

MOLECULAR ASSESSMENT OF HISTOPATHOLOGICAL STAGING IN SQUAMOUS-CELL CARCINOMA OF THE HEAD AND NECK

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Abstract Background. Surgical oncologists rely heavily on the histopathological assessment of surgical margins to ensure total excision of the tumor in patients with head and neck cancer. However, current techniques may not detect small numbers of cancer cells at the margins of resection or in cervical lymph nodes.

Methods. We used molecular techniques to determine whether clonal populations of infiltrating tumor cells harboring mutations of the p53 gene could be detected in histopathologically negative surgical margins and cervical lymph nodes of patients with squamous-cell carcinoma of the head and neck.

Results. We identified 25 patients with primary squamous-cell carcinoma of the head and neck containing a p53 mutation who appeared to have had complete tumor resection on the basis of a negative histopathological assessment. In 13 of these 25 patients, molecular analysis was positive for a p53 mutation in at least one tumor mar-

gin. In 5 of 13 patients with positive margins by this method (38 percent), the carcinoma has recurred locally, as compared with none of 12 patients with negative margins ($P=0.02$ by the log-rank test). Furthermore, molecular analysis identified neoplastic cells in 6 of 28 lymph nodes (21 percent) that were initially negative by histopathological assessment.

Conclusions. Among specimens initially believed to be negative on light microscopy, a substantial percentage of the surgical margins and lymph nodes from patients with squamous-cell carcinoma of the head and neck contained p53 mutations specific for the primary tumor. Patients with these positive margins appear to have a substantially increased risk of local recurrence. Molecular analysis of surgical margins and lymph nodes can augment standard histopathological assessment and may improve the prediction of local tumor recurrence. (N Engl J Med 1995;332:429-35.)

SQUAMOUS-CELL carcinoma of the head and neck is one of the most common cancers, with a global incidence of 500,000 cases per year.¹ Surgical resection is the principal treatment for the majority of advanced-stage carcinomas of the upper aerodigestive tract and a frequent choice in treating early lesions as well. The single most important prognostic factor for squamous-cell carcinoma of the head and neck is complete surgical removal of the neoplasm, because it is generally believed that failure to eradicate the primary tumor is the leading cause of death from this type of cancer.²⁻⁵ When gross tumor remains, local recurrence is likely, leading ultimately to death. Similarly, if microscopic cancer is present at a margin of resection, the rate of local recurrence increases substantially and the survival rate decreases.^{4,6-17} Local recurrence occurs in up to half of patients with even microscopically negative surgical margins, and in these patients it is the leading cause of treatment failure.^{4,5,11} The presence of metastatic squamous-cell cancer in cervical lymph nodes also increases the risk of locoregional recurrence and distant meta-

static spread and correlates with a 50 percent decrease in survival.^{5,9,18-28} The earliest stages of metastasis to the neck can be difficult to identify by light microscopy.^{18,20} Small foci of metastatic cancer, called micrometastases, are often missed because of sampling problems^{18,20}; a single 5- μ m section through a 1-cm lymph node samples only 1/2000 of the node.

Using an assay based on the polymerase chain reaction (PCR) that has the capacity to detect 1 mutant cancer cell among 10,000 normal cells, we sought to determine whether microscopically occult neoplastic cells could be identified in surgical margins and lymph nodes obtained during operations for head and neck cancer.²⁹⁻³¹ This molecular assay relies on the detection of mutations of the p53 gene, the most common specific genetic alteration in human cancer.³² It has been used successfully to detect tumor cells in the stool of patients with colorectal cancer, the urine of patients with bladder cancer, and the sputum of patients with lung cancer.^{29,31,33} Cytologic analysis failed to detect tumor cells in any of these samples. In the current study we determined whether molecular analysis could be more precise than the standard histopathological assessment of cancer in surgical margins and lymph nodes.

