Featured Article

Tetranucleotide Microsatellite Instability in Surgical Margins for Prediction of Local Recurrence of Head and Neck Squamous Cell Carcinoma

Stephane Temam,1,4 Odile Casiraghi,2 Jean-Baptiste Lahaye,3 Jacques Bosq,2 Xian Zhou,5 Morbize Julieron,4 Gerard Mamelle,1 J. Jack Lee,5 Li Mao,4 Bernard Luboinski,1 Jean Benard,3 and Francois Janot1

Departments of 1Head and Neck Surgery, 2Pathology, and 3Genetics, Institut Gustave-Roussy, Villejuif, France, and Departments of 4Thoracic/Head and Neck Medical Oncology and 5Biostatistics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Purpose: Postoperative radiotherapy is used to prevent local recurrence of head and neck squamous cell carcinoma in patients with positive surgical margins. We sought to determine whether tetranucleotide microsatellite instability could be detected in surgical margins and used to predict local recurrence.

Experimental Design: We prospectively collected tumor and surgical margin specimens from patients with head and neck squamous cell carcinoma who had undergone surgical resection at Institut Gustave-Roussy during a 1-year period. Margins were considered positive if extensive pathological examination revealed either carcinoma within 5 mm or dysplasia. We tested five tetranucleotide microsatellite markers (UT5085, L17686, D95753, ACTBP2, and CSF1R) in the tumor specimens and paired surgical margins of the patients whose margins were negative on pathological examination.

Results: Pathological examination revealed that among the 76 patients, 22 had positive margins; therefore, these patients were excluded. Of the 54 remaining patients, 26 (48%) had tumors informative for markers UT5085, L17686, or both; the other 3 markers were not informative. Seven (27%) of the 26 informative tumors had the same instability pattern in the surgical margins. At a median follow-up of 26 months, 5 of the 7 local recurrences occurred in patients with molecularly positive surgical margins. A strong, independent association was found between positive surgical margins and local recurrence (P = 0.01; hazard ratio, 6.49).

Conclusions: Tetranucleotide microsatellite instability in surgical margins may be a useful biomarker to predict local recurrence of head and neck squamous cell carcinoma in patients with apparently disease-free margins.

INTRODUCTION

Of >500,000 new cases of head and neck squamous cell carcinoma (HNSCC) diagnosed annually worldwide, ~23,000 are diagnosed in France, of which the incidence of HNSCC is among the highest in the world (1). The high incidence of this malignancy in France probably owes to the greater prevalence of combined smoking and alcohol consumption in this country than in other nations (2, 3).

Surgical resection of primary tumors and neck lymph nodes remains a major therapeutic approach for HNSCC. Postoperative radiotherapy improves local and regional control for patients with locally advanced tumors; surgical margins found, on pathological examination, to be positive for malignancy; and nodal metastasis, especially with extracapsular spread (4, 5). However, whether postoperative radiotherapy is effective for patients with midsize tumors, histopathologically negative surgical margins, and no node involvement remains unclear, although 15–20% of these patients develop local or regional recurrence after surgery (6).

Many molecular markers discovered during the past 10 years have been shown to permit distinction between malignant and nonmalignant tissues (7). Some of the markers can be used to determine premalignant conditions before the appearance of a histopathologically evident abnormality (8). In the first report of molecular assessment of surgical margins, which was published in 1995, Brennan et al. (9), assessing p53 mutations of patients with HNSCC, demonstrated that this marker was valuable in predicting local and regional control. Several investigators subsequently analyzed surgical margins using p53 mutations (10), microsatellite alterations (11), and eIF4E overexpression (12) as markers and in each case showed an association between positive surgical margins and tumor recurrence.

