

p53 Expression and Mutations in Squamous Cell Carcinoma of the Head and Neck: Expression Correlates with the Patients' Use of Tobacco and Alcohol

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ABSTRACT: P53 expression was assessed in 93 head and neck squamous cell carcinoma patients using CM-1, DO-7, and DO-1 antibodies. Sixty eight percent was found to have positive nuclear staining. The frequency of p53 mutations were investigated in 13 patients using single-stranded conformational polymorphism (SSCP) analysis of DNA fragments that had been amplified by the polymerase chain reaction (PCR). P53 gene mutations were analyzed by SSCP in exons 4 to 7 of 13 patients, and nine were found to have mutations in exon 4; two of these patients also had a mutation in exon 5. In the group of 93 patients, p53 overexpression was found to correlate with the patients' history of heavy smoking ($p < 0.01$). We also analyzed the drinking and smoking history of 71 of these patients by logistic regression analysis and found that heavy smoking correlates with p53 overexpression ($p < 0.05$), but heavy drinking was not found to be significant. However when both smoking and drinking histories were assessed together, a correlation was found ($p < 0.05$). Future work may indicate that specific p53 mutations are associated with patients who have a history of heavy drinking and smoking.

KEYWORDS: p53 tumor suppressor gene, expression, mutations (SSCP), head and neck cancer, smoking, drinking, survival.

I. INTRODUCTION

Squamous cell carcinoma of the head and neck accounts for about 2% of all solid malignancies in the Western world. However, it is one of the most distressing diseases because its surgical treatment often results in dysfunction and disfigurement.¹ Furthermore, the success of treating this disease has not improved in the last 25 years.² Molecular investigations into head and neck cancer were started in the last 8 years, and now a number of oncogenes, *ras*, *myc*, *erbB-1*, and the p53 tumor suppressor gene, have been implicated in the development of this disease.³

Initial investigations into p53 head and neck cancer indicated that about 60% of these cancers

overexpressed this gene⁴⁻⁸ but no correlations have been found between p53 overexpression and any of the clinicopathological parameters.⁴ In addition, p53 mutations have been demonstrated by single-stranded conformational polymorphism (SSCP) analysis and sequencing in a range of head and neck tumor specimens and cell lines.^{6,9-13}

The p53 gene product was initially identified as a host cell protein bound to the large T-antigen, the dominant transforming oncogene of the SV40 virus.¹⁴ Normal levels of p53 act as tumor suppressor genes, but mutations in the p53 gene may convert it into a dominant gene. However, they may remain as recessive or become null mutations.^{15,16} Inactivation of the p53 gene may

also be caused by deletions,¹⁷ binding to viral oncoproteins,¹⁸ or by binding to host proteins.¹⁹ A turning point in our understanding of this tumor suppressor gene occurred with the publications of Oliner et al.,¹⁹ Farmer et al.,²⁰ and Yin et al.,²¹ which has led to the recently proposed models for p53 function.²¹⁻²³ There is overwhelming epidemiological evidence for a correlation between heavy smoking and drinking in head and neck cancer.²⁴⁻²⁷ However, there is little genetic information available that links exposure of tobacco and alcohol to head and neck cancer. The aim of this study was to assess the level of p53 expression and mutations in head and neck cancer patients and correlate the results with a range of clinicopathological parameters and with the patients' tobacco and alcohol usage.

II. MATERIALS AND METHODS

A. Tumor Specimens and Patient Details

Ninety-three patients with squamous cell carcinoma of the head and neck (oral, laryngeal, pharyngeal, and nodal specimens) were selected for this study from the Department of Otorhinolaryngology, University of Liverpool. Tumor tissue samples obtained from surgical specimens were frozen in liquid nitrogen. The tissue sections used in the immunohistochemical investigations in this study had been fixed in formalin and embedded in paraffin wax. Serial sections of 5 μ m thickness were cut and processed for immunohistochemistry. The following clinicopathological data were available: sex, age, site of primary tumor, TNM staging using the UICC²⁸ convention, site of recurrence, histopathological differentiation, the pathology of lymph node metastasis, and follow-up. The smoking pattern was classified as nonsmokers, moderate smokers (under 20 cigarettes per day), and heavy smokers (over 20 cigarettes per day) or equivalent quantities of pipe tobacco. Patients who had stopped smoking for more than 5 years were also considered separately. The patients' drinking history was also recorded: nondrinker, moderate drinker (less than 21 units per week), and heavy drinker (over 21 units per week).

