco smoke would be expected to vary considerably between the URT and LRT.
Additionally, the upper aerodigestive tract is exposed to different known environmental risk factors not associated with the LRT. Some of these risk factors include alcohol, sodium nitrites, and smokeless tobacco products. The fact that the p53 mutational profile observed for URT tumors was more similar to that previously reported in gastric cancer than to LRT tumors would suggest that these additional factors may play a role in determining the underlying mutational events of these tumors. There is evidence to suggest that tumors of the head and neck are associated with both alcohol and tobacco smoke (42), and that there may be a synergistic effect between the two.

Environmental agents that induce specific mutational events may exert a general field carcinization effect on the exposed tissues (43, 44). Detection of critical mutational events in these exposed tissues, at an early stage (preneoplastic lesions), may lead to earlier diagnosis and therapeutic intervention and to a better overall prognosis. A recent report has demonstrated that elevated levels of p53 are present in premalignant lesions of the head and neck (45). We are pursuing further investigation to determine whether mutations of the p53 gene are associated with early lesions of the respiratory tract.

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