Microsatellites are small repetitive nucleotide sequences that are highly polymorphic in the general population allowing the distinction between maternal and paternal alleles. The most frequent microsatellite alteration in tumors is loss of heterozygosity (LOH), the loss of one allele, which is usually considered a major mechanism for inactivating tumor suppressor genes (13). Another type of microsatellite alteration is microsatellite instability (MSI), or the insertion or deletion...
of repeat DNA microsatellite sequences (14). The mechanism causing MSI in sporadic cancers is unclear and appears to differ from that causing MSI in familial colorectal cancers (15). Because MSI can be easily detected in tissues containing clonal expanded cells, such as tumor tissues, it may be a sensitive molecular marker for the detection of cancer cells in tissues with a substantial background population of normal cells (7, 16–18).

The present study, which was performed in a French population, was designed to test whether MSI can detect residual tumor cells in histopathologically negative surgical margins of patients who have undergone curative surgery and whether surgical margins found to be molecularly positive on the basis of MSI can predict local or regional recurrence in these patients.

PATIENTS AND METHODS

Sample Collection and Histopathologic Examination. Between January 1 and December 31, 2000, we prospectively collected tumor biopsy specimens, peripheral blood lymphocytes, and surgical margin specimens from 76 patients treated for HNSCC at Gustave-Roussy Institut (Villejuif, France). The Institutional Review Board approved the study, and informed consent was obtained from all of the patients.

In each case, the surgeon collected surgical margin specimens (i.e., mucosa and deep tissues) from the edges of the surgical defect for frozen section examination or from the edges of the excised surgical specimen (Fig. 1). The specimens were immediately stored in liquid nitrogen. Tissue from the margins (mucosa and deep tissue) of the surgical specimens were stained and fixed in formalin. Two pathologists (O. C. and J. B.) then performed extensive pathological examination with multiple sections on the fixed surgical specimen and frozen surgical margins. In each case, the distance between the tumor and surgical margins was recorded (Fig. 1), and it had to be >5 mm. We also looked for dysplasia in each surgical margin according to WHO criteria (19). Patients with close margins or all grades of dysplasia were considered to have pathologically positive margins.

Molecular analysis of the specimens with negative margins was performed and follow-up data of the patients analyzed. The patients with positive margins were excluded from the analysis.

Study Design. Tumor staging (pTNM Union International Contre Cancer 1997) was performed on the basis of findings of physical examination, panendoscopy, and head and neck computed tomography scan, magnetic resonance imaging, or both and modified by the additional evidence acquired from surgery and from pathological examination. All of the patients included in this study were judged to be metastases-free before surgery on the basis of clinical and biochemical examinations and chest radiography. Computed tomography examination of the chest, ultrason examination of the liver, and nuclear bone scan were performed in patients with N3 nodes or nodes located in the lower part of the neck. All of the patients underwent unilateral or bilateral neck dissection depending on tumor sites and node extension.

The indication for postoperative radiotherapy depended on tumor stage, tumor site, and node invasion. Radiotherapy targeted the tumor and neck and was performed as soon as clinical conditions permitted and always within 7 weeks after surgery.

Every patient had the same follow-up schedule: clinical examination and flexible endoscopic examination performed every 3 months during the first 2 years and every 6 months thereafter until 5 years after the end of treatment. In addition, a head and neck computed tomography scan was obtained 4–6 months after the end of treatment and a chest X-ray every 6 months until 5 years had elapsed. In cases of suspected recurrence, biopsy was performed. Nodal and metastatic evolutions were evaluated on the basis of clinical examination and computed tomography scan. Panendoscopy with biopsy was performed when needed.

Analysis of MSI. All of the tumor biopsy specimens included in the analysis were diagnosed as invasive HNSCC and had a proportion of tumor cells >70%. Serial 40-μm-thick frozen sections of each surgical margin specimen were performed with H&E histopathologic control of the first and the last 5-μm-thick section. A second pathologist, blinded to the clinical data, reviewed all of the slides to confirm complete excision of the tumors.

DNA from tumor and surgical margin specimens was extracted using the QIAamp Tissu kit (Qiagen, Courtaboeuf, France). Fresh blood was collected from patients in EDTA tubes, and lymphocytes were separated to extract normal control DNA with the QIAamp Blood kit. DNA quality was verified using GeneQuant II (Amersham Pharmacia Biotech, Cambridge, United Kingdom).

