ORIGINAL ARTICLE

Association Between *p53* Gene Mutations and Tobacco and Alcohol Exposure in Laryngeal Squamous Cell Carcinoma

Domenica Ronchetti, PhD; Cesare B. Neglia, MD; Bruno M. Cesana, MD; Nadia Carboni, BSc; Antonino Neri, MD; Giancarlo Pruneri, MD; Lorenzo Pignataro, MD

Objectives: To analyze the relationship between *p53* gene mutations, tobacco smoke, and alcohol consumption in patients with laryngeal squamous cell carcinoma.

Design: We analyzed *p53* gene mutations in exons 5 through 8 by polymerase chain reaction–single-strand conformation polymorphism analysis in a cohort of 84 patients with laryngeal squamous cell carcinoma.

Setting: University hospital.

Results: *p53* gene mutations were detected in 24 (28.6%) cases (95% confidence interval, 19.3%-39.5%), and the GC to TA transversion (33%) was the most common type of mutation (95% confidence interval, 15.6%-55.3%). Most mutations mapped to the *p53* DNA-binding domain, which is necessary for the physiological activity of *p53* as a tumor suppressor. A statistically significant association was found between *p53* mutations and exposure to tobacco smoke ($P=.001$), which was the only variable significantly associated with *p53* mutations in a multivariate model. The association with alcohol consumption was only at a borderline level of significance ($P=.065$).

Conclusion: Our data document that a smoking habit is the only independent variable associated with an increased risk of *p53* mutations in the laryngeal mucosa.

Arch Otolaryngol Head Neck Surg. 2004;130:303-306

From the Department of Otolaryngology (Drs Ronchetti, Neglia, and Pignataro), the Epidemiology Unit (Dr Cesana), the Third Division of Internal Medicine (Ms Carboni), and the Laboratory of Experimental Hematology and Molecular Genetics (Dr Neri), Ospedale Maggiore–Istituto di Ricovero e Cura a Carattere Scientifico, University of Milan, and the Division of Pathology and Laboratory Medicine, European Institute of Oncology (Dr Pruneri), Milan, Italy.
The authors have no relevant financial interest in this article.

THE *P53* TUMOR SUPPRESSOR gene encodes for a 53-kD nuclear phosphoprotein that plays a central role in cell-cycle control and apoptosis.[1] Missense mutations of the *p53* tumor suppressor gene cause most of the common cancerous genetic lesions in humans.[2] Accordingly, *p53* gene mutations have been reported in approximately one third of the head and neck carcinomas analyzed.[3-12]

Cigarette smoking and alcohol drinking are the most important risk factors for laryngeal squamous cell carcinoma (LSCC)[13] and are thought to act synergistically.[14] Studies have demonstrated the relationship between *p53* gene mutations and tobacco exposure in patients with tumors originating from different sites of the head and neck region,[6,9,10] and, in 2 small series, in patients with LSCC.[11,12] By contrast, the association between alcohol consumption and *p53* gene mutations in head and neck cancer has not been fully explored.[6,9]

