

## ASSOCIATION BETWEEN CIGARETTE SMOKING AND MUTATION OF THE p53 GENE IN SQUAMOUS-CELL CARCINOMA OF THE HEAD AND NECK

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**Abstract Background.** Although epidemiologic studies have long associated tobacco and alcohol use with the development of squamous-cell carcinoma of the head and neck, the molecular targets of these carcinogens have yet to be identified. We performed a molecular analysis to determine the pattern of mutations in the p53 gene in neoplasms from patients with squamous-cell carcinoma of the head and neck and a history of tobacco or alcohol use.

**Methods.** Sequence analysis of the conserved regions of the p53 gene was performed in tumor samples from 129 patients with primary squamous-cell carcinoma of the head and neck. We then used statistical analysis to identify any patient characteristics associated with mutation of the p53 gene.

**Results.** We found p53 mutations in 42 percent of the patients (54 of 129). Fifty-eight percent of the patients who smoked cigarettes and used alcohol (37 of 64; 95 percent confidence interval, 45 to 70 percent), 33 percent of the patients who smoked but abstained from alcohol (13 of 39; 95 percent confidence interval, 19 to 50 percent), and 17 percent of the patients who neither smoked

nor drank alcohol (4 of 24, 95 percent confidence interval, 5 to 37 percent) had p53 mutations ( $P=0.001$ ). (Two patients used alcohol but did not smoke, and neither had a p53 mutation.) Furthermore, 100 percent of the mutations in the patients who neither drank nor smoked occurred at sites containing cytidine phosphate guanosine dinucleotides (potentially representing endogenous mutations) within the p53 gene (5 of 5 mutations; 95 percent confidence interval, 48 to 100 percent), whereas only 23 percent of those in cigarette smokers consisted of such changes (12 of 53 mutations; 95 percent confidence interval, 12 to 36 percent;  $P=0.001$ ).

**Conclusions.** In our study, a history of tobacco and alcohol use was associated with a high frequency of p53 mutations in patients with squamous-cell carcinoma of the head and neck. Preliminary evidence linked cigarette smoking to p53 mutations at nonendogenous mutation sites. Our findings suggest a role for tobacco in the molecular progression of squamous-cell carcinoma of the head and neck and support the epidemiologic evidence that abstinence from smoking is important to prevent head and neck cancer. (N Engl J Med 1995;332:712-7.)

**E**PIDEMIOLOGIC data have strongly linked cigarette smoking and alcohol consumption to the development of certain cancers.<sup>1,2</sup> Smoking is the most common cause of cancer-related death in the United States, and tobacco and alcohol use accounts for one third of all cancer-related deaths.<sup>1,2</sup> Tobacco and alcohol are important etiologic agents in squamous-cell carcinoma of the head and neck.<sup>3-6</sup> A large-scale prospective study determined that the relative risk of death due to cancer among smokers older than 35 years of age, as compared with nonsmokers, was 27.5 for oral and pharyngeal cancer and 10.5 for laryngeal cancer.<sup>7</sup> Repeated exposure to specific carcinogens in cigarette smoke may cause multiple neoplastic lesions in the mucosa of the aerodigestive tract (field carcinogenesis).<sup>8,9</sup> The upper aerodigestive tract, the only area in the body in which the alimentary tract and the airways form a common conduit, is an ideal site for evaluating the independent and synergistic effects of tobacco and alcohol.

The molecular targets of cigarette smoke and alcohol

have not been firmly identified. Carcinogens may leave unique "fingerprints" in the form of specific mutations that cause the initiation or progression of cancer.<sup>10,11</sup> Mutation of the p53 gene, the most common genetic alteration in human cancer, has been linked to tobacco smoking in squamous-cell carcinoma of the head and neck, as well as esophageal, lung, and bladder cancer.<sup>11,12</sup> However, this conclusion rests on studies involving small numbers of patients and often immunohistochemical evaluation,<sup>13-23</sup> a method that, because of its high false positive and false negative rates, does not always identify mutations of the p53 gene.<sup>24-27</sup> Although technically difficult and time consuming, molecular sequencing is the gold standard for detecting p53 mutations. It is the only means of identifying the pattern of p53 mutations that may result from exposure to carcinogens.<sup>11</sup> We collected samples of invasive squamous-cell carcinomas of the head and neck, sequenced the p53 gene, and attempted to determine whether any clinical characteristics correlated with mutation of the gene.

