Patterns of p53 Gene Mutations in Head and Neck Cancer: Full-Length Gene Sequencing and Results of Primary Radiotherapy

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ABSTRACT

p53 gene alterations are common in head and neck cancers, but their prognostic value has not been clearly established. Despite evidence in other cancers that sequencing of the entire p53 coding region provides prognostic information, full-length p53 gene sequencing has rarely been performed in head and neck cancers. In this study, p53 was assessed in a series of 42 pretreatment biopsies from patients with laryngeal carcinomas by full-length gene sequencing and by immunohistochemistry (IHC). Associations among p53 genotype, protein expression, and local recurrence were assessed in a series of 42 pretreatment biopsies from patients for a minimum of 5 years. DNA was extracted from formalin-fixed, paraffin-embedded biopsies, and exons 2–11 of the p53 gene were individually amplified by PCR and then directly sequenced. IHC was performed to detect mutant and wild-type p53 protein using the DO7 monoclonal antibody. p21 protein expression was assessed using the EA1 monoclonal antibody. Twenty genetic alterations were observed in 42 tumors (48%). Four of these alterations (20%) occurred outside exons 5–8. There was a significant association between p53 gene and protein status (χ² = 4.18, P = 0.04), although the correlation was weak (β coefficient = −0.327). Although local relapse following radiation was significantly associated with nodal status, no correlations were observed between p53 status and local control, suggests that this marker is not as powerful as traditional prognostic factors, such as lymph node status.

INTRODUCTION

HNSCC is the sixth most common cancer in the world, accounting for 4% of cancers in men and 2% of cancers in women (1). Worldwide, ~500,000 new cases of HNSCC are diagnosed annually (2). The current management for HNSCC uses radiation therapy and surgery, either alone or in combination (3). Despite treatment advances that have resulted in reductions in patient morbidity, overall 5-year survival rates for HNSCC have remained stubbornly low, at ~30–40%, for the past several decades (4, 5). These results are clearly disappointing and support the need for further studies on the biology of this disease.

Alteration of the p53 gene is the single most commonly reported genetic abnormality in a number of cancers, including those arising from the head and neck (6, 7). Normally, p53 encodes for a nuclear phosphoprotein, which acts as a sequence-specific transcription factor and is involved in cell cycle regulation and cellular response to DNA damage (8). In this setting, p53 protein levels rise, leading to either cell cycle arrest or apoptosis (9, 10). p53-dependent G1-S arrest is mediated, in part, by transactivation of the p21waf1/cip1 gene, which, in turn, promotes cell cycle arrest by binding to and inhibiting cyclin-dependent kinases (11).

In tumors, the prevalence of p53 alterations varies, ranging from 10 to 60% in most cancers and topping 80% in some subsets (7). In HNSCC the reported frequency of p53 alterations ranges from 20 to 90%, depending on the methodologies to assess p53, type of tumor material, and heterogeneity of tumor sites examined. In most studies, investigators have determined the levels of p53 protein using IHC, on the basis that mutant protein is stable but that the half-life of wild-type protein is too short to permit detection (12, 13). Although IHC can provide important information about protein expression, some studies have shown an inconsistent relationship between p53 protein levels and gene status (14, 15). A number of explanations may account for these discrepancies, including differences in antigen retrieval techniques as they are applied to routinely processed tissue specimens, the effects of non-missense mutations on protein expression, and alternate mechanisms of p53 protein stabilization (16–18). Fewer studies have examined p53 at the gene level, in part because it is more laborious, technically more demanding, and frequently not applicable to routinely processed...
tumor biopsies. Moreover, studies that have examined p53 at the gene level have frequently restricted their analysis to traditional “hot spot” regions in exons 5–8 (19–21). Early studies on a narrow range of tumors showed that the majority of p53 mutations occurred within the highly conserved regions in exons 5–8 that code for the DNA-binding domain (7). However, it has been shown that, in some tumor types, up to 25% of gene alterations may occur outside exons 5–8, emphasizing the need for examination of all coding exons (17, 22). Moreover, in some cancers, full-length sequencing to identify p53 gene alteration can provide significant prognostic information (23). Despite this, the prevalence of mutations throughout the entire p53 coding region has not been examined in head and neck tumors from a single anatomical site, such as the larynx.