METHODS

Study Population

Invasive squamous-cell carcinomas of the head and neck were resected surgically at Johns Hopkins Hospital with the approval of the institutional review board, and portions of the neoplasms were collected with the consent of the patient. After the primary tumor was removed and the margins were examined by study of frozen sections to confirm the adequacy of resection, additional normal-appearing tissue was removed from the edges of the surgical defect. Portions of lymph nodes obtained from neck-dissection specimens that were not

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used for diagnostic histopathological analysis were fresh-frozen. DNA was prepared from all tissues in a separate laboratory to avoid any possibility of PCR contamination.²⁹

Histopathological Examination

Portions of the primary carcinomas, the surgical margins, and the lymph nodes were processed and sectioned in an identical manner to guarantee an accurate histopathological assessment before the molecular analysis was performed. The frozen specimens were embedded in Optimum Cold Temperature medium (OCT, Tissue-Tek, Miles, Elkhart, Ind.), a polyglycol embedding medium, and the frozen specimen block was evenly planed with a cryostat, resulting in a smooth surface for sectioning. First, two sections 5 μ m thick were obtained for hematoxylin-and-eosin staining and examination by light microscopy. The slides were interpreted in a blinded fashion as negative, positive, or nondiagnostic for the presence of squamous-cell carcinoma by a pathologist not involved in the initial assessment. Next, 20 sections 12 μ m thick were cut and placed in a mixture of sodium dodecyl sulfate and proteinase K for DNA analysis. The tissue DNA was extracted with phenol and chloroform and precipitated with ethanol.³⁴ A second set of 2 sections was obtained and stained with hematoxylin and eosin, followed by a second set of 20 sections 12 μ m thick for DNA analysis, and then a third set of 2 sections for staining with hematoxylin and eosin. Thus, 240 μ m of tissue for DNA analysis from each margin was immediately sandwiched between sections examined by light microscopy.

Sequencing of the p53 Gene

A 1.8-kb fragment of the p53 gene encompassing exons 5 to 9 was amplified from the fresh-frozen DNA in the primary tumor by PCR and cloned and sequenced as described elsewhere.^{29,34,35} The products of the sequencing reactions were then separated by elec-

trophoresis on gels consisting of 8 M urea and 6 percent polyacrylamide, fixed, and exposed to film.

Molecular Probing

Patients found to have p53 mutations in their primary tumors were selected for further analysis. DNA extracted from the sectioned margins and lymph nodes was used to amplify exons 5 to 9 of the p53 gene by PCR.^{29,30} The PCR products were then cloned into a bacteriophage vector and amplified further in *Escherichia coli*.²⁹ From 500 to 10,000 clones were then transferred to nylon membranes and hybridized with oligonucleotide probes end-labeled with phosphorus-32.²⁹⁻³¹ These probes were unique and specific for the mutant p53 base pair found by sequencing the amplified region of the p53 gene in each patient's primary tumor (the oligonucleotide probes for each specific p53 mutation are available on request). After hybridization, the membranes were washed stringently at 54 to 60°C to detect only mutant-specific binding of the probes.²⁹ The membranes were then exposed to x-ray film; hybridizing plaques identified the presence of a mutant p53 gene.²⁹⁻³¹ Assuming that each cancer cell contained two copies of the mutant p53 allele, we estimated the percentage of clonal (mutated) tumor cells in each specimen by counting the number of labeled plaques and dividing this number by the total number of plaques present on each plate that contained the inserted p53 DNA fragment (all plaques that hybridized to a wild-type p53 probe).

The assay included positive and negative controls for each margin and lymph node examined. The positive control was the amplified p53 gene product derived from the patient's primary carcinoma; Southern blot analysis was used to detect hybridization of the product to its mutant-specific oligonucleotide probe. The negative control included "cloned" PCR products from reactions devoid of DNA and cloned p53 products derived from patients with different p53 mutations in the primary tumor. All positive assays were repeated.

Statistical Analysis

The data on surgical margins and characteristics of the patients were entered into a standard spreadsheet program (Quattro-Pro, Borland International, Scotts Valley, Calif.) and statistically analyzed with JMP 3.0 (SAS Institute, Cary, N.C.). The probability of a local recurrence of cancer was analyzed with respect to the results of the molecular analysis by the log-rank test.

RESULTS

Study Population

Sixty-nine patients with invasive squamous-cell carcinoma of the head and neck who were scheduled for tumor resection at Johns Hopkins Hospital entered the study. By sequencing the DNA of the primary tumor, we identified 30 patients (43 percent) who had mutations of the p53 gene in their neoplasms. This group of patients consisted of 13 women and 17 men with an average age of 63 years (range, 46 to 85). Twenty-nine of the 30 patients were heavy tobacco smokers, and 25 had a history of heavy alcohol consumption. Most of the patients had advanced-stage or recurrent squamous-cell carcinoma of the head and neck, as is typical in a tertiary referral center.