## METHODS

### Patients

One hundred forty-four consecutive patients with squamous-cell carcinoma of the head and neck who were undergoing biopsy or surgical resection at Johns Hopkins Medical institutions were prospectively entered into the study, which was approved by the appropriate institutional review board. Sixty-nine of these patients had been part of a previous study investigating the value of p53 as a molecular marker of occult tumor cells in pathological samples.<sup>28</sup> Demographic

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data were collected from the hospital charts, the cancer registry, and interviews with the patient and treating physician as necessary. Demographic data on each patient were collected by staff members who had no knowledge of the status or the type of p53 mutation present in the patient's tumor.

The history of use of tobacco and alcohol was carefully documented. Nonsmokers and nondrinkers were defined as patients who never used, rarely used, or had stopped using tobacco and alcohol, respectively, more than 20 years before being treated for head and neck cancer. Smokers and drinkers were defined as patients with moderate or heavy use of cigarettes (at least 20 pack-years) and alcohol (one or more drinks per day — one drink being defined as containing approximately 10 g of alcohol, which is equal to 1 oz [30 ml] of 86-proof hard liquor, one 3.6-oz [108-ml] glass of wine containing 12 percent alcohol, or one 12-oz [360-ml] can of beer), respectively, during the 20 years preceding their treatment for head and neck cancer.<sup>4</sup> We intended to stratify these patients according to whether they had quit using tobacco or alcohol more than 15, 10, or 5 years before treatment or were still using them at the time of our study.

The perioperative data included the tumor–node–metastasis stage of the head and neck cancer (stage I, II, III, or IV according to the staging system of the American Joint Committee on Cancer<sup>29</sup>), the site of the primary tumor, and the pathological grade of the neoplasm on light-microscopical examination. All patients were assigned to subgroups according to whether the cancer was newly diagnosed or recurrent at the time of evaluation of the p53 gene.

### Molecular Analysis

With the patient's consent, portions of the invasive tumors were collected in the operating room and immediately frozen in liquid nitrogen. The frozen specimens were microdissected to remove normal tissue (only specimens containing more than 50 percent neoplastic cells were included in the analysis), and DNA was isolated.<sup>30</sup> A 1.8-kb fragment of the p53 gene encompassing exons 5 through 9 was amplified from the frozen primary-tumor DNA by the polymerase chain reaction,<sup>31</sup> cloned, and then sequenced.<sup>32</sup> The results were confirmed with repeated amplification, cloning, and sequencing of the tumor DNA (a complete list of the specific p53 mutations in these patients is available on request).

### Statistical Analysis

The clinical and pathological findings were analyzed with respect to p53 mutations with use of the chi-square and Fisher's exact tests. The relation of multiple patient characteristics to mutations of the p53 gene was also examined by logistic regression. We used JMP 3.0 statistical software (SAS Institute, Cary, N.C.).

## RESULTS

### Characteristics of the Patients

One hundred forty-four consecutive patients with invasive squamous-cell carcinoma of the head and neck were enrolled in the study. Three patients were excluded because of a lack of demographic data (their hospital charts could not be located), and 12 other patients were excluded because cigarette-smoking and alcohol-consumption histories were not available. The demographic data were analyzed separately for the 102 patients with newly diagnosed cancer and the 27 patients with recurrent cancer (Table 1).

Of the patients with newly diagnosed squamous-cell carcinoma of the head and neck, 88 percent (90 of 102) presented with advanced stage III or IV cancer, as is typical in most tertiary cancer centers. The most common primary sites were the larynx, the oral cavity, and the oropharynx. Light-microscopical examination of

**Table 1. Characteristics of the Patients with Newly Diagnosed or Recurrent Squamous-Cell Carcinoma of the Head and Neck.**

CHARACTERISTIC	NEWLY DIAGNOSED CANCER (N = 102)	RECURRENT CANCER (N = 27)
Age (yr)*	63±13.6	63±9.7
Sex (%)		
Male	79	56
Female	21	44
Clinical stage (%)†		
I	3	39
II	9	42
III	30	4
IV	58	15
Site of primary tumor		
Oral cavity	27	26
Oropharynx	27	18
Hypopharynx	9	4
Larynx	35	52
Nasopharynx	1	0
Unknown	1	0
Pathological grade on light microscopy (%)‡		
Well differentiated	25	24
Moderately differentiated	51	60
Poorly differentiated	24	16
Cigarette smoker (%)	79	82
Alcohol drinker (%)	57	30

\*Values are means ±SD.