Mutations in the p53 gene may provide cells with a growth advantage by decoupling them from the normal restrictions imposed on cellular proliferation in response to DNA damage (24, 25). Consequently, cells with p53 mutations may be more resistant to therapies that depend on generating DNA damage to kill cancerous cells. The importance of p53 status and response to radiation therapy in tumor samples is supported by studies of tumors arising in the esophagus, breast, and prostate (23, 26). For example, Prendergast et al. (27) showed that, despite the low prevalence of p53 alterations in pretreatment tumor specimens, p53 mutations and altered protein expression were significant predictors of local treatment failure in a group of 18 node-positive prostate cancers treated by radiation therapy. Results of studies examining the importance of p53 alterations in HNSCC treated by radiation therapy are conflicting; some studies have shown p53 alterations predict local control, but others show no relationship (28–32). Differing results may arise from the pooling of tumors from sites throughout the head and neck, incomplete analyses, and inconsistent treatment regimes. The aim of this study was to determine the prevalence of p53 gene alterations in a series of laryngeal carcinomas by direct sequencing of all coding exons. We also examined the relationship between p53 gene alterations and tumor response to radiation therapy, as assessed by local control.

MATERIALS AND METHODS

Case Selection. Between June 1984 and December 1990, 42 unselected patients with cancer of the larynx were managed by the multidisciplinary head and neck site group at the Toronto-Sunnybrook Regional Cancer Center with a policy of primary radiotherapy, under which surgery was reserved for salvage. The diagnostic work-up included examination under anesthesia with biopsy (if not already performed by the referring physician), complete physical examination, complete blood counts, renal and liver function tests, chest X-ray, and laryngeal tomograms and/or CT scan of the head and neck. An ultrasound of the liver was performed in patients with abnormal liver function tests, and bone scans were performed in patients with unexplained skeletal pain. Patients were staged according to the Union International Contre Cancer Tumor-node-metastasis classification. There were, in total, 42 pretreatment, formalin-fixed, paraffin-embedded tissue biopsies obtained.

Radiation Therapy. All patients were immobilized in a plastic mask and treated with 60Co on a 6-MV linear accelerator.

The primary tumor and bilateral neck nodes were irradiated through lateral opposed photon beams. The supraclavicular nodes were treated with a matched anterior photon field. The initial treatment volumes, including primary tumor, involved nodes, and potential sites of microscopic spread, were treated to a dose of 4600 cGy in 23 fractions over 4.5 weeks to the larynx and regional nodes. Sites of gross residual disease that were ≤3 cm in maximum diameter were boosted with an additional 2000 cGy in 10 fractions over 2 weeks, whereas those that were >3 cm were boosted with an additional 2400 cGy in 12 fractions over 2.5 weeks. All patients, therefore, received a total dose of either 6600 or 7000 cGy.

Monitoring and Follow-Up. Patients were assessed clinically at monthly intervals. A CT scan of the head and neck was obtained 8–12 weeks posttreatment. Complete responders were followed every 3 months for the first 2 years, every 6 months for the next 3 years, and annually thereafter. Chest X-rays were performed at 3 and 12 months and annually thereafter. Suspected sites of local recurrence were evaluated with CT scan and confirmed by aspiration cytology or by biopsy. Disease identified on metastatic survey was not biopsied.

Radiation Therapy Study Cohort. Correlations between p53 status (gene and protein) and treatment outcomes with radiotherapy were limited to a subset of 35 patients who were followed for a minimum follow-up of 5 years or until they experienced an in-treatment field recurrence. This cohort was characterized by a median age of 62 years (range, 45–76 years) and a male:female ratio of 4:1. Distribution by tumor-node-metastasis stage is summarized in Table 1.