A panel of five tetranucleotide microsatellites was used. The
tetranucleotide microsatellite UTS085 was amplified with primers referenced in the GenBank sequence database (accession no. GDB 309286), as described by Coulet et al. (20): (a) UTS085A, 5’-AAAGTGGGATAAGGCAGC-3’; and (b) UTS085B, 5’-AGATGCAACAACACATACAGC-3’. The other four tetranucleotide microsatellites (D9S753, ACTBP2, CSF1R, and L17686) were amplified with the primers described by Xu et al. (21). The primer (a) was labeled in blue by 6-carboxy-fluorescin. Amplification was performed in a 20-μl reaction volume with 0.5 units of Hot-Start TaqDNA polymerase (Qiagen), 0.2 μM deoxynucleotide triphosphate, 3.2 pmol of each forward and reverse primer, 2 mM MgCl₂, and 2 μl of 10× Qiagen buffer. A total of 100 ng of DNA was used as a template for each sample. The mixtures were denatured for 20 min at 95°C, followed by 35 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s. A final elongation was performed for 10 min at 72°C. Amplified products were run in polyacrylamide sequencing gels, ELLIOSEQ gel (Laboratoire Ellios Bio Media, Paris, France), mixed with 0.5 μl of GENESCAN-500 ROX (Applied Biosystems, Inc., Warrington, United Kingdom) and 2.5 μl of formamide blue after a 2-min denaturing step at 94°C. PCR products were detected on the gel by laser fluorescence on an ABI prism 377 DNA sequencer (Applied Biosystems, Inc.). Data were analyzed using Genescan analysis (Applied Biosystems, Inc.).

DNA from all of the tumor specimens and lymphocytes from the corresponding patients were amplified to select tumors with MSI, which was defined as the presence of a new pattern in at least one extra band in tumor DNA compared with lymphocyte DNA. Patients with at least one MSI on one marker in tumor were considered informative patients and were included in the analysis of surgical margins, in which DNA was compared among the tumor specimen, surgical margin specimen, and lymphocytes. A positive surgical margin was defined as the finding of a pattern similar to that in the tumor.

Statistical Analysis. Univariate analyses using Fisher’s exact test and Student’s t test were performed for categoric and continuous data, respectively. Clinical and biological characteristics were analyzed for their association with time to local recurrence using Cox proportional hazards models. Estimates of survival curves were calculated according to the Kaplan-Meier product-limit method and were calculated from the time of surgery to the time of death or the last follow-up visit. Times to local recurrence for various prognostic groups were compared using the log-rank test. The Cox proportional hazards regression model was used to assess the prognostic effect of patient characteristics and molecular markers to estimate local disease-free survival; a confirmatory Cox proportional hazards regression model was performed to assess the need for transformation on the basis of Martingale residual plots. Predictive variables with P values of <0.10 for the univariate Cox proportional hazards model were included in a multivariable model. In this model, we implemented the rule of a backward elimination with a P of 0.05, thereby allowing any variable previously deleted to be included in the final model if its P was <0.05. All of the computations were carried performed using SAS (Cary, NC) and S-plus 2000 software (Seattle, WA).

RESULTS

During the study period, 76 patients underwent surgery of the primary tumor and had specimens suitable for analysis. Extensive pathological examination revealed that 22 (29%) of the patients had positive surgical margins (i.e., carcinoma ≤5 mm from the surgical margin or findings of all grades of dysplasia, according to WHO criteria, in the surgical margin); these patients were excluded from the analysis.

The 54 patients with negative surgical margins were 46 men and 9 women ranging in age from 31 to 79 years (median, 58 years). MSI was detected in the tumor specimens of 26 patients (48%) with at least one of the markers analyzed. The clinical characteristics of these 26 patients are shown in Table 1. Six tumors contained MSI with UTS085 only, 7 tumors with L17786 only, and 13 tumors with both markers. The other markers (D9S753, ACTBP2, and CSF1R) were not informative for tumors in any patient and, therefore, were excluded from additional analysis of the surgical margins.