†According to the tumor–node–metastasis staging system of the American Joint Committee on Cancer.<sup>29</sup>

‡The degree of differentiation was known for 102 patients with newly diagnosed cancer and 25 patients with recurrent cancer.

the operative specimens, available for 86 patients, revealed that approximately half the neoplasms were moderately differentiated; the others were evenly divided between well-differentiated and poorly differentiated cancers. Most patients with newly diagnosed cancer currently smoked cigarettes (81 of 102, or 79 percent) or had smoked within the past 20 years (a history of at least 20 pack-years). Fifty-seven percent of these patients (58 of 102) also reported moderate to heavy intake of alcohol within the past 20 years. Very few patients had stopped using either tobacco or alcohol within the past 20 years; therefore, we did not subdivide the groups according to whether they had stopped smoking or drinking alcohol 5, 10, or 15 years before the study began.

Twenty-one percent of the patients (27 of 129) presented with previously treated recurrent squamous-cell carcinoma of the head and neck (Table 1). Eighty-one percent of these patients (21 of 26) had been classified as having stage I or II lesions when the original diagnosis was made (in 1 patient there was no documentation of the original stage). The stage of the recurrent neoplasms was not revised to reflect the occurrence of more advanced tumors, even though the patients typically presented with extensive locoregional disease. The primary sites of the recurrent and newly diagnosed neoplasms were similar, and the degrees of histologic differentiation (available for 127 patients) were also similar. Among the patients with recurrent cancer, 82

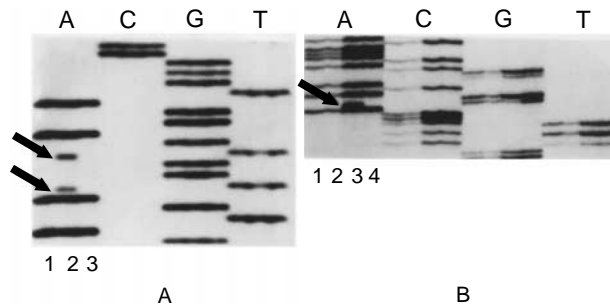


Figure 1. Autoradiographs of Mutations of the p53 Gene in Patients with Squamous-Cell Carcinoma of the Head and Neck.

Sequencing analysis of DNA from tumor samples is shown with the lanes grouped together to facilitate the identification of abnormal mutant bands (three tumors are shown in Panel A, and four tumors in Panel B). In Panel A, a tandem mutation consisting of base-pair changes (TA→AT) at codons 253 and 254 (changing threonine to serine and isoleucine to phenylalanine) is shown in lane 2 (arrows). In Panel B, a change in a single base pair (GC→AT) at codon 278 (changing proline to a stop codon) is evident in lane 3 (arrow).

percent (22 of 27) smoked and 30 percent (8 of 27) were moderate-to-heavy users of alcohol.

#### Molecular Analysis

The p53 gene was sequenced in tumor specimens from 129 patients with squamous-cell carcinoma of the head and neck, and 42 percent of the neoplasms had at least one mutation of the p53 gene (Fig. 1). Four of these tumors had tandem mutations of the p53 gene (2 apparently unrelated mutations), yielding a total of 58 mutations in 54 head and neck cancers (Table 2). The most common p53 mutations were GC→AT, GC→TA, and AT→GC. Twenty-eight percent of the p53 mutations (16 of 58) included splice sites, frame shifts, deletions, or stops. These changes would be predicted to encode truncated p53 proteins that immunohistochemical analysis usually fails to detect.

#### Statistical Analysis

Logistic-regression analysis did not reveal significant correlations between the presence or absence of p53

mutations ( $P>0.50$ ), the tumor-node-metastasis stage ( $P>0.50$ ), the pathological tumor grade ( $P>0.50$ ), or the primary site of the neoplasm in patients with either newly diagnosed cancer ( $P=0.70$ ) or recurrent cancer ( $P=0.10$ ).