Also, a group of patients with benign and potentially malignant head and neck lesions were included in this study. These included fibromas, keratosis, hyperkeratosis, leukoplakias, and specimens of normal tissues.

B. DNA Extraction

Genomic DNA was extracted from the tumor specimens by methods previously described.²⁹

C. PCR-SSCP Analysis

The primers for the p53 gene containing exons 4 to 9 (2.9 kb) were amplified by the polymerase chain reaction (PCR) using the primers in Table I. The PCR reaction (50 μ l) contained 250 ng of genomic DNA, 0.4 μ M of each respective primer, 2.5 units of Taq polymerase, 5 μ l of 10X PCR buffer (100 mM Tris-HCL pH 8.3), and 0.2 mM dNTP mixture. The reactions were initially denatured at 98°C for 7 min and then allowed to cool to 90°C. The Taq polymerase was then added to the samples and the samples placed in a thermocycler. The DNA was amplified for 30 cycles consisting of 95°C for 1 min, 58°C for 1 min, and 70°C for 4 min. Five microliters of PCR samples were analyzed by electrophoresis on a 0.8% ultrapure agarose gel (BRL, U.S.) and the amplified products visualized under UV light after staining with ethidium bromide.

D. Nested PCR-SSCP Analysis

One microliter of each sample's first PCR reaction mixture (amplified DNA size, 2.9 kb) was combined with 5 μ l of 10X PCR buffer, 1 μ Ci [α -³²P]dCTP (3000 Ci/mmol; Amersham, U.K.), 1 μ l dNTP mixture (0.2 mM dATP, 0.02 mM dCTP, 0.2 mM dGTP, and 0.2 mM dTTP), and 0.4 μ M of both primers for a specific exon in a final volume of 50 μ l (Table I). After an initial denaturation step at 98°C for 5 min, 2.5 units of Taq polymerase were added to each reaction tube, which was then placed in the thermocycler for 30 cycles. The temperatures and times for the reaction cycles of exons 4, 5, 6, and 7 were 95°C (25 s), 62°C (25 s), and 70°C (30 s),

TABLE I
Primers for PCR Amplification

Amplified gene	Primer sequence	
p53 Exon 4-9	5'-GTAGGAATTCGTCCCAAGCAATGGATGAT-3'	(sense)
	5'-CATCGAATTCTGGAAACTTTCCACCTTGAT-3'	(antisense)
Primers for p53 exons 4-9 - PCR - SSCP		
Exon 4	PU4: 5'-AATGGATGATTGATGCTGTCC-3'	(sense)
	PD4: 5'-CTCAGGGCAACTGACCGTGC-3'	(antisense)
Exon 5	PU5: 5'-GACTTTCAACTGTCTC-3'	(sense)
	PD5: 5'-CTGGGACCCTGGGCAAC-3'	(antisense)
Exon 6	PU6: 5'-GAGACGACAGGGCTGGTT-3'	(sense)
	PD6: 5'-CCACTGACAACCACCTT-3'	(antisense)
Exon 7	PU7: 5'-GTGTTGTCTCCTAGGTTGGC-3'	(sense)
	PD7: 5'-AAGTGGCTCCTGACCTGGAG-3'	(antisense)

respectively. Five microliters of the PCR-amplified product was then diluted with 40 μ l of buffer A (0.1% SDS, 10 mM EDTA), and 5 μ l of this mixture was added to an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) (United States Biochemicals, Ohio). Samples were denatured at 98°C for 7 min and placed immediately on ice to prevent renaturation before being run on 1 mm \times 20 \times 24 cm, 0.6X hydrolink polyacrylamide nondenaturing gels. The DNA was electrophoresed in TBE (0.09 M Tris base, 0.09 boric acid, and 2.5 mM EDTA) running buffer at 12 to 15 W at room temperature. Gels were dried and exposed to XAR-5 film (Kodak) for 12 to 24 h.