A total of 113 surgical margin specimens from the 26 informative patients (mean, 4 specimens; range, 3–8 specimens) were analyzed for markers UTS085 and L17786. Seven of the 26 patients (27%) had at least one positive margin (Fig. 2) determined by the two markers, including 2 patients with at least one positive margin with UTS085 only and 5 with at least one positive margin with both markers. Three patients had two positive margins in the same surgical specimen. There were eight mucosal positive margins and two deep positive margins. No statistically significant association between any clinical characteristic and surgical margin status was found (Table 2).

During the follow-up period (median, 26 months; range, 5–42 months), 7 of the 26 informative patients (27%) had local recurrence, including 5 with nodal relapse. Five of the 7 patients with molecularly positive surgical margins developed local recurrence, compared with only 2 of the 19 patients without molecularly positive margins. MSI positivity of the surgical margins was strongly associated with local recurrence (P = 0.006; Fisher exact test), as shown in Table 2, and with time to local recurrence (P = 0.01; Kaplan-Meier analysis with log-rank test), as shown in Fig. 3. No second primary tumor occurred.

Sex, pT stage, pN stage, histopathologic gravity sign (i.e., perineural invasion, angiolymphatic invasion, or both), postoperative radiotherapy, and tumor site were not associated significantly with time to local recurrence. Cox regression analysis of time to local recurrence showed that a positive surgical margin was a strong and independent risk factor (P = 0.03; hazard ratio, 6.49; 95% confidence interval, 1.26–33.57). Age, sex, stage (i.e., limited versus advanced), pT stage (1 or 2 versus 3 or 4), pN stage (0–2a versus 2b–3), histopathologic gravity signs, and postoperative radiotherapy had no correlation with time to local recurrence (yielding P values ranging from 0.27 to 0.96) and were not selected in a multivariate stepwise logistic regression analysis (Table 3).

DISCUSSION

We found that using tetranucleotide microsatellite markers to detect residual cancer cells in histopathologically negative
surgical margin specimens from patients with HNSCC was predictive of local recurrence. A considerable number (27%) of patients with negative findings on extensive pathological examination had molecularly positive surgical margins, and the probability of local recurrence was significantly higher in these patients than in those with molecularly negative surgical margins.

Our results are consistent with the report by Brennan et al. (9), who found p53 mutation-positive surgical margins in 10 of 22 patients (excluding 3 patients who were found to have positive margins on pathological reexamination); all three local relapses in that study occurred in patients with p53 mutation-positive margins. Using the same p53 phage plaque assay to detect p53 mutations with very high sensitivity, Partridge et al. (10) found positive surgical margins in 6 of 11 patients analyzed; four of the five local recurrences occurred in this group.

Although predicting local recurrence by p53 mutations seems promising, MSI is potentially more useful in clinical settings. The complexity of the p53 mutation assays described limits their use in routine clinical practice, whereas microsatellite alterations can be easily detected in tumor tissues and, therefore, represent useful markers for detecting minimal residual tumor cells (7, 17). LOH analysis has also been used to detect tumor DNA in many types of cancer (7), including HNSCC (11, 18, 22, 23). However, LOH analysis is not a very sensitive assay, because this abnormality may be undetectable in specimens whose proportion of tumor cells is 30% or fewer. In contrast, by dilutional calibration, it had been shown that MSI might detect 1 tumor cell among 200 normal cells (18). We expect that by using a laser fluorescence detection assay on an ABI prism 377 DNA sequencer, as in our study, the sensitivity was slightly higher.
In the present analysis, we studied only tetranucleotide repeats to avoid PCR stutter and false-positive results. However, compared with LOH and p53 mutations, MSI is less frequent in HNSCC. Using a five-marker panel, the rate of informative patients was only 48%. These findings confirm the results of Xu et al. (21), who found 56% of informative HNSCC with these markers. However, unlike Xu et al. (21), we found that of the five markers tested, three (D9S753, ACTBP2, and CSF1R) did not detect instability in our series. It is possible that different markers are needed for patients with different malignancies or risk factors. Additional tetranucleotide markers or other markers must