We obtained a total of 78 surgical margins from the 30 patients (an average of 2.6 margins per patient) and 33 cervical lymph nodes from 6 patients (an average of 5.5 nodes per patient). Five patients were found to have positive surgical margins in the operating room at the time of the final histopathological assessment and were excluded from further analysis. The 72 margins containing no evidence of microscopic carcinoma (as documented on the final pathological reports of the cancer operations in the remaining 25 patients) were

Table 1. Characteristics of the Study Patients with Squamous-Cell Carcinoma of the Head and Neck.

PATIENT No.	AGE (YR)/SEX	SITE OF PRIMARY TUMOR	STAGE*	p53 MUTATION†
1	66/F	Larynx	T3N0M0	CTG→CCG at 257
2	59/M	Larynx	T1N0M0(R)	CGC→CAC at 175
3	67/M	Larynx	T3N0M0	ACC→TCC at 253, ATC→TTC at 254
4	46/M	Hypopharynx	T2N2aM0	TAT→TGT at 220
5	58/F	Oropharynx	T4N1M0	GAC→CAC at 281
6	49/F	Oropharynx	T3N0M0	TGT→TAT at 275
7	66/M	Oropharynx	T3N2aM0	GGT→GAT at 187
8	51/M	Hypopharynx	T4N1M0	CAT→CGT at 193
9	63/M	Oropharynx	T3N2bM0	CGA→TGA at 306
10	85/F	Oral cavity	T4N0M0	CGG→GGG at 248
11	56/M	Hypopharynx	T4N1M0	ATC→TTC at 255
12	62/F	Oropharynx	T1N1M0	CCT→CGT at 278
13	56/F	Hypopharynx	T4N2aM0	GAG→TAG at 298
14	57/M	Larynx	T2N0M0(R)	GAC→GAG at 228
15	59/M	Oropharynx	T4N2bM0	TAT→TGT at 220
16	65/M	Hypopharynx	T3N2bM0	CGC→CAC at 175
17	65/M	Oral cavity	T1N0M0	TAT→GAT at 205
18	70/F	Oropharynx	T3N0M0(R)	CCT→TCT at 278
19	62/F	Oropharynx	T2N0M0	CGG→TGG at 282
20	72/M	Larynx	T4N1M0	CGG→CAG at 248
21	80/M	Larynx	T3N0M0	TAC→CAC at 163
22	68/M	Larynx	T4N2cM0	AAG→TAG at 291
23	66/M	Oral cavity	T4N2aM0	CGT→CTT at 273
24	72/F	Oral cavity	T1N0M0(R)	AGG→GGG at 249
25	65/M	Oral cavity	T3N1M0	AAG→TAG at 164

*According to the tumor-node-metastasis (TNM) staging system of the American Joint Committee on Cancer.³⁶ R denotes recurrent tumor.

†The change in nucleotides is shown, followed by the position of the mutation in the DNA sequence.

Table 2. Molecular Analysis of Surgical Margins.*

PATIENT No.	SURGICAL MARGINS	HISTOPATHOLOGICAL ASSESSMENT	MUTANT-SPECIFIC PROBING	PERCENTAGE OF MUTANT CLONES
1	M1	Negative	Positive	0.35
	M2	Negative	Positive	0.5
	M3	Negative	Negative	—
	M4	Negative	Positive	0.1
	M5	Negative	Positive	0.2
	M6	Negative	Positive	5.0
2	M1	Negative	Positive	0.1
	M2	Negative	Positive	0.25
	M3	Negative	Positive	0.05
	M4	Negative	Positive	0.2
3	M1	Negative	Negative	—
	M2	Positive	Positive	15.0
4	M1	Negative	Positive	5.0
5	M1–M4	Negative	Negative	—
	M5	Negative	Positive	28.0
6	M1–M3	Negative	Negative	—
7	M1, M2	Negative	Negative	—
8	M1	Negative	Negative	—
	M2	Negative	Positive	4.0
9	M1	Negative	Positive	0.2
	M2	Negative	Positive	0.5
	M3	Negative	Negative	—
	M4	Nondiagnostic	Positive	5.0
	M5	Positive	Positive	10.0
10	M1	Negative	Positive	0.4
	M2	Negative	Positive	1.3
11	M1, M2	Negative	Negative	—
	M3	Nondiagnostic	Positive	0.2
	M4	Nondiagnostic	Positive	0.7
	M1	Negative	Negative	—
12	M2	Negative	Negative	—
	M1	Negative	Negative	—
13	M2	Positive	Positive	10.0
	M1–M8	Negative	Negative	—
15	M1	Negative	Negative	—
	M2	Negative	Positive	0.25
16	M1	Negative	Negative	—
17	M1–M4	Negative	Negative	—
18	M1	Negative	Negative	—
19	M1, M2	Negative	Negative	—
20	M1–M3	Negative	Negative	—
21	M1–M4	Negative	Negative	—
22	M1	Negative	Negative	—
23	M1	Negative	Negative	—
24	M1	Negative	Positive	0.4
	M2	Negative	Negative	—
25	M1, M2	Negative	Negative	—
	M3	Negative	Positive	1.0