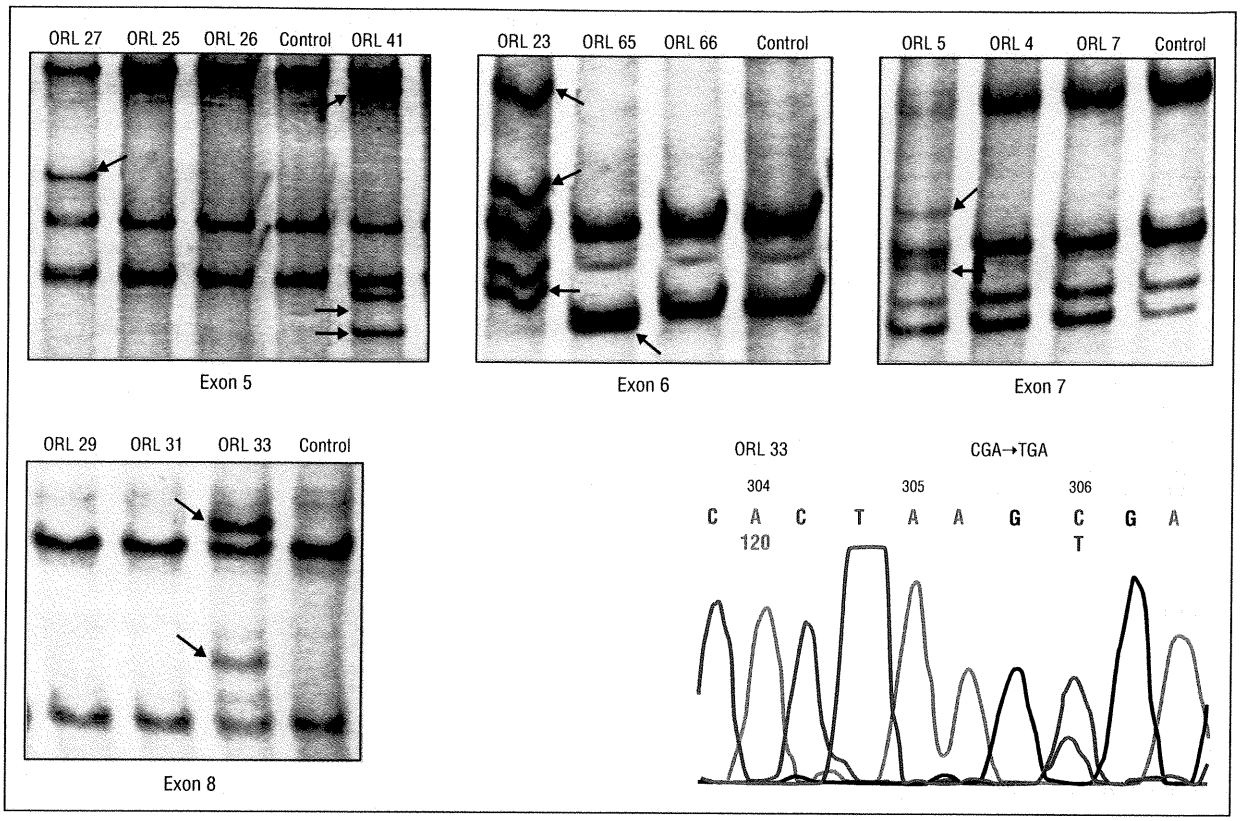
The aim of the present work was to analyze the association between the exposure to the traditional risk factors of tobacco smoke and alcohol consumption and the prevalence of *p53* mutations in a homogeneous series of 84 patients with LSCC.

METHODS

PATIENT SELECTION

Samples of primary LSCC from 84 consecutive patients were collected from the files of Clinica Otorinolaringoiatrica I at the University of Milan between 1993 and 1997. Findings concerning 18 patients from this series have been included in a previous report.[15] The patients only had primary LSCC, had no history of previous malignancies, and had not previously received radiotherapy or chemotherapy. All underwent surgical removal of their tumors by partial or total laryngectomy. The study procedures were in accordance with the ethical standards of the Helsinki Declaration of 1975.[16]

All patients had been exposed to tobacco smoking. Details concerning tobacco and alcohol exposure were obtained by means of a specific questionnaire systematically included in the medical records. It included questions about smoking habits, age when starting smoking, mean daily cigarette consumption,



Representative examples of single-strand conformation polymorphism analysis of exons 5, 6, 7, and 8 of the *p53* gene; migrating fragments not observed in controls are indicated by arrows. Bottom right, Direct sequencing of polymerase chain reaction–amplified fragments from patient 33, whose electropherogram shows the C→T mutation at codon 306. ORL indicates otorhinolaryngology patient.

types of drink consumed, and mean daily consumption of alcoholic beverages. Pack-years, a measure of cumulative smoking, was calculated as the number of years a patient had smoked multiplied by the daily number of packs smoked. The patients were divided into 2 groups according to their number of pack-years of tobacco consumption (≤45 or >45 pack-years). Information regarding alcohol consumption was expressed as number of drinks per day before diagnosis (1 drink corresponding to 150 mL of wine or 30 mL of hard liquor). Patients were classified as moderate drinkers or nondrinkers (≤7 drinks per day) or as heavy drinkers (>7 drinks per day).