E. Immunohistochemical Analysis

Immunohistochemical analysis of the p53 protein was undertaken using the CM-1 antibody in all cases;³⁰ DO-7 and DO-1 antibodies were used in the majority of the cases.³¹ The rabbit antiserum CM-1 has been raised against full-length p53 expressed in *Escherichia coli* using a modified T7-based expression vector such that the first methionine of p53 was the initiation codon. This resulted in an expression vector that encodes a full-length p53 protein that is not fused to any

other protein sequences.³⁰ The CM-1 antibody was used at a concentration of 1 in 50, and DO-7 was used at 1 in 100. The DO-1 and DO-7 antibodies were prepared from splenotypes from BALB/c mice, hyperimmunized with recombinant human wild-type protein, and fused with a nonproducing mouse myeloma cell line.³¹ The DO-1 antibody was kindly provided by Professor D. Lane and used at a 1 in 100 dilution of culture supernatant. The avidin-biotin (ABC) technique was used to stain the paraffin-embedded sections. In negative controls, p53 CM-1, DO-7, and DO-1 were omitted from the first stage and replaced with the same concentration of a polyclonal mouse immunoglobulin (Sigma, Pool). Three cell lines were used as controls for p53 staining: spontaneously immortalized rat 208F cells were used as negative controls; positive controls included the MCF-7 human breast cancer cell line and their transfected derivative RFV53HO6-3 cells, which carry the mutant mouse p53 gene carrying valine instead of alanine at amino acid 135.³² P53 staining was scored as (1) negative or equivocal, (2) moderate, and (3) intense staining.

F. Statistical Analysis

Quantitative data were analyzed by χ^2 or Fisher's exact test and weighted logistic regression

analysis³³ where appropriate. Survival curves were drawn up using the Kaplan-Meier product limit estimate.³⁴ Differences between survival times were analyzed by the log rank method.³⁵

III. RESULTS

Ninety-three head and neck cancer patients were assessed for p53 levels of expression using a combination of antibodies (CM-1, DO-7, and DO-1). Sixty-eight percent of the specimens were found to have positive nuclear staining and 17% had intense staining. No correlations were found between p53 staining and age, sex, site of the primary tumor, tumor stage, or site of the recurrence. Neither was any correlation found between p53 expression and survival when calculated from the date of diagnosis of the disease (Figure 1).

Thirteen of these patients were analyzed for mutations in exons 4 to 7, as the majority of previous studies have shown that the mutations in human tumors are found in this region.³⁶ We first determined the optimal conditions for PCR amplification of the 2.9-kb p53 gene fragment (Figure 2) and for the nested PCR amplification of the individual exons, 4 to 7 (Figure 3). In order to exclude mutations caused by *Taq* polymerase, the SSCP analysis was carried out on two different occasions for each tumor sample. The SSCP results are shown in Figures 4 and 5. Nine of the 13 tumor samples investigated showed extra bands, which indicates the presence of mutations. The same fragment as the wild-type control was also observed in the mutated specimens at varying intensities. This is due to the fact that the tumor specimens contain both normal and neoplastic cells. Nine of the p53 mutations were found in