*The surgical margins (M) studied in a given patient are numbered consecutively beginning with M1. The histopathological examinations were performed by surgical histopathologists on the hospital staff, and the slides were interpreted as positive, negative, or nondiagnostic (in which case there was probably an electrocautery artifact) for squamous-cell carcinoma. The percentage of mutant clones is equal to the number of mutant-specific clones divided by the total number of clones containing the inserted p53 DNA fragment, as described in the Methods section.

submitted for molecular analysis. The characteristics of the 25 patients are shown in Table 1.

Surgical Margins

The 72 apparently negative surgical margins from the 25 patients were probed with the specific p53 mu-

tant oligonucleotide derived from the primary tumors (Table 2). In 13 of the 25 patients (52 percent), the amplified p53 region from at least one surgical margin hybridized to the tumor-specific probe, demonstrating the presence of neoplastic cells containing mutations (Fig. 1 and 2). The estimated percentage of cells with mutations in the surgical margins ranged from 0.05 percent to 28.0 percent (Table 2). The PCR products from the surgical margins of the remaining 12 patients did not hybridize to the mutant-specific probes, suggesting that those margins did not harbor neoplastic cells (Fig. 1).

Additional slides from the 25 patients with histologically negative surgical margins from the operating room were reexamined in a blinded fashion with standard light microscopy by a second pathologist. In three of these patients, 1 surgical margin was positive for squamous-cell carcinoma (66 margins were negative, and 3 were nondiagnostic). The PCR products of the p53 gene in these three margins showed substantial mutant-specific hybridization, each having an estimated population of at least 5 percent neoplastic cells (Table 2). Moreover, two of these three patients whose cancers were reclassified by light microscopy had local recurrences of cancer (as described below under Treatment Outcome). Figure 3 shows representative sections of histologically positive, nondiagnostic, and negative margins.

Cervical Lymph Nodes

Sandwich sections of 33 cervical lymph nodes from six patients with squamous-cell carcinoma of the head and neck were also examined by a pathologist before the molecular analyses were performed. Only 5 of the 33 lymph nodes (15 percent) had microscopical evidence of metastatic cancer. However, molecular analysis identified mutant p53 genes in the PCR products from 11 nodes (33 percent). Therefore, of the 28 lymph nodes that were negative by light microscopy, 6 (21 percent) were found by molecular analysis to contain neoplastic cells. All lymph nodes diagnosed as positive for squamous-cell carcinoma by light microscopy were estimated to contain at least 5.0 percent mutant cells (Table 3). Four of the five patients with occult metastases identified by molecular probes would have had the stage of their head and neck cancers upgraded if the staging had included molecular analysis.

On Southern blot analysis, the amplified products of the p53 gene derived from the primary-tumor DNA in all patients hybridized with their individually synthesized oligonucleotide probes.³¹ In addition, these samples consistently did not hybridize with oligonucleotide probes derived from the sequences of different p53 mutations.

Treatment Outcome

All patients received standard adjuvant treatment as required, including postoperative radiation therapy. At follow-up, 5 of 13 patients (38 percent) with positive margins by molecular analysis had biopsy-proved recurrences of carcinoma (Fig. 4). All five recurrences

occurred by the 7th month, and the median follow-up for the remaining eight patients was 17 months (range, 10 to 26). However, none of the 12 patients whose surgical margins were negative by the same technique had recurrent disease ($P=0.02$ by the log-rank test). The median follow-up in these 12 patients was 13 months (range, 8 to 27). It is noteworthy that the location of tumor margins that were positive by molecular analysis accurately predicted the site of local recurrence in all five patients with recurrences. For example, Patient 5 had a recurrence of her right-alveolar-ridge carcinoma approximately six months after the surgical margin from the right alveolar ridge was shown to be positive by molecular analysis. This tumor recurred despite a full course of postoperative radiation therapy.