Genomic DNA Preparation from Paraffin Sections. Three 5-μm sections of each sample were cut with a microtome and placed on glass slides. Each slide was heated to 65°C to melt the paraffin, and then the tissue was removed with a scalpel and placed in 12 μl of 10× lysis buffer [100 mM Tris-HCl (pH 8.0), 500 mM KCl, 25 mM MgCl2, and 4.5% Tween 20], 98 μl of distilled H2O, and 10 μl of 20 mg/ml proteinase K (Sigma). Following overnight incubation at 55°C, the proteinase K was inactivated by heating the samples for 5 min at 94°C. PCR was then performed on the solubilized DNA recovered in the supernatant.

PCR Amplification. Exons 2–11 of the p53 gene were amplified separately using p53 Gene Analysis Kits (Visible Genetics Inc., Toronto, Canada). Briefly, 3 μl of genomic DNA were added to a 25-μl PCR mixture according to manufacturer’s instructions and then cycled through 35 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s on a thermo-
PCR products were then visualized by electrophoresis of 2 μl of reaction product in a 1% agarose gel.

**Cycle Sequencing.** PCR products were sequenced using nested CY5.5-labeled primers provided in the p53 Gene Analysis Kit (Visible Genetics Inc.). Two to 8 μl of each PCR product were used in a sequencing reaction according to manufacturer’s instructions, and the products were analyzed using the MicroGene Blaster Automated DNA electrophoresis unit (Visible Genetics Inc.). All samples were initially screened by sequencing in either the 5' or 3' directions. All gene alterations were confirmed by repeat sequencing in the opposite direction and then reconfirmed by further reamplification and direct sequencing steps.

**IHC.** Briefly, 5-μm serial sections were cut and mounted on glass slides coated with 2% aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO) in acetone. Sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersion in 0.3% methanolic peroxide for 15 min. Immunoreactivity of the target antigens was enhanced using pressurized heat antigen retrieval (pressure cooking; Ref. 33). The sections were placed in a pressure cooker containing 0.01 M sodium citrate buffer (pH 6.0), heated to 130°C for 2 min, and then cooled. p53 protein expression was determined by incubating the tissue sections with the monoclonal antibody DO7 (Novocastra, Newcastle, United Kingdom) diluted 1:500 in 1×TBS. This antibody recognizes both wild-type and mutant forms of the p53 protein. For analysis of p21 protein expression, tissue sections were incubated with EA1 monoclonal antibody (Oncogene Sciences, Cambridge, MA) diluted 1:200 in TBS. All incubations were carried out overnight at 4°C. The sections were then incubated with a biotinylated secondary antibody for 60 min, followed by the application of preformed avidin-biotin complex (Dakopatts, Denmark) for 60 min. The

**Table 2** Summary of p53 gene mutations and results of IHC for p53 and p21 proteins

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<td>42 wt</td>
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[^a]: del, deletion; ins, insertion; ND, not done; wt, wild type.

[^b]: Immunohistochemical staining categories: 0, 0–5% tumor cells stained; +, 5–9%; ++, 10–24%; ++++, 25–49%; ++++, ≥50%.
bound complexes were visualized by the application of a 0.05% solution of 3,3’-diaminobenzidine (pH 7.6; Sigma) containing 0.3% hydrogen peroxide as a substrate. Following incubation, the sections were washed and then lightly counterstained with hematoxylin, dehydrated, and coverslipped.

**Quantification.** Scoring of p53 and p21 immunostaining was performed by counting the number of cells showing nuclear staining as a proportion of the total tumor cell population, expressed categorically as follows: 0, 0–9% tumor cells stained; +, 10–24% tumor cells stained; ++, 25–49% tumor cells stained; and ++++, ≥50% tumor cells stained. For p53, tumors showing >10% positive cells were considered to show abnormal protein staining.

**Statistical Analysis.** Associations between p53 gene and protein status and with p21 protein expression were assessed by \( \chi^2 \) analysis. The strength of association was assessed by the correlation coefficient. For the radiation therapy cohort, Kaplan-Meier method was used to model time to tumor recurrence as a function of p53 mutational status and p53 protein expression.