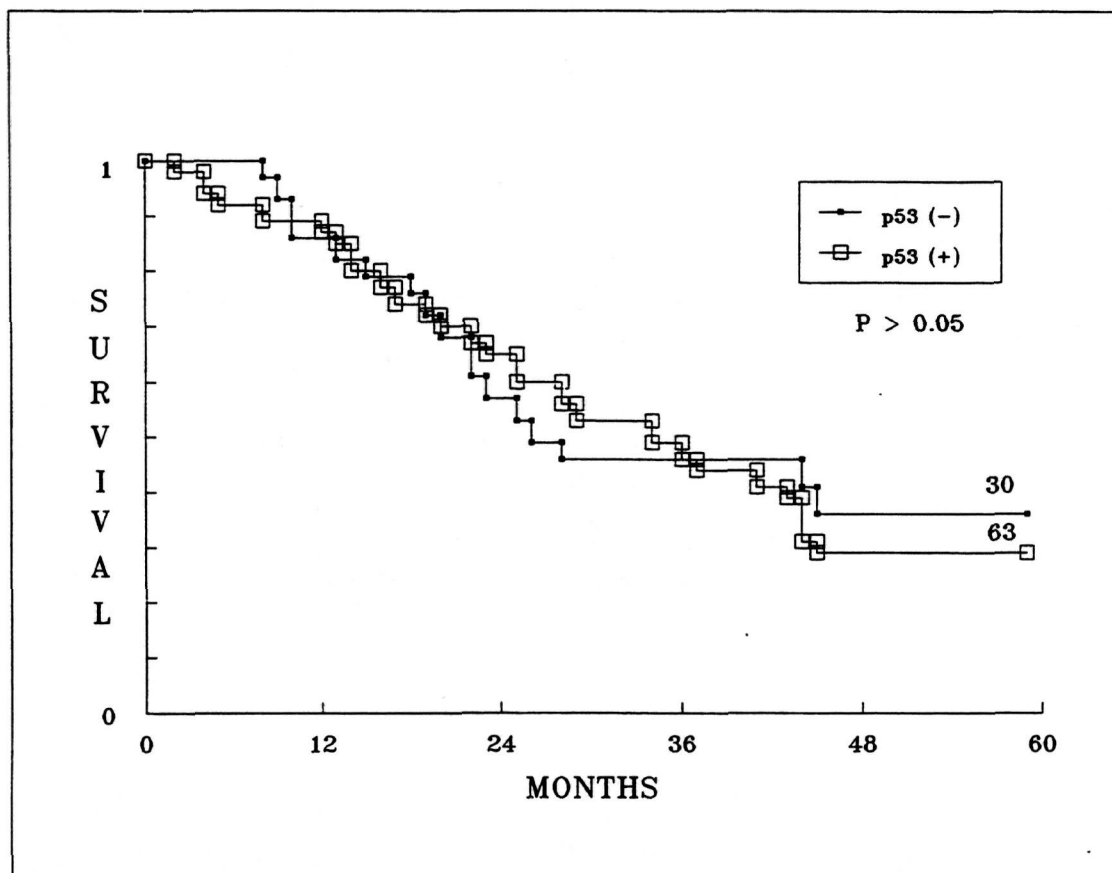


FIGURE 1. Survival curve of 93 head and neck squamous cell carcinoma patients with positive and negative p53 expression, drawn up using the Kaplan-Meier product limit estimate.

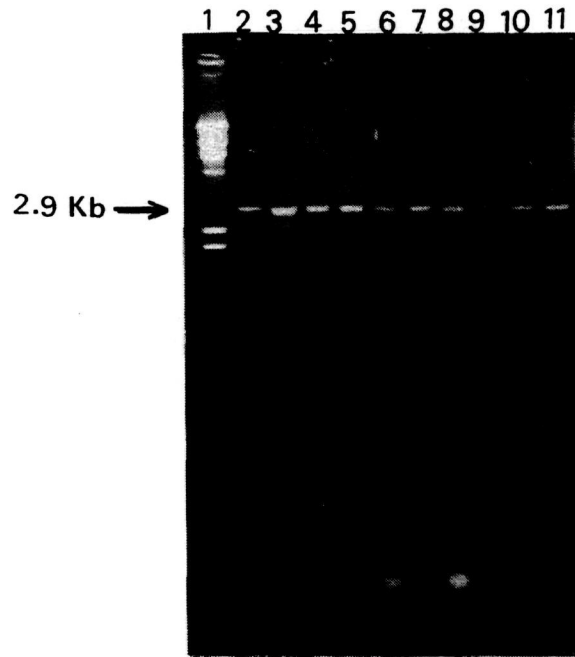


FIGURE 2. The 2.9-kb p53 gene fragment generated by PCR amplification. Lane 1, λ -DNA/HindIII size markers; lanes 2 to 11, 2.9-kb p53 gene fragment generated after 30 cycles of PCR using DNA from patients with squamous cell carcinoma of the head and neck. Electrophoresis was on 0.8% agarose gel in 0.5X TBE buffer.

exon 4 (tumor numbers 1, 5, 6, 7, 9, 10, 11, 12, and 13). However, two of these tumors also had a mutation in exon 5 (1, 5). No mutations were found in exons 6 or 7 (data not shown). All but one of the 13 patients analyzed for p53 mutations by SSCP analysis were found to overexpress p53. One patient with negative p53 staining was found to have a mutation in exon 4 and, furthermore, was a nonsmoker.

In an ongoing study of smoking and p53 levels of expression, we have demonstrated in this group of 93 patients that there is a significant difference between p53 expression in nonsmokers and heavy smokers ($p < 0.01$) (Table II). It is of note that 10 of 12 patients, who have stopped smoking for 5 years or more, overexpress p53. These data include 73 patients who have been previously reported by us.⁴ In this study, we also investigated the patients' drinking history, and the data were available on 81 of these patients. We analyzed the smoking and drinking

history of these 72 patients by logistic regression analysis by two methods: (1) excluding those patients with a "stopped-smoking" history and (2) including this group of stopped-smoking patients. The analysis of the smoking and drinking history of the 63 patients (excluding the stopped-smoking group) by logistic regression analysis demonstrated that heavy smoking correlates with p53 overexpression ($Z = 2.01$; $p < 0.05$), but heavy drinking was not found to be significant ($Z = 0.5$; $p > 0.05$). However, when both smoking and drinking histories were assessed together, a correlation was found ($Z = 2.15$; $p < 0.05$), which indicates that these two environmental factors are probably linked to aberrant expression of p53 in the etiology of head and neck cancer (Figure 6). The analysis of the smoking and drinking history of the 72 patients (including the stopped-smoking group with the heavy smokers) indicated a correlation between smoking and drinking ($Z = 2.29$; $p < 0.05$).