DISCUSSION

We have demonstrated by the molecular detection of tumor-specific p53 mutations that 52 percent (13 of 25) of our patients with squamous-cell carcinoma of the head and neck who underwent cancer resections presumed to be complete actually had positive surgical margins. The high incidence of residual tumor cells in these margins closely approximated the percentage of patients who have local recurrences after resection of

head and neck cancer.^{4,5,11} It is still unclear whether epithelial cells with clonal p53 mutations can appear phenotypically normal, although normal-appearing cells can show positive staining with anti-p53 antibodies.^{37,38}

The standard surgical approach for large head and neck cancers is excision of the primary lesion, followed by sampling of the periphery of the resultant defect with multiple intraoperative frozen sections to ensure complete removal of the tumor.¹⁰ However, this technique is subject to sampling errors inherent in the examination of thin sections of a large piece of tissue and interpretive errors by the pathologist.¹⁰⁻¹³ Handling of the surgical specimen by the surgeon and the pathologist, another source of error, may result in a margin that is difficult to interpret (nondiagnostic), as was noted with regard to the three margins that appeared to have been damaged by electrocautery during their collection.

An alternative technique for the analysis of margins is Mohs' chemosurgery, which has the potential to sample tumor margins more thoroughly.¹² This time-consuming technique has demonstrated that in 70 percent of head and neck carcinomas microscopic "fingers" of tumor, 10 to 20 cells wide, extend at least 1 cm away from the gross disease.¹¹⁻¹³ Sampling techniques using frozen sections may miss these minute microscopic ex-

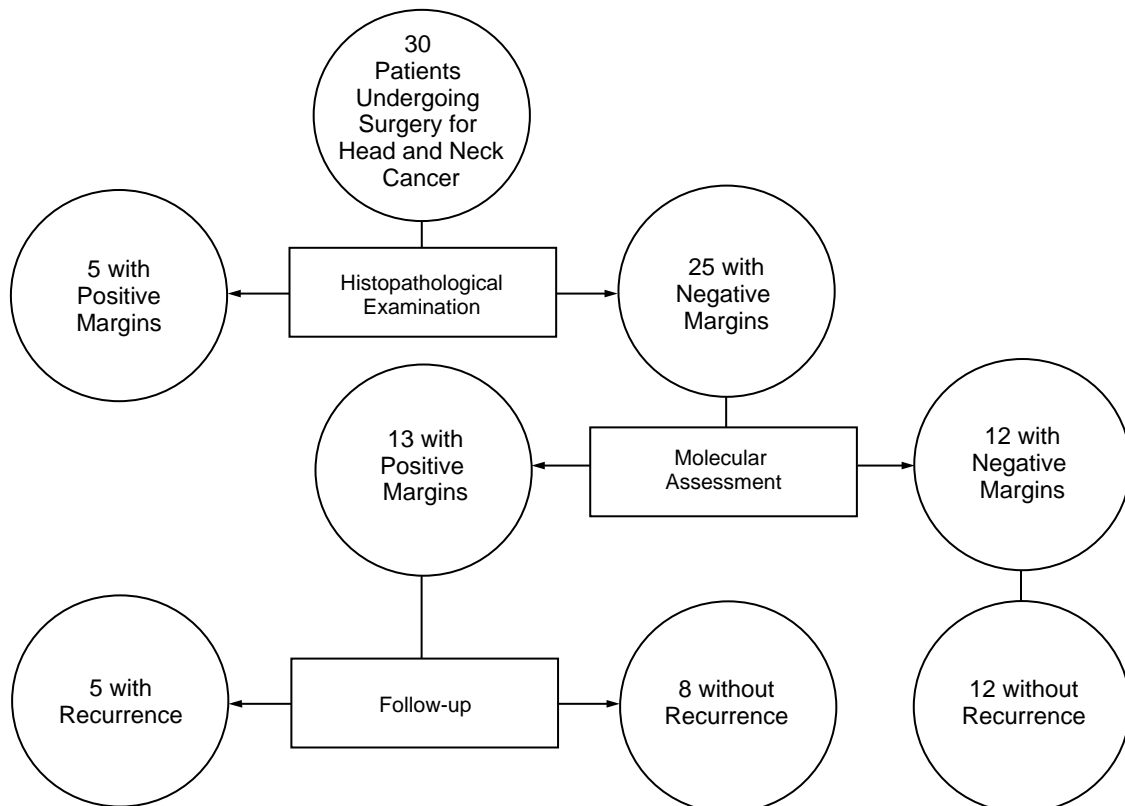


Figure 1. Molecular Analysis and Histopathological Assessment of the Surgical Margins of 25 Patients with Squamous-Cell Carcinoma of the Head and Neck Who Underwent Resection Intended to Be Curative.