### RESULTS

We identified p53 gene alterations in 20 of 42 (48%) biopsies of laryngeal carcinoma. These results are summarized in Tables 2 and 3. These alterations were primarily point mutations resulting in nonconservative amino acid substitutions (Fig. 1a). In addition, there were three insertions/deletions but no nonsense or splice site alterations (Fig. 1b). Genetic alterations were detected throughout the gene, from codon 85 to 355. Of the 20 alterations, four (20%) were outside exons 5–8. The pattern of gene mutations outside these exons differed from the pattern inside. In contrast to the changes inside exons 5–8, which were mostly missense changes, the gene alterations outside exons 5–8 were mostly insertions and deletions. The proportion of tumor versus normal tissue in each biopsy ranged from 10 to 90% (Table 2) with p53 mutations detected in tumors throughout this range.

Using IHC, p53 protein overexpression (Fig. 2) was observed in 11 of 21 (52%) of samples without detectable p53 gene alterations showed overexpression of the protein.

p21 staining was seen in the nuclei of tumor cells (Fig. 3). There was a heterogeneous distribution of p21 staining in tumor cells, with the proportion of positive cells ranging from 5 to 75% in the tumors. There were no correlations between the levels of p21 protein and p53 protein expression (\( \chi^2 = 3.457, P = 0.33 \)) or p53 gene status (\( \chi^2 = 1.627, P = 0.65 \)).

The radiation therapy study cohort of 35 patients treated with radical radiation therapy was followed for a median of 4 years. The 5-year cause-specific, disease-free, and overall survival rates were 91, 77, and 63%, respectively. Local recurrence was documented in 8 patients (Table 5). A univariate analysis of prognostic factors identified T and N stages as significant predictors of local failure. In this analysis, neither the p53 gene status (Fig. 4) nor the combination of p53 gene and IHC status (Fig. 5) emerged as a significant predictor of local failure.

### DISCUSSION

p53 gene alterations are the single most common genetic abnormality in all cancers, including those arising in the head and neck. The reported prevalence of p53 mutations in head and neck cancers varies from 20 to 91% (34–37), and this range may reflect the heterogeneity of tumors that arise in this region (38). Our study examined a cohort of tumors from a well-defined anatomical site with the head and neck, the larynx, and found gene alterations in 48% of patients. These data support the concept that alterations of the p53 gene play an important role in the development of a large proportion of tumors at this site (19, 39).
Initial studies of the p53 gene showed that most mutations were clustered within the conserved regions encoding the DNA-binding domain (8). We found that 20% of all p53 gene mutations occur outside exons 5–8. This is similar to the frequency of 33% in these regions that was found in the only other study to examine HNSCC for p53 gene mutations outside the DNA-binding domain (37). Moreover, these results are similar to studies in breast (17, 23, 40), hepatocellular (41), ovarian, and non-small cell lung (22) carcinomas showing that 10–25% of p53 gene alterations occur outside traditionally examined hot spot regions.

Similar to tumors at other sites, we found the pattern of mutations inside and outside exons 5–8 differed. Hartmann et al. (22) examined the promoter region and exons 1–11 of the p53 gene in 194 primary breast cancers and found that 18 of 82 mutations were outside exons 5–8. This is similar to the frequency of 33% in these regions that was found in the only other study to examine HNSCC for p53 gene mutations outside the DNA-binding domain (37). Moreover, these results are similar to studies in breast (17, 23, 40), hepatocellular (41), ovarian, and non-small cell lung (22) carcinomas showing that 10–25% of p53 gene alterations occur outside traditionally examined hot spot regions.

Table 4  Comparison between results of p53 mutational analysis and p53 protein levels, as assessed by IHCa

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<th>p53 wild-type</th>
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<tr>
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<tr>
<td>Total</td>
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*χ² = 4.179; P = 0.041; Φ coefficient = −0.327.
*a, >10% tumor cells showing nuclear staining.