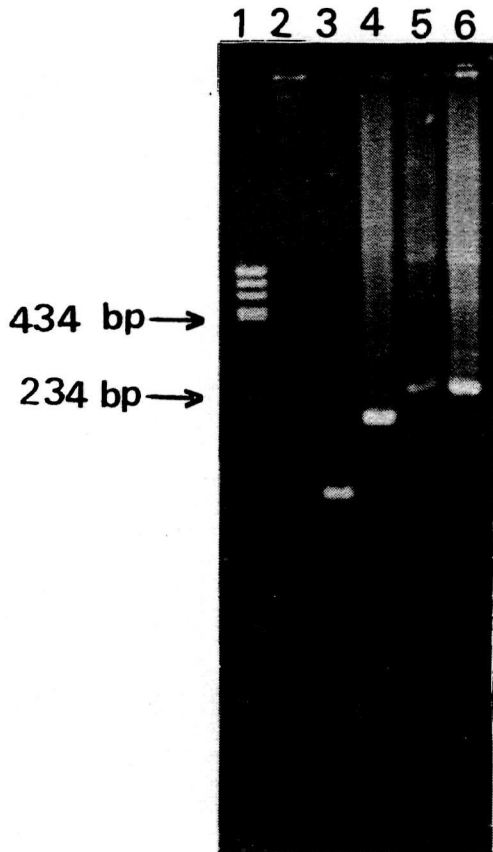


FIGURE 3. The p53 gene exon fragments generated by nested PCR amplification. Lane 1, pBR322/HaeIII/BAP size markers; lane 2, exon 9; lane 3, exon 7; lane 4, exon 6; lane 5, exon 5; lane 6, exon 4. The exons of the p53 gene were generated after 30 cycles of nested PCR using the 2.9-kb fragment as template. Electrophoresis was on 2% agarose gel in 0.5X TBE buffer.

A group of patients who have had biopsies for benign and potentially malignant head and neck lesions were also analyzed for p53 levels of expression (i.e., fibromas, keratosis, hyperkeratosis, and leukoplakias). None of these biopsies or normal specimens was found to overexpress the p53 protein.

IV. DISCUSSION

The role of p53 in the development of human cancers has now been demonstrated in a wide range of tumors;³⁶ however, the stage at which

p53 has its effect on the initiation or progression of the disease remains unclear. A number of reports have considered this to be a late event in the development of colonic cancer,^{37,38} breast cancer,³⁹⁻⁴¹ and lung cancer.^{42,43} However, Chiba et al.⁴⁴ considered that mutations in the p53 gene occurred frequently in the early stages of non-small-cell lung cancers. We have previously argued that because the stopped-smoking group of head and neck cancer patients were found to have high levels of p53 expression, alterations in this gene may be one of the early events in the development of head and neck cancer.⁴ The updated data on smoking history and the stopped-smoking groups in this paper continue to support this hypothesis. It is of particular note that Suzuki et al.⁴⁵ have recently demonstrated a statistical correlation between p53 mutations and lifetime cigarette consumption in non-small-cell lung cancer patients in Japan.

A number of research groups have analyzed p53 mutations in head and neck squamous cell carcinomas from patients in the U.S.,¹¹ Japan,⁹ Italy,¹⁰ and the U.K.^{6,46} All these groups except that of Brachman et al.¹¹ have confined their analysis of the p53 gene to exons 5 to 8, a region previously shown to contain the majority of p53 mutations in squamous cell carcinomas from other anatomical sites. However, we have found a completely different pattern of mutations in the head and neck cancers from patients living in the northwest of England. All of the tumors that showed a p53 mutation by SSCP analysis in our study were found in exon 4, except for two tumors that also had mutations in exon 5. Sequencing of exon 4 in these tumors has presently been undertaken to determine if these mutations are biologically significant; and, as polymorphisms in certain exons have been previously reported, this possibility has to be excluded.^{47,48} On analyzing the p53 exon regions, where mutations have been reported in different parts of the world, no consensus emerges: the majority of the mutations in the U.S. are found in exon 7,^{11,12} whereas exons 5 and 8 are the most common in Japan,^{9,13} and exon 4 in the U.K. No pattern emerged from the Italian study¹⁰ (Table III). Furthermore, on examination of the nucleotide transversion pattern of these p53 mutations in head and neck cancer patients, again