Thirteen of the 25 patients (52 percent) had neoplastic cells in the margins of the resected tissue that were not detected on histopathological examination. After a median follow-up of 17 months (range, 10 to 27), 5 of the 13 patients with positive margins by molecular analysis had local recurrences, whereas none of the 12 patients with negative margins by molecular analysis had a local recurrence.

All five patients with positive surgical margins by histopathological examination had persistent locoregional cancer.

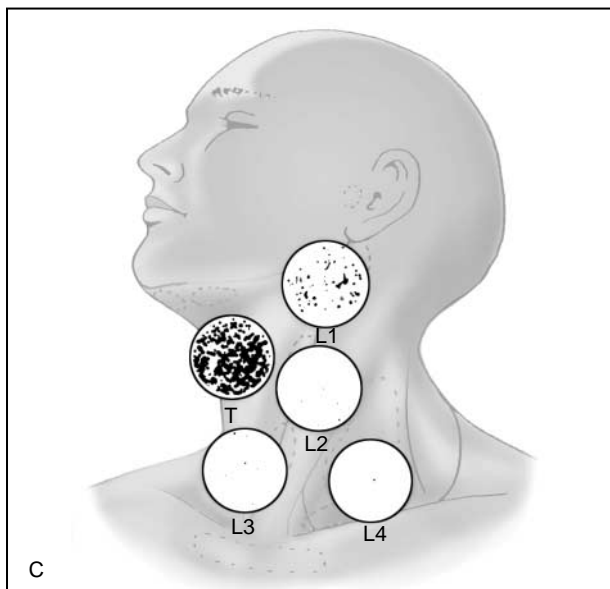
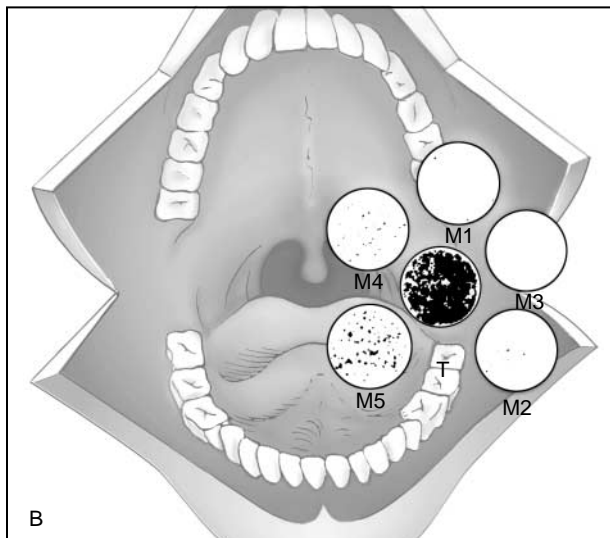
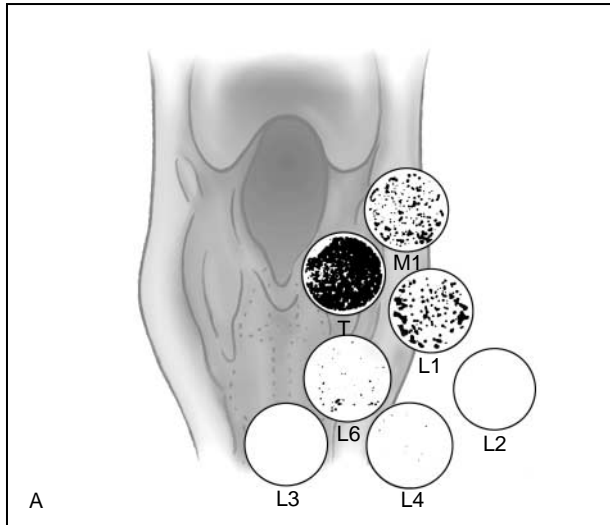


Table 3. Molecular Analysis of Cervical Lymph Nodes.*

PATIENT No.	LYMPH NODES	HISTOPATHOLOGICAL ASSESSMENT	MOLECULAR PROBING	PERCENTAGE OF MUTANT CLONES
1	L1	Positive	Positive	10.0
2	L1-L10	Negative	Negative	—
	L11	Negative	Positive	0.35
3	L1-L3	Negative	Negative	—
4	L1	Positive	Positive	10.0
	L2, L3	Negative	Negative	—
	L4	Negative	Positive	1.0
	L5	Negative	Negative	—
	L6	Negative	Positive	2.0
	L7, L8	Negative	Negative	—
5	L1-L4	Negative	Negative	—
	L5	Positive	Positive	11.0
	L6	Positive	Positive	5.0
16	L1	Positive	Positive	5.0
	L2	Negative	Positive	0.4
	L3	Negative	Positive	0.3
	L4	Negative	Positive	0.7

*The cervical lymph nodes (L) studied in a given patient are numbered consecutively beginning with L1. The histopathologic examinations were performed by surgical histopathologists on the hospital staff, and the slides were interpreted as positive, negative, or nondiagnostic for squamous-cell carcinoma.