We found a statistically significant association between p53 mutational status and protein overexpression, although the correlation was weak (Φ coefficient = −0.327). We found that 17% (3 of 18) of tumor samples that contained p53 gene mutations did not show any protein expression by IHC. Two of the three false-negative samples (samples 12 and 37) harbored frameshift insertions or deletions that drastically altered amino acid composition and led to premature protein truncations. These alterations, therefore, may not stabilize the protein sufficiently to permit detection by IHC. Alternatively, negative staining of p53 mutant tumor samples may represent inability of the antibody to react with its epitope due to structural alterations of the translated protein. The third false-negative sample (sample 4) contained a missense mutations that would be predicted to result in p53 protein stabilization but failed to express p53 protein by IHC. One possible explanation for this is that wild-type protein may cause destabilization of mutant p53 protein in its tetrameric form in cells (40).

In contrast, 11 of 21 (52%) of tumors without detectable gene alterations overexpressed p53 protein. There are a number of possibilities for this phenomenon.
of explanations for discrepancy between wild-type p53 gene status and elevated protein levels. Cells that have stained positive may carry p53 mutations that, due to low copy numbers, may be below the threshold of detection. Alternatively, wild-type p53 protein may be stabilized either by binding to cellular or viral proteins or by disturbances in the ubiquination pathway that would otherwise degrade p53 (8). The functional significance of overexpressed wild-type p53 protein in the development of laryngeal carcinomas is not known because IHC cannot differentiate functionally active from inactive protein (13). Finally, technical issues related to the use of routinely processed biopsy material may also account for a proportion of cases showing overexpressed protein detected by IHC in the absence of a p53 gene mutation (31, 42).

p21 plays an important role as a p53-response element following DNA damage. Furthermore, a number of studies have now suggested that p21 is important in cell differentiation and senescence pathways by showing that the topographical distribution in a number of tissues, including esophageal epithelia, is consistent with proliferation arrest and differentiation (43). We found no correlation between p53 gene status or protein levels and p21 protein levels. This is similar to other studies, including those examining laryngeal carcinomas that also found an inconsistent relationship between p21 protein levels and p53 status (44). The relationship between p21 and p53 in cancers, however, may be tissue and tumor type specific. In low-grade gliomas, p21 protein levels are independent of p53 status, but this is not so for glioblastoma multiforme (45). Other tissues in which p21 levels are independent of p53 include ovarian carcinoma (46) and pancreatic carcinoma (47). In contrast, in colorectal cancer, there is a correlation between p21 levels and wild-type p53 (48). We also found that p21 levels were independent of tumor grade. Two other studies have reported conflicting findings regarding p21 and tumor differentiation in head and neck cancers, with one study supporting this association (44) and another finding no relationship (49). In addition, we found no relationship between p21 levels in laryngeal carcinoma and any clinical parameter or prognostic parameter associated with the disease. Our results differ from those of Erber et al. (49), who found that overexpression of p21 protein was associated with increased disease recurrence, shorter disease-free survival, and shortened overall survival and may reflect the smaller number of treatment failures in our series.

Despite evidence in other cancers that p53 alterations at either the genetic or the protein levels are predictive of treatment failure following radiation therapy, their predictive value in HNSCC has not been established (23, 26). In this study, no correlation was identified between p53 status at the gene or protein level and local recurrence following radiation therapy. Similar findings were reported in a study of p53 protein levels in 90 laryngeal carcinomas treated by radiation therapy (31). Similarly, Awwad et al. (50) analyzed 79 head and neck carcinomas treated with radiation and found no association between p53 protein overexpression and overall and disease survival. Our results differ from two recent studies showing a strong correlation between the presence of p53 gene mutations in cancers from a number of different sites in the head and neck and response to radiation therapy (28, 32). The lack of correlation in our study may reflect the smaller number of patients studied in comparison to the two studies showing significance or may show true tumor site differences. Analysis of a larger cohort of patients with laryngeal carcinomas is, therefore, needed to validate this difference.
In conclusion, we have examined all coding exons of the \textit{p53} gene in squamous cell carcinomas from the larynx and found a high prevalence of \textit{p53} gene alterations. We found that a large proportion of gene alterations in these tumors occurred outside exons 5–8 and that the pattern of gene alterations differed outside compared with within this region. We found that there was weak correlation between \textit{p53} gene status and protein expression and that \textit{p53} status at the gene or protein level did not correlate with response to radiation therapy, as assessed by local control.