By contrast, 47 percent of the tumors obtained from smokers in the group with newly diagnosed cancer (38 of 81) had p53 mutations, whereas only 14 percent of the tumors from nonsmokers (3 of 21) had mutations of the p53 gene ( $P=0.006$ ). A significant association between alcohol use and mutation of the p53 gene was also found in patients with newly diagnosed cancer. Mutations of the p53 gene were found in 55 percent of the carcinomas from the patients who drank alcohol (32 of 58), but in only 20 percent of the tumors from patients who did not drink (9 of 44,  $P<0.001$ ). The association of cigarette smoking ( $P=0.34$ ) and alcohol use ( $P=0.42$ ) with mutation of the p53 gene was not significant in the population of patients with recurrent squamous-cell carcinoma of the head and neck, possibly because of the small number of patients in that group ( $n=27$ ). Twelve of the 22 smokers with recurrent cancer had mutations of the p53 gene, whereas this was true for only 1 of the 5 nonsmokers with recurrent cancer. Tumors from 5 of the 8 alcohol drinkers with recurrent cancer had p53 mutations, whereas tumors from 8 of the 19 nondrinkers with recurrent cancer had such mutations.

The characteristics of the patients with newly diagnosed cancer were similar to those of the patients with recurrent cancer (Table 1). Almost all the patients with recurrent cancer (25 of 27) had received radiation therapy before undergoing a second tumor resection. In these patients, we did not see the deletions of the p53 gene that exposure to radiation can cause.<sup>33-36</sup> Moreover, the proportions of p53 mutations in the patients with primary (42 percent) and recurrent (50 percent) tumors and the pattern of these mutations were almost identical. Consequently, the two groups were combined for a more detailed analysis of smoking and drinking habits. In the total population of 129 patients, the association of smoking and drinking with mutations of the p53 gene was stronger than in the subgroups (Fig. 2). Tumors from patients with head and neck cancer who smoked cigarettes and drank alcohol had a 58 percent incidence of p53 mutations (95 percent confidence interval, 45 to 70 percent), those from patients who only smoked had a 33 percent incidence of p53 mutations (95 percent confidence interval, 19 to 50 percent), and those from patients who neither smoked nor drank had a 17 percent incidence of p53 mutations (95 percent confidence interval, 5 to 37 percent;  $P=0.001$ ). Only two nonsmoking patients used alcohol, and neither had a p53 mutation.

Sites containing cytidine phosphate guanosine (CpG) dinucleotides are susceptible to endogenous mechanisms of mutation. Methylation at these sites can lead to spontaneous deamination and the misincorporation of nucleotides on the complementary DNA strand. All

Table 2. p53 Mutations Identified in 54 Patients with Squamous-Cell Carcinoma of the Head and Neck.\*

BASE-PAIR CHANGE	p53 MUTATIONS	
	NO. IDENTIFIED	% OF TOTAL†
GC→CG	5	9
GC→TA	12	21
GC→AT	18	31
AT→TA	7	12
AT→GC	9	16
AT→CG	3	5
Frame shift	4	7

\*A total of 58 mutations were identified in tumor specimens from 54 patients. Frame shifts are deletions or insertions of one or more base pairs.

†Because of rounding, the values total 101 percent.

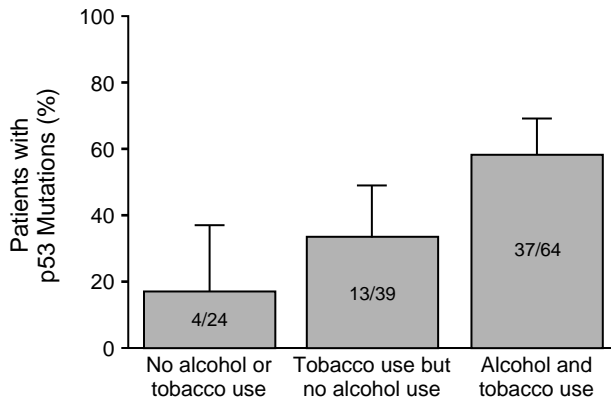


Figure 2. Association of p53 Gene Mutations with Cigarette Smoking and Alcohol Consumption in 129 Patients with Squamous-Cell Carcinoma of the Head and Neck.