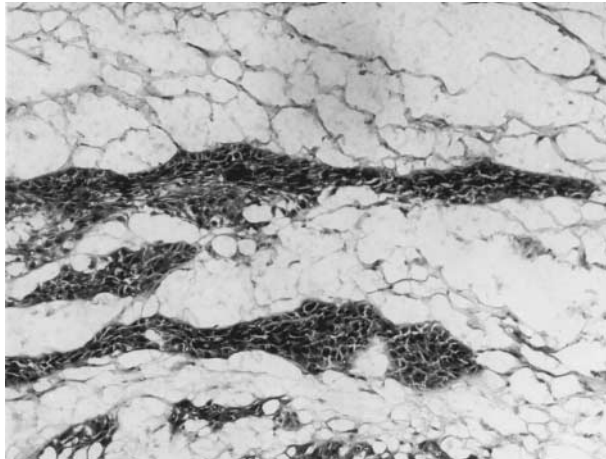
tensions of tumor.¹¹⁻¹³ Thus, it is not surprising that a second light-microscopical examination found residual tumor in 3 of the 72 apparently negative surgical margins sent for molecular analysis. The ability to sample a much larger amount of tissue than a pathologist can examine under the microscope is a major strength of the molecular assay for p53 mutations.

The most important prognostic factor in patients with head and neck squamous-cell carcinoma is the completeness of surgical removal of the tumor.³ A consistent finding is that the presence of microscopic cancer at the surgical margins substantially reduces local control of disease and patient survival.^{7-17,39} The most effective treatment for positive surgical margins is reoperation, with the excision of additional tissue.^{4,6,8,14,16} When excessive morbidity would result from reoperation, adjuvant radiotherapy is a frequently chosen alternative.¹⁴ Molecular recognition of tumor cells in apparently tumor-free tissue may identify patients who would benefit from reoperation or radiotherapy. Moreover, patients with negative surgical margins by molecular analysis may need only close follow-up examinations.

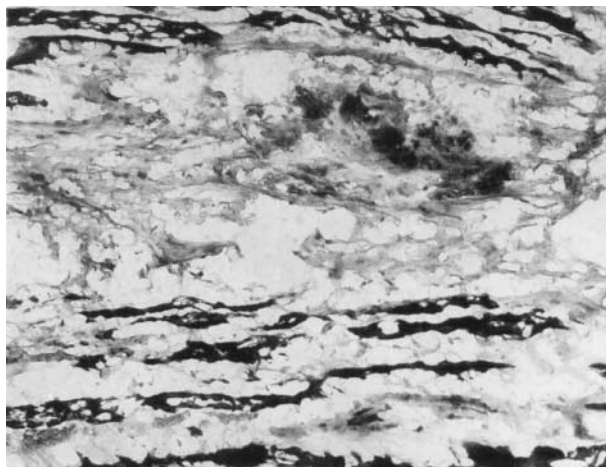
We are currently using the molecular analysis of surgical margins in the evaluation and postoperative follow-up of our surgical patients. The first step in the evaluation of head and neck cancer is endoscopy, with

Figure 2. Molecular Analysis of Surgical Margins and Lymph Nodes.

Insets show autoradiographs of plaques hybridized with mutant-specific oligomers derived from each patient's primary tumor. Hybridizing clones (black dots) are shown in the surgical margins (M) and lymph nodes (L) and in the primary tumor (T), which was used as a positive control. In Panel A (Patient 4, hypopharynx), the assay was positive in one margin (M1) and three lymph nodes (L1, L4, and L6). It was negative (empty circles) in L2 and L3. In Panel B (Patient 9, oropharynx), the assay was positive in M1, M2, M4, and M5 and negative in M3. In Panel C (Patient 16, hypopharynx), the assay was positive in all four lymph nodes to varying degrees. Data on each patient and estimated percentages of tumor cells in the margins and lymph nodes are shown in Tables 1, 2, and 3.