\begin{table}
\centering
\caption{Summary of \textit{p53} status and clinical features for the radiation therapy cohort (total of 35 patients)}
\begin{tabular}{cccccccc}
\hline
Patient reference no. & Site & Stage & Relapse$^{a}$ & Gene alteration & p53 IHC$^{b}$ & p21 IHC$^{c}$ \\
\hline
1 & Glottis & I & None & Mutant & Positive & ++ \\
2 & Supraglottis & II & None & Mutant & Positive & +++ \\
3 & Glottis & II & None & Wild-type & Negative & + \\
4 & Glottis & 0 & None & Mutant & Negative & 0 \\
5 & Glottis & III & None & Wild-type & Negative & +++ \\
6 & Glottis & I & None & Wild-type & Negative & +++ \\
7 & Supraglottis & III & Local & Wild-type & Negative & +++ \\
8 & Supraglottis & I & None & Mutant & Positive & + \\
9 & Glottis & I & Local & Mutant & Positive & ++ \\
10 & Glottis & I & None & Mutant & Positive & + \\
11 & Supraglottis & IV & None & Mutant & Positive & 0 \\
12 & Glottis & I & None & Mutant & Negative & + \\
13 & Glottis & I & Local & Wild-type & Negative & + \\
14 & Glottis & I & None & Mutant & Positive & + \\
15 & Glottis & I & None & Wild-type & Positive & + \\
16 & Glottis & 0 & None & Wild-type & Positive & 0 \\
17 & Glottis & IV & Local & Wild-type & Positive & ++ \\
18 & Glottis & II & None & Mutant & Positive & + \\
19 & Glottis & I & None & Mutant & Positive & + \\
20 & Glottis & I & Local & Mutant & Positive & ++ \\
21 & Supraglottis & IV & None & Wild-type & Negative & 0 \\
22 & Glottis & I & None & Wild-type & Negative & + \\
23 & Glottis & I & None & Wild-type & ND & ND \\
24 & Supraglottis & IV & Local and regional & Mutant & Positive & + \\
25 & Supraglottis & III & None & Wild-type & Negative & ++ \\
26 & Supraglottis & III & Local and distant & Wild-type & Positive & + \\
27 & Supraglottis & IV & None & Mutant & Positive & ND \\
28 & Glottis & II & None & Mutant & Positive & ++ \\
29 & Glottis & I & None & Mutant & Positive & ++ \\
30 & Glottis & I & None & Mutant & Positive & ++ \\
31 & Glottis & I & None & Mutant & Positive & ++ \\
32 & Glottis & I & None & Mutant & Positive & ++ \\
33 & Glottis & I & None & Mutant & Positive & ++ \\
34 & Supraglottis & I & None & Wild-type & Negative & 0 \\
35 & Supraglottis & III & Local and distant & Mutant & Positive & +++ \\
36 & Supraglottis & IV & None & Mutant & Negative & +++ \\
37 & Supraglottis & IV & None & Mutant & Positive & +++ \\
38 & Glottis & I & None & Mutant & Positive & ++ \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a}All patients were followed for 5 years after radiation therapy.
\textsuperscript{b}Positive, >10\% of cells showed \textit{p53} protein expression in the nucleus of tumor cells; Negative, no or \leq10\% of tumor cells showed staining for \textit{p53} protein by IHC; ND, not done.
\textsuperscript{c}0, 0–9\% tumor cells stained; +, 10–24\%; ++, 25–49\%; ++++, \geq50\%; ND, not done.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Kaplan-Meier analysis of local control for 35 patients treated with radical radiotherapy according to \textit{p53} gene status.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Kaplan-Meier analysis of local control for 35 patients treated with radical radiotherapy according to \textit{p53} gene and protein status.}
\end{figure}
REFERENCES


Patterns of \textit{p53} Gene Mutations in Head and Neck Cancer: Full-Length Gene Sequencing and Results of Primary Radiotherapy

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