The frequency of p53 gene mutations in patients with invasive squamous-cell carcinoma of the head and neck was related to the patients' exposure to cigarette tobacco and alcohol (P=0.001). Cigarette smokers who drank alcohol were 3.5 times more likely than nonsmokers who abstained from alcohol to have mutations of the p53 gene. The T bars represent the upper 95 percent confidence limit. Two nonsmokers who drank alcohol were excluded from the analysis (neither had a p53 mutation).

of the mutations in tumors from the patients with head and neck cancer and p53 mutations who neither smoked nor drank occurred at CpG sites (5 of 5 mutations; 95 percent confidence interval, 48 to 100 percent), but such mutations were found in only 23 percent of the tumors from patients with cancer and p53 mutations who smoked cigarettes (12 of 53 mutations; 95 percent confidence interval, 12 to 36 percent; P=0.001).

**DISCUSSION**

Patterns of mutations have been associated with certain environmental carcinogens.<sup>10,11,37-39</sup> We sequenced the p53 gene in tumor specimens from 129 patients with squamous-cell carcinoma of the head and neck and found that mutations of the gene correlated strongly with cigarette smoking, either alone or in combination with alcohol consumption. These mutations were 3.5 times more common among patients who both smoked cigarettes and drank alcohol than among patients who neither smoked nor drank.

A significant minority of the patients (19 percent) neither smoked nor drank, and 30 percent smoked but abstained from alcohol. We could thus analyze tobacco and alcohol use as independent risk factors for mutation of the p53 gene. Since only two patients drank alcohol but did not smoke, we could not evaluate the effect of alcohol in the absence of smoking.

Preliminary data link mutation of the p53 gene with cigarette smoking in patients with lung carcinoma.<sup>17-20</sup> Most of the studies have used immunohistochemical analyses to evaluate the p53 protein; this method has substantial false positive and false negative rates as compared with those for molecular sequencing.<sup>24-27,40</sup> In

our patients, 28 percent of the p53 mutations could have resulted in a truncated p53 protein, which would not stain with labeled anti-p53 antibodies. Another study linking exposure to carcinogens with p53 mutations found 14 mutations, most of which were GC→TA.<sup>17</sup> The authors suggested that benzo[a]pyrene in tobacco smoke specifically causes GC→TA mutations in the p53 gene.<sup>17</sup> In esophageal cancer, another neoplasm related to smoking and alcohol consumption, a wide range of p53 mutations has been found, most commonly GC→AT and GC→TA.<sup>15,16</sup> Mutations of the p53 gene in patients with bladder cancer who smoked typically consisted of GC→CG and AT→GC.<sup>22,41</sup> These mutations may result from the aromatic amines and N-[4-(5-nitro-2-furyl)-2-thioxolyl]formamide, both of which are present at increased levels in urothelial cells in cigarette smokers. All the patients in these earlier studies were cigarette smokers, and their cancers had a wide spectrum of base-pair changes in the p53 gene, similar to those in our patients with squamous-cell carcinoma of the head and neck who used tobacco and alcohol (Fig. 3).

Because both endogenous and exogenous mutagens

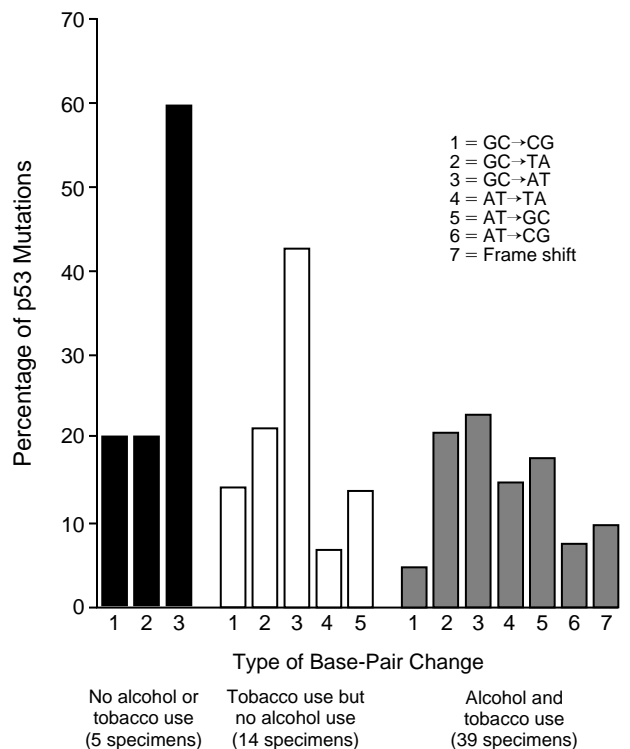


Figure 3. The Pattern of p53 Base-Pair Mutations Resulting from Exposure to the Carcinogens in Cigarette Tobacco and Alcohol.