A



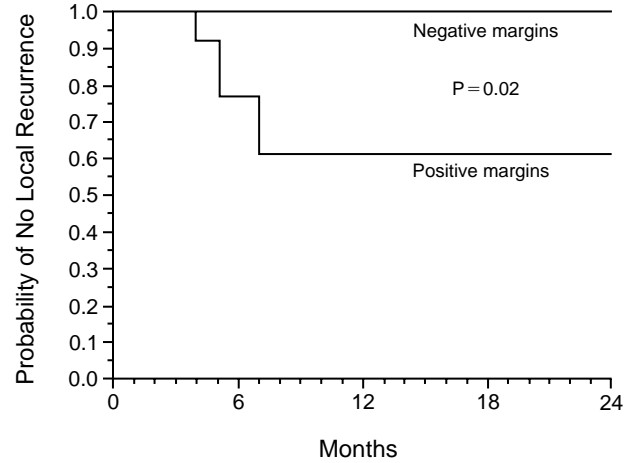
B



C

Figure 3. Photomicrographs of Histopathologically Assessed Surgical Margins.

Hematoxylin-and-eosin staining of positive (Panel A), nondiagnostic (Panel B), and negative (Panel C) surgical margins is shown. These margins were all positive by molecular analysis. The calculated percentages of neoplastic cells were 10 percent in Panel A (M2 from Patient 13), 5 percent in Panel B (M4 from Patient 9), and 0.25 percent in Panel C (M2 from Patient 15).



No. AT RISK

Negative	12	12	8	5	4
Positive	13	10	7	4	2

Figure 4. Probability of Having No Local Recurrence, According to the Results of the Molecular Assay.

Kaplan-Meier curves are shown for the probability of having no local recurrence in the 25 study patients with surgical margins that were negative by light microscopy but were reevaluated with molecular probes. Data on patients who died of metastatic disease (without a local recurrence) or remained alive without local disease were censored in the analysis. The probability of having no local recurrence in patients with positive margins by the molecular assessment was significantly lower than that in patients with negative margins ($P=0.02$ by the log-rank test).

biopsy of the cancer. While the surgeon awaits the final results of biopsy, the definitive surgical resection is typically scheduled to take place one to two weeks after the biopsy. During this period, the p53 gene in the primary tumor is sequenced, the p53 mutation identified, and a unique molecular probe synthesized. The patient then undergoes surgical resection of the cancer, and the surgical margins and lymph-node sections are sent to the pathologist and the molecular laboratory. The molecular assay takes three days, after which a decision is made about further treatment.

The results of the examination of lymph nodes by molecular analysis were noteworthy. Because of the discovery of tumor cells in apparently benign lymph nodes, four of these patients would have been designated as having a more advanced stage of carcinoma. If the results of molecular analysis had been applied, the cancers in these patients would have been restaged from N0, N1, N2b, and N2a to N1, N2c, N2c, and N2b, respectively (according to the staging system of the American Joint Committee on Cancer).³⁶ In current practice, the examination of frozen sections of lymph nodes is critical, because the presence, number, and location of metastatic lymph nodes and evidence of extracapsular tumor spread correlate with locoregional recurrence, distant metastatic spread, and survival.^{5,9,19,21,28,40,41} However, pathologists can miss the early stages of metastatic disease in lymph nodes.²⁰ The application of molecular analysis to clinical trials may help stratify patients more precisely.

A current limitation of the technique we have described is that p53 mutations are present in only half of head and neck cancers.^{34,37} However, other genetic changes in squamous-cell carcinoma of the head and neck may provide additional markers for similar analysis. For example, inactivation of the retinoblastoma gene, which has been implicated in approximately 20 percent of head and neck cancers with loss of chromosome 13q, may serve as a second molecular marker for occult squamous-cell carcinoma of the head and neck.^{42,43} Moreover, the ease by which other clonal markers can be identified without sequence analysis may allow the detection of other genetic alterations.⁴⁴ A second limitation is that this technically challenging assay requires approximately three days to complete. Alternative molecular techniques, including other PCR-based assays⁴⁴⁻⁴⁶ and tests using ligation in detection or amplification,⁴⁷ are being developed to detect mutant cells.

A prospective multi-institutional trial has recently been initiated to evaluate the efficacy of the molecular analysis of surgical margins and lymph nodes in surgery to treat head and neck cancer. Because histopathological assessment is so important for staging, prognosis, and therapeutic intervention in most kinds of tumors, the addition of molecular analysis may have far-reaching implications.

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