p53 EXON 4

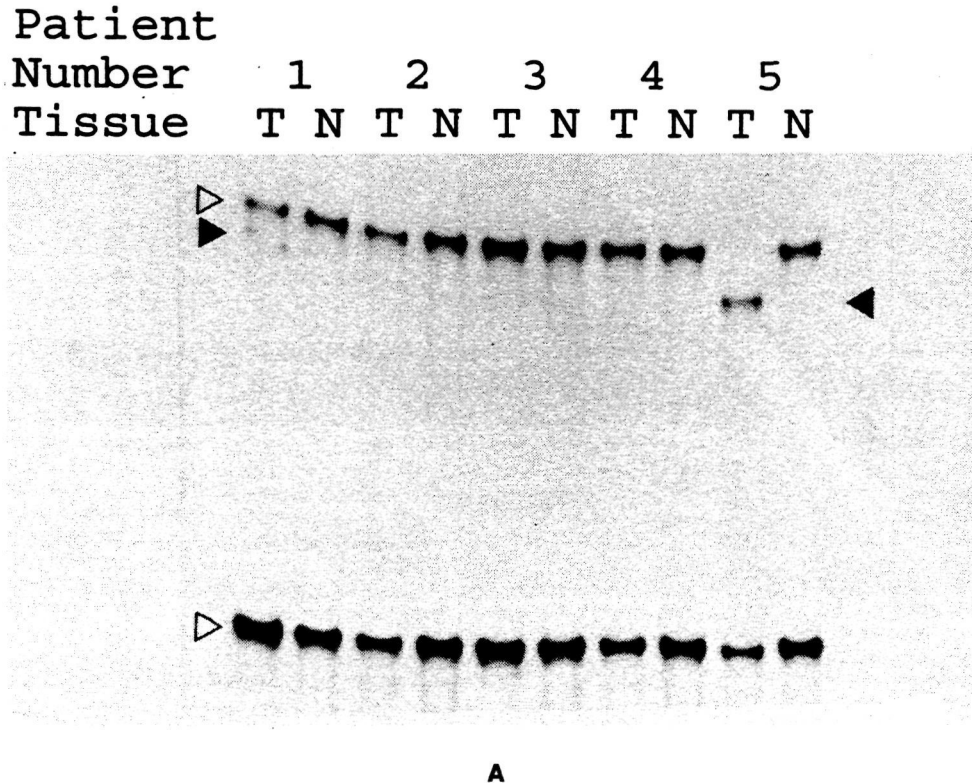


FIGURE 4. SSCP analysis of exon 4 of the p53 gene of the head and neck cancer specimens. An aliquot of the PCR amplified and ^{32}P -labeled samples were loaded on 0.6X hydrolink polyacrylamide nondenaturing gel. The DNA was electrophoresed according to the conditions described in Section II. (A) DNA from patient 1 (lane 1) shows one additional band (solid arrow), while DNA from patient 5 (lane 9) shows altered mobility (open arrow) compared with normal control DNA samples (lanes 2, 4, 6, 8, 10); (B) DNA from patients 6, 7, 9, 10, 11, 12, and 13 (lanes 1, 3, 7, 9, 11, 13, and 15) show one additional band (solid arrow) compared with normal control DNA samples (lanes 2, 4, 6, 8, 10, 12, 14, and 16) (open arrows). T, tumor; N, normal.

no consensus emerges. In the U.S., Somers et al.¹² found that 63% (7/11) of tumors had G-T transversions, whereas Sakai et al.¹³ reported that 50% of their specimens in Japan had G-A transversions. Because the type of mutation is often associated with specific mutagens, it would appear that there are particular carcinogens in different parts of the world causing similar cancers of the head and neck. This may be explained by the presence of regionally distinct carcinogens in both tobacco and alcohol interacting with local environmental cofactors in the development of this disease.