Cigarette smokers who abstained from alcohol had five types of base-pair changes: AT→TA, AT→GC, GC→AT, GC→TA, and GC→CG. Cigarette smokers who drank alcohol had the widest spectrum of alterations, involving all types of potential base-pair changes, including frame shifts. Frame shifts involve insertions or deletions of one or more base pairs.

generate specific kinds of base substitutions at preferred sites, the spectrum of p53 mutations in tumors may provide information about their cause.<sup>38,39,42</sup> The pattern of p53 mutations in our patients was notable in two respects. First, the highest incidence of mutations was associated with exposure to tobacco and alcohol. In patients in Papua New Guinea who had squamous-cell carcinoma of the head and neck predominantly associated with betel-nut chewing,<sup>43</sup> the incidence of p53 mutations was much lower (10 percent), suggesting that tobacco (and perhaps alcohol) may produce carcinogens that increase the frequency of such mutations. Second, the location of the changes within the p53 gene with respect to CpG sites was also associated with exposure to cigarette tobacco and alcohol. These changes at CpG sites have been implicated as endogenous mutational "hot spots" resulting from methylation and deamination of cytosine by cellular enzymatic processes.<sup>42,44</sup> Consequently, a higher percentage of changes at CpG sites would be expected in patients whose mutations occurred without substantial exposure to environmental carcinogens. In colon cancer, a neoplasm not associated with smoking, the frequency of p53 mutations is also high, but most mutations occur at endogenous CpG sites.<sup>42</sup> In the group of patients who neither smoked nor drank, we also detected a predominance of mutations at CpG sites. However, the small number of subjects in this group means that these findings must be regarded as preliminary.

Critical studies have shown that the loss of the protective p53 cellular mechanism allows the evolution of a clonal population of cells with a selective growth advantage that may eventually result in the progression of cancer.<sup>45,46</sup> Moreover, inactivation of the p53 gene may be an important step in the progression of preinvasive lesions of the head and neck.<sup>23</sup> The different types of base-pair changes in squamous-cell carcinoma of the head and neck suggest the involvement of many of the tobacco toxins thus far identified, although experiments in animals have suggested that tobacco-specific nitrosamine derived from nicotine may be the main culprit.<sup>1</sup> Our results also suggest that alcohol may augment the effects of tobacco by further increasing the frequency of p53 mutations. Researchers have suggested that alcohol may cause mucosal injury and increase the absorption of the mutagenic toxins present in cigarette smoke.<sup>3</sup> Alcohol consumption may also directly cause carcinogenesis by inducing microsomal enzymes involved in the metabolism of carcinogens by contributing to nutritional deficiencies, and by introducing carcinogenic impurities that may have contaminated alcoholic beverages.<sup>3</sup>

We have demonstrated that cigarette smoking and alcohol consumption increase the frequency of p53 mutations. Although such mutations also occur in cancers that are not related to smoking, our findings provide further evidence that such mutations are generally re-

stricted to endogenous hot spots in nonsmokers and that cigarette smoke may have a propensity to inactivate the p53 gene. Because inactivation of the gene appears critical for the progression of many head and neck cancers, our molecular data strongly support the epidemiologic evidence that abstinence from smoking is important for the prevention of such cancers. Moreover, the link between exposure to tobacco and p53 mutations in squamous-cell carcinoma of the head and neck raises the possibility that a specific carcinogenic exposure can serve as the etiologic agent in a particular patient's cancer.