Patient follow-up ranged from 4 to 92 months (mean±SD, 40.2±22.5 months; median, 36.5 months). All patients received clinical and fibroscopic examinations every 3 months during the first year after surgery, every 6 months during the second year, and every year thereafter.

PCR-SSCP ANALYSIS

p53 gene mutations were evaluated by the polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis. Exons 5 through 8 were separately amplified using specific oligonucleotide primers. Briefly, PCR was performed using 100 ng of genomic DNA, 10mM Tris-hydrochloric acid (pH 8.8), 50mM potassium chloride, 1mM magnesium chloride, 100µM of each of the deoxynucleotide triphosphates, 0.25 U *Taq* DNA polymerase, and 10 pmol of each sense and antisense primer in a final volume of 25 µL. Thirty cycles of denaturation (15 seconds at 94°C), annealing (1 minute at 65°C for reactions amplifying exons 5, 6, or 7 and 1 minute at 58°C for those amplifying exon 8), and extension (1 minute at 72°C) were performed on an automated heating block (Perkin Elmer Cetus, Norwalk, Conn). Then, 10 µL of the reaction mixture was mixed in a 1:1 ratio with a denaturing stop solution. Samples were heated at 95°C for 5 minutes, chilled on ice, and immediately loaded on a 6% acrylamide TBE gel. The samples were run at a constant power of 6 to 8 W for 14 hours at room temperature. Detection was performed by the silver staining method.

SEQUENCING ANALYSIS

Polymerase chain reaction products showing aberrant migration patterns were analyzed in both directions using the direct sequencing method and the 5′ and 3′ primers used in the PCR-SSCP analysis. The DNA fragments were purified by agarose gel extraction and sequenced using the Big Dye Terminator Sequencing Kit in an ABI Prism 310 automated sequencer (Perkin Elmer Cetus) (**Figure**).

STATISTICAL ANALYSIS

The associations between clinicopathological parameters and *p53* mutations were evaluated using the χ^2 test, and the χ^2 test for trend in the case of ordinal variables with more than 2 classes.[17]

Multiple logistic regression was carried out to assess the association between drinking (≤7 or >7 drinks per day) and smoking (≤45 or >45 pack-years), their interaction, and the occurrence of *p53* gene mutations.

The prognostic relevance of the patients' baseline characteristics to overall mortality (death due to cancer and other causes) was assessed by survival analysis according to the product-limit estimate of the cumulative probability function.[18] The

Table 1. Main Characteristics of Patients With Laryngeal Squamous Cell Carcinoma Who Had Mutated and Wild-type *p53* Genes

Characteristics	No. (%) Mutated *p53*	No. (%) Wild-type *p53*	*P* Value
Sex			
Male	23 (29.5)	55 (70.5)	
Female	1 (16.7)	5 (83.3)	.5
Age, y			
<60	8 (24.2)	25 (75.8)	
≥60	16 (31.4)	35 (68.6)	.65
Pack-years smoking			
≤45	5 (9.4)	48 (90.6)	
>45	19 (61.3)	12 (38.7)	.001
Alcohol consumption			
None to moderate (≤7 drinks)	6 (16.7)	30 (83.3)	
Heavy (>7 drinks)	18 (37.5)	30 (62.5)	.070
Anatomical site			
Supraglottic	16 (32.0)	34 (68.0)	
Glottic	8 (23.5)	26 (76.5)	.55
Tumor extension			
T1-T2	8 (20.0)	32 (80.0)	
T3-T4	16 (36.4)	28 (63.6)	.16
Node metastases			
N0	17 (30.9)	38 (69.1)	
N1	7 (24.1)	22 (75.9)	.70
Histological grade			
G1	1 (9.1)	10 (90.9)	
G2	12 (70.8)	27 (69.2)	.30
G3	11 (32.4)	23 (67.6)	.23*
Clinical stage			
I-II	8 (24.2)	25 (75.8)	
III-IV	16 (31.4)	35 (68.6)	.646

*Calculated with the χ^2 test for trend.