The SSCP method for analysis of p53 mutations has also been used to examine p53 mutations in oral squamous cell carcinomas,¹³ where 63% of the tumors were found to have mutations in the region of the p53 gene assessed (i.e., exons 5 to 8). We found a similar figure (60%) in the group of patients analyzed by SSCP (exons 4 to 7). However, it is most likely that all research groups to date are missing mutations by confining their investigations to specific regions of the p53 gene. In the future, it will be necessary to analyze all the p53 exons by SSCP and confirm the results by sequencing to determine

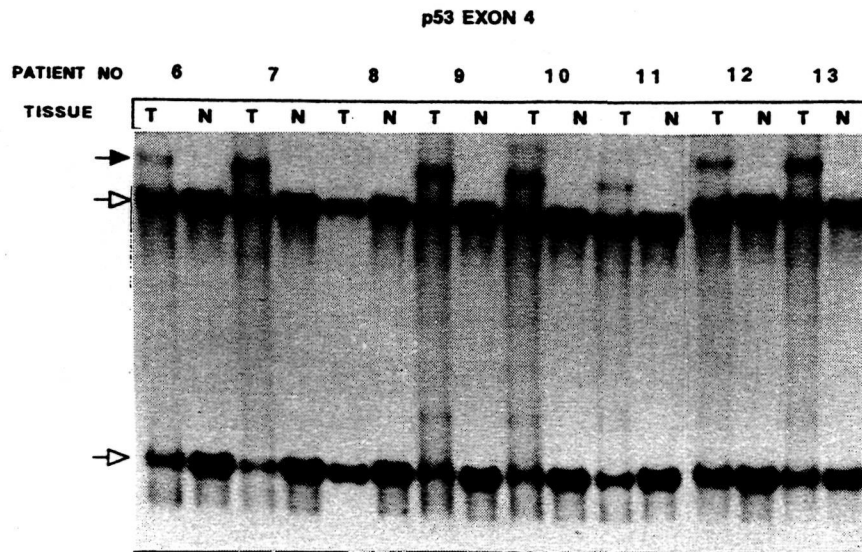


FIGURE 4B

p53 EXON 5

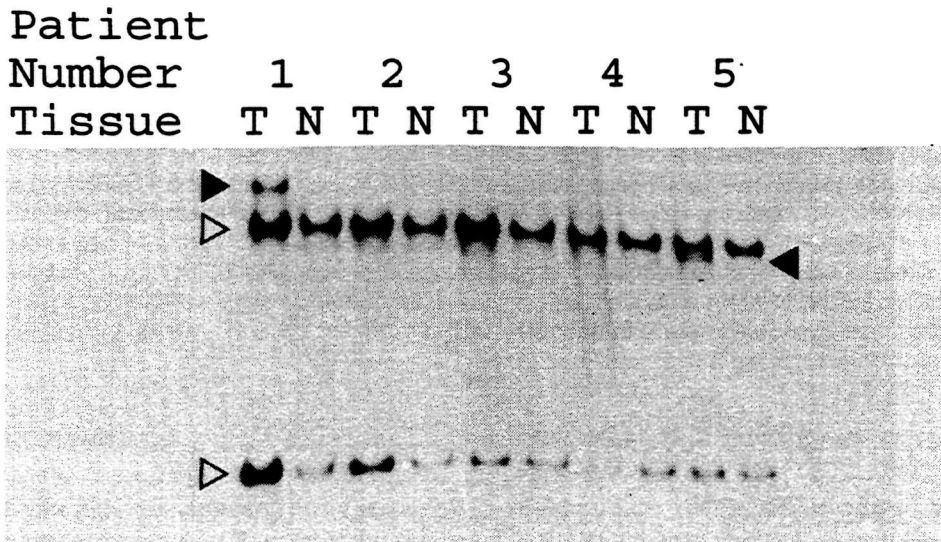


FIGURE 5. SSCP analysis of exon 5 of the p53 gene of the head and neck cancer specimens. DNA from patients 1 and 5 (lanes 1 and 9) show one additional band (solid arrow) compared with normal tissue samples (open arrows) (lanes 2, 4, 6, 8, and 10).

the complete picture; we are currently pursuing this approach.

In addition, a comparison of the p53 expression data with the site of the mutation will provide

further insight into the mechanisms of the role of the p53 gene in the disease. Bodner et al.⁴⁹ very elegantly demonstrated that the expression of the p53 protein in lung cancer patients correlates with

TABLE II
P53 Expression and Smoking History
in 93 Patients with Squamous Cell
Carcinoma of the Head and Neck

Smoking history	p53 expression ^b		
	-	+	++
Nonsmoker	8	4	0
Stopped smoking			
>5 years	2	7	3
Moderate smoker	11	10	3
Heavy smoker	9	26	10
Total	30	47	16

Note: Fishers Exact Test: nonsmoker and heavy smoker ($p < 0.01$); stopped smoker and heavy smoker ($p > 0.05$).