## REFERENCES

1. Carbone D. Smoking and cancer. *Am J Med* 1992;93:Suppl 1A:13S-17S.
2. Cullen J, Greenwald P. Prevention of cancer. In: Edelman BA, Michelson L, eds. *Handbook of prevention*. New York: Plenum Press, 1986:308-41.
3. Choi SY, Kahyo H. Effect of cigarette smoking and alcohol consumption in the aetiology of cancer of the oral cavity, pharynx and larynx. *Int J Epidemiol* 1991;20:878-85.
4. Mashberg A, Boffetta P, Winkelmann R, Garfinkel L. Tobacco smoking, alcohol drinking, and cancer of the oral cavity and oropharynx among U.S. veterans. *Cancer* 1993;72:1369-75.
5. Christen AG. The impact of tobacco use and cessation on oral and dental diseases and conditions. *Am J Med* 1992;93:Suppl 1A:25S-31S.
6. Boffetta P, Mashberg A, Winkelmann R, Garfinkel L. Carcinogenic effect of tobacco smoking and alcohol drinking on anatomic sites of the oral cavity and oropharynx. *Int J Cancer* 1992;52:530-3.
7. Department of Health and Human Services. Reducing the health consequences of smoking: 25 years of progress: a report of the Surgeon General: 1989 executive summary. Washington, D.C.: Government Printing Office, 1989. (DHHS publication no. (CDC) 89-8411.)
8. Slaughter DP, Southwick HW, Smejkal W. "Field cancerization" in oral stratified squamous epithelium: clinical implications of multicentric origin. *Cancer* 1953;6:963-8.
9. Hong WK, Lippman SM, Wolf GT. Recent advances in head and neck cancer—larynx preservation and cancer chemoprevention. *Cancer Res* 1993;53:5113-20.
10. Vogelstein B, Kinzler KW. Carcinogens leave fingerprints. *Nature* 1992;355:209-10.
11. Harris CC, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* 1993;329:1318-27.
12. Lesmes GR. Summary and conclusions. *Am J Med* 1992;93:Suppl 1A:55S-56S.
13. Field JK, Spandidos DA, Malliri A, Gosney JR, Yiagnosis M, Stell PM. Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of the head and neck. *Br J Cancer* 1991;64:573-7.
14. Maestro R, Dolcetti R, Gasparotto D, et al. High frequency of p53 gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. *Oncogene* 1992;7:1159-66.
15. Hollstein MC, Peri L, Mandard AM, et al. Genetic analysis of human esophageal tumors from two high incidence geographic areas: frequent p53 base substitutions and absence of ras mutations. *Cancer Res* 1991;51:4102-6.
16. Wagata T, Shibagaki I, Imamura M, et al. Loss of 17p, mutation of the p53 gene, and overexpression of p53 protein in esophageal squamous cell carcinomas. *Cancer Res* 1993;53:846-50.
17. Puisieux A, Lim S, Groopman J, Ozturk M. Selective targeting of p53 gene mutational hotspots in human cancers by etiologically defined carcinogens. *Cancer Res* 1991;51:6185-9.
18. Bongiorno PF, Whyte RI, Lesser EJ, Moore JH, Orringer MB, Beer DG. Alterations of K-ras, p53, and erbB-2/neu in human lung adenocarcinomas. *J Thorac Cardiovasc Surg* 1994;107:590-5.
19. Suzuki H, Takahashi T, Kuroishi T, et al. p53 Mutations in non-small cell lung cancer in Japan: association between mutations and smoking. *Cancer Res* 1992;52:734-6.
20. Gosney JR, Gosney MA, Butt SA, Field JK. Over-expression of p53 protein and cigarette smoking in bronchial carcinoma. *Int J Oncol* 1993;2:1071-4.
21. Zhang ZF, Sarkis AS, Cordon-Cardo C, et al. Tobacco smoking, occupation, and p53 nuclear overexpression in early stage bladder cancer. *Cancer Epidemiol Biomarkers Prev* 1994;3:19-24.

22. Spruck CH III, Rideout WM III, Olumi AF, et al. Distinct pattern of p53 mutations in bladder cancer: relationship to tobacco usage. *Cancer Res* 1993; 53:1162-6. [Erratum, *Cancer Res* 1993;53:Suppl:2427.]
23. Boyle JO, Hakim J, Koch W, et al. The incidence of p53 mutations increases with progression of head and neck cancer. *Cancer Res* 1993;53:4477-80.
24. Duffy MJ. Cellular oncogenes and suppressor genes as prognostic markers in cancer. *Clin Biochem* 1993;26:439-47.
25. Fisher CJ, Gillett CE, Vojtesek B, Barnes DM, Millis RR. Problems with p53 immunohistochemical staining: the effect of fixation and variation in the methods of evaluation. *Br J Cancer* 1994;69:26-31.
26. Wynford-Thomas D. p53 In tumour pathology: can we trust immunocytochemistry? *J Pathol* 1992;166:329-30.
27. Cunningham J, Lust JA, Schaid DJ, et al. Expression of p53 and 17p allelic loss in colorectal carcinoma. *Cancer Res* 1992;52:1974-80.
28. Brennan JA, Mao L, Hruban RH, et al. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1995;332:429-35.
29. Beahrs OH, Henson DE, Hutter RVP, Myers MH, eds. Manual for staging of cancer. 3rd ed. Philadelphia: J.B. Lippincott, 1988:27-62.
30. Baker SJ, Preisinger AC, Jessup JM, et al. p53 Mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res* 1990;50:7717-22.
31. Sidransky D, Von Eschenbach A, Tsai YC, et al. Identification of p53 gene mutations in bladder cancers and urine samples. *Science* 1991;252:706-9.
32. Buchman GW, Schester DM, Raschtas A. Rapid and efficient cloning of PCR products using the clone amp system. *Focus* 1992;14:41-5.
33. Breimer LH. Ionizing radiation-induced mutagenesis. *Br J Cancer* 1988;57: 6-18.
34. Renan MJ. Point mutations, deletions, and radiation carcinogenesis. *Radiat Res* 1992;131:227-8.
35. Brachman DG, Hallahan DE, Beckett MA, Yandell DW, Weichselbaum RR. p53 Gene mutations and abnormal retinoblastoma protein in radiation-induced human sarcomas. *Cancer Res* 1991;51:6393-6.
36. Vahakangas KH, Samet JM, Metcalf RA, et al. Mutations of p53 and ras genes in radon-associated lung cancer from uranium miners. *Lancet* 1992; 339:576-80.
37. Strauss BS. The origin of point mutations in human tumor cells. *Cancer Res* 1992;52:249-53.
38. Borek C. Molecular mechanisms in cancer induction and prevention. *Environ Health Perspect* 1993;101:Suppl 3:237-45.
39. Wogan GN. Molecular epidemiology in cancer risk assessment and prevention: recent progress and avenues for future research. *Environ Health Perspect* 1992;98:167-78.
40. Baas IO, Mulder JWR, Offerhaus GJA, Vogelstein B, Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994;172:5-12.
41. Habuchi T, Takahashi R, Yamada H, et al. Influence of cigarette smoking and schistosomiasis on p53 gene mutation in urothelial cancer. *Cancer Res* 1993; 53:3795-9.
42. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 Mutations in human cancers. *Science* 1991;253:49-53.
43. Thomas S, Brennan J, Martel G, et al. Mutations in the conserved regions of p53 are infrequent in betel-associated oral cancers from Papua New Guinea. *Cancer Res* 1994;54:3588-93.
44. Duncan BK, Miller JH. Mutagenic deamination of cytosine residues in DNA. *Nature* 1980;287:560-1.
45. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-67.
46. Sidransky D, Mikkelsen T, Schwecheimer K, Rosenblum ML, Cavanaugh W, Vogelstein B. Clonal expansion of p53 mutant cells is associated with brain tumour progression. *Nature* 1992;355:846-7.

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#### IMAGES IN CLINICAL MEDICINE

Images in Clinical Medicine, a weekly *Journal* feature, presents clinically important visual images, emphasizing those a doctor might encounter in an average day at the office, the emergency department, or the hospital. If you have an original unpublished, high-quality color or black-and-white photograph representing such a typical image that you would like considered for publication, send it with a descriptive legend to Kim Eagle, M.D., University of Michigan Medical Center, Division of Cardiology, 3910 Taubman Center, Box 0366, 1500 East Medical Center Drive, Ann Arbor, MI 48109. For details about the size and labeling of the photographs, the requirements for the legend, and authorship, please contact Dr. Eagle at 313-936-5275 (phone) or 313-936-5256 (fax), or the *New England Journal of Medicine* at [images@edit.nejm.org](mailto:images@edit.nejm.org) (e-mail).