Table 2. *p53* Gene Mutations in a Group of Biopsy Specimens From Laryngeal Squamous Cell Carcinomas

Specimen No.	Mutation	Codon	Type of Mutation
			Transition
2	Missense	267	CG→TA
5	Missense	248	GC→AT
22	Missense	177	CG→TA
23	Missense	196	CG→TA
27	Missense	175	GC→AT
30	Missense	238	GC→AT
32	Missense	278	CG→TA
33	Nonsence	306	CG→TA
69	Missense	239	AT→GC
70	Missense	214	AT→GC
			Transversion
39	Splicing	187	GC→TA
41	Missense	176	GC→TA
46	Missense	176	GC→TA
50	Missense	245	GC→TA
51	Missense	157	GC→TA
54	Missense	277	GC→TA
56	Missense	273	GC→TA
61	Missense	237	GC→CG
63	Missense	141	GC→CG
65	Missense	193	AT→TA
80	Splicing	187	GC→TA
73	Insertion	177	
75	Insertion	162	
81	Deletion	259	

survival function of the different categories of the investigated variables were compared using the log-rank test.[19]

RESULTS

Samples of LSCC from 84 patients were evaluated for *p53* mutations in exons 5 through 8 by PCR-SSCP analysis.

The main clinical and pathologic characteristics of the study patients are shown in **Table 1**. Their mean age was 62.5±9.3 years (range, 35-81 years); 78 (92.8%) were male; 53 (63.1%) had smoked less than 45 pack-years and 31 (36.9%) had smoked more than 45 pack-years; and 36 were considered moderate drinkers (≤7 drinks per day for 34 patients) or nondrinkers, and 48 were heavy drinkers (>7 drinks per day). The clinical staging and identification of the anatomical site of the tumors were based on the International Union Against Cancer TNM classification of malignant tumors. Fifty tumors (59.5%) were supraglottic and 34 (40.5%) were glottic. Thirty-three patients (39.3%) had stage I or II tumors and 51 (60.7%) had stage III or IV tumors. According to the histological typing of Shanmugaratnam et al,[20] there were 11 (13.1%) well-differentiated (G1), 39 (46.4%) moderately differentiated (G2), and 34 (40.5%) poorly differentiated (G3) carcinomas.

Altered mobility due to structural aberration of the DNA fragments amplified via PCR were detected in 24 patients (28.6%). Eighteen patients in the present series had been previously tested for *p53* gene mutation[15] and 5 cases bore *p53* gene mutations (there were 2 mutations in exon 5 and 1 mutation in exons 6, 7, and 8). Nineteen of the 66 newly tested patients showed *p53* gene mutation (seven in exon 5, three in exon 6, five in exon 7, and four in exon 8). The 24 *p53* mutations were confirmed and characterized by direct DNA sequencing. Most of them (21/24) were single-base substitutions, 2 were insertions, and 1 was a deletion (**Table 2**).

Most (14/24) of the *p53* mutations mapped to the protein-DNA binding surface, whose integrity is necessary for the physiological activity of *p53* as a tumor suppressor. In particular, there were 9 mutations in the large loop L2 (residues 163-195), and 5 mutations in the loop L3 (residues 236-251).

As shown in Table 2, the point mutations were represented by 10 transitions and 11 transversions, and the G→T transversion was the most common (33%) mutational event.

Among the clinicopathological characteristics evaluated, only tobacco smoke ($P=.001$) and alcohol consumption—at a borderline statistical significance level ($P=.065$)—were significantly associated with the occurrence of *p53* gene mutations (Table 1). No statistically significant interaction ($P=.3$) was found when grouping the risk factors of tobacco smoking and alcohol consumption. With multivariate analysis, tobacco smoking was the only independent variable found to be significantly associated with *p53* gene mutations (odds ratio, 15.23; 95% confidence interval, 4.13-56.15; $P<.001$).

During follow-up, 36 tumors (42.9%) recurred. Of the 84 patients, 29 (34.5%) died of the disease and 2 (2.4%) died of other causes. All 53 surviving patients are free of disease.

No statistical difference in overall survival was found between the patients with wild-type and mutated *p53* gene (62.5% vs 63.3%; $P=.94$). Although patients with *p53*-mutated tumors (50.0%) showed a higher probability of recurrence than patients with *p53* wild type (40.0%), this trend was not statistically significant ($P=.47$).

COMMENT

We evaluated the relationship between tobacco smoking and alcohol consumption and *p53* gene mutations in a homogeneous and well-characterized series of laryngeal carcinomas. We analyzed *p53* gene mutations in exons 5 through 8, the "hot-spot" region in which most of the mutations occur.[21] Although we did not investigate exons 4, 9, and 10, in which approximately 15% of *p53* gene mutations are expected,[21] we document, in accordance with previous findings,[10,11] that *p53* gene mutations occur in about one third of cases. In keeping with previous data,[22] we found that most (58.3%) of the *p53* mutations map to the protein-DNA binding surface. Interestingly, we found 1 GC→AT transition at codon 248, and 2 GC→TA transversions at codons 273 and 157. These sites have been previously reported as the "hot spots" for *p53* gene mutations in lung cancer and other smoke-induced neoplasms, and to act in vitro as selective binding sites for benzo[a]pyrene adducts.[23] We also document 5 *p53* mutations at codons 175 through 177 and 3 at codons 237 through 239. Likewise, analyzing 35 cases of LSCC, Zhang et al[11] found that 37% of the *p53* mutations occur in the 238 through 248 region, and 8.6% in the 173 through 176 region. Taken together, these data suggest that these regions may also be considered "hot spots" for *p53* mutation in LSCC.

Previous studies have ascertained the association between *p53* gene mutation and tobacco smoke and alcohol consumption in patients with head and neck carcinoma,[6,9,10,24] but few data are available for LSCC.[11,12] We document a significant direct correlation between *p53* inactivation and tobacco exposure, since approximately 80% of mutations were detected in patients who had smoked more than 45 pack-years. It has been suggested that alcohol consumption may act synergistically with tobacco smoking in inducing *p53* mutations in patients with head and neck cancer[6,9] Although we found that heavy drinking was nearly significantly associated with *p53* mutations at univariate analysis, tobacco smoking was the only independent predictor of *p53* mutations in a multivariate model. These data suggest that the mutagenic role of alcohol consumption is relatively less important than tobacco smoke in laryngeal tumorigenesis.

Finally, in agreement with with previous studies, we did not detect any association between *p53* mutations and prognosis, which further confirms that the assessment of *p53* gene mutations cannot be used as a reliable prognostic marker for LSCC.[25]

Submitted for publication December 30, 2002; final revision received June 20, 2003; accepted July 14, 2003.

This work was supported by a grant from the Associazione Italiana Ricerca sul Cancro (Dr Neri) and a 1999 Ricerca Corrente grant from the Ministero della Salute.

Corresponding author and reprints: Lorenzo Pignataro, MD, Dipartimento di Scienze Otorinolaringologiche, Ospedale Maggiore, IRCCS, Via F. Sforza 35, 20122 Milano, Italy (e-mail: lorenzo.pignataro@unimi.it).

REFERENCES

1. Lowe SW, Ruley HE, Jacks T, et al. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell.* 1993;74:957-967.
2. Stratton MR. The p53 gene in human cancer. *Eur J Cancer.* 1992;28:293-295.
3. Brachman DG, Beckett M, Graves D, et al. p53 mutation does not correlate with radiosensitivity in 24 head and neck cancer cell lines. *Cancer Res.* 1993;53:3667-3669.
4. Boyle JO, Hakim J, Koch WM, et al. The incidence of p53 mutations increases with progression of head and neck cancer. *Cancer Res.* 1993;53:4817-4822.
5. Ahomadegbe JC, Barrois M, Fogel S, et al. High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumors and metastases, absence of correlation with clinical outcome: frequent protein overexpression in normal epithelium and in early non-invasive lesions. *Oncogene.* 1995; 10:1217-1227.
6. Field JK, Zoumpourlis V, Spandidos DA, Jones AS. p53 expression and mutations in squamous cell carcinoma of the head and neck: expression correlates with the patients'use of tobacco and alcohol. *Cancer Detect Prev.* 1994;18:197-208.
7. Koch WM, Brennan JA, Zahurak M, et al. p53 mutations in locoregional treatment failure in head and neck squamous cell carcinoma. *J Natl Cancer Inst.* 1996; 88:1580-1586.
8. Olshan AF, Weissler MC, Pei H, Conway K. p53 mutations in head and neck cancer: new data and evaluation of mutational spectra. *Cancer Epidemiol Biomarkers Prev.* 1997;6:499-504.
9. Brennan JA, Boyle JO, Koch WM, et al. Association between cigarette smoking and mutation of the p53 gene in squamous cell carcinoma of the head and neck. *N Engl J Med.* 1995;332:712-717.
10. Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. Head and neck cancer in nonsmokers: a distinct clinical and molecular entity. *Laryngoscope.* 1999; 109:1544-1551.
11. Zhang LF, Hemminki K, Szyfter K, Szyfter W, Soderkvist P. p53 mutations in larynx cancer. *Carcinogenesis.* 1994;15:2949-2951.
12. Sarkar FH, Sakr WA, Li YW, Jacobs J, Crissman JD. Tumor suppressor p53 gene mutation in squamous cell carcinoma of the larynx. *Diagn Mol Pathol.* 1996;5: 201-205.
13. Cattaruzza MS, Maisonneuve P, Boyle P. Epidemiology of laryngeal cancer. *Eur J Cancer B Oral Oncol.* 1996;32B:293-305.
14. Flanders WD, Rothman KJ. Interaction of alcohol and tobacco in laryngeal cancer. *Am J Epidemiol.* 1982;115:371-379.
15. Pruneri G, Pignataro L, Fracchiolla NS, et al. p53 protein expression in laryngeal squamous cell carcinomas bearing wild-type and mutated p53 gene. *Histopathology.* 1996;28:513-519.
16. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. *Bull World Health Organ.* 2001;79:373-374.
17. Armitage P, Berry G. *Statistical Methods in Medical Research.* London, England: Blackwell Scientific Publications; 1987:125-132.
18. Kaplan EL, Meier P. Non parametric estimation from incomplete observations. *J Am Stat Assoc.* 1958;53:457-481.
19. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep.* 1966;50:163-170.
20. Shanmugaratnam K, Sobin LH. *Histological Typing of Tumours of the Upper Respiratory Tract and Ear.* Geneva, Switzerland: World Health Organization; 1991.
21. Soussi T, Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer.* 2001;1:233-240.
22. Cho Y, Svetlana G, Jeffrey PD, Pabletich NP. Kristal structure of a p53 tumor suppressor-DNA complex:understanding tumorigenic mutations. *Science.* 1994[23]; 265:346-355.
23. Denissenko M, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in p53. *Science.* 1996;18:430-432.
24. Lazarus P, Stern J, Zwiebel N, Fair A, Richie JP Jr, Shantz S. Relationship between p53 mutation incidence in oral cavity squamous cell carcinomas and patient tobacco use. *Carcinogenesis.* 1996;17:733-739.
25. Assimakopoulos D, Kolettas E, Zagorianakou N. Prognostic significance of p53 in the cancer of the larynx. *Anticancer Res.* 2000;20:3555-3564.