^a Moderate smoker, <20 cigarettes per day; heavy smoker, >20 cigarettes per day; stopped smoking, 5 to 18 years before developing a head and neck cancer.

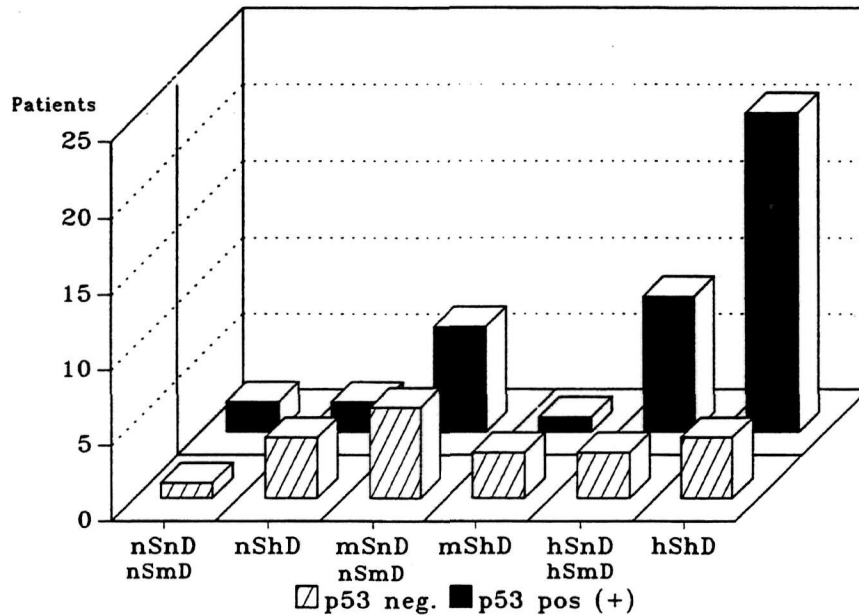


FIGURE 6. p53 overexpression correlates with the patients' history of heavy drinking and heavy smoking ($Z = 2.15$; $p < 0.05$). nSnD = nonsmoker, nondrinker, etc.

the class of the gene mutation. All the positively staining tumors were shown to have mutations in exons 5 to 8, whereas the negative-staining tumors

were shown to have mutations outside these exons. These authors argued that the high-expressor group may represent the expression pattern of the

TABLE III
p53 Mutations in Head and Neck Cancer Classified by Exon and Geographic Region

Geographic region	p53 exons										Ref.
	2	3	4	5	6	7	8	9	10	11	
U.S.	—	—	—	4	5	4	2	1	—	—	11
U.S.	NT	NT	NT	2	1	6	2	NT	NT	NT	12
Japan	NT	NT	NT	1	4	4	5	NT	NT	NT	9
Japan	NT	NT	NT	7	—	—	5	NT	NT	NT	13
Italy	NT	NT	NT	1	2	2	1	NT	NT	NT	10
U.K.	NT	NT	NT	1	NT	1	NT	NT	NT	NT	6
U.K.	NT	NT	9	2	—	—	NT	NT	NT	NT	This study

dominant negative mechanism of p53 inactivation, whereas the low-expressor group may represent the expression of a recessive mechanism of inactivation of the p53 gene. Alternatively, these two expressor classes may represent mutations caused by different groups of mutagens, and thus the p53 overexpressor group in the head and neck patients reported in this study, which correlates with the smoking and drinking history, may represent the activity of specific mutagens in the disease process.

In this investigation, no correlations were found between p53 overexpression and any of the clinicopathological parameters, and this is the largest study reported to date in head and neck cancer. Furthermore, no correlation has been reported between p53 expression and survival in this group of patients. However, in an investigation into p53 expression in "end-stage disease" in squamous cell carcinoma of the head and neck, we have shown that there is a correlation between p53 overexpression and a worse clinical outcome, indicating that p53 may also play a role in the late stages of the disease process.⁵⁰

Investigations into the role of the p53 tumor suppressor gene in the pathogenesis of cancer has shown that this gene plays a number of diverse roles in the development of the disease.^{20,21,51-53} It is now apparent that p53 may also have a number of roles in the development of head and neck cancer based on the previously described results — the relationship between p53 expression in the stopped-smoking group of patients and the correlation between survival and end-stage disease.^{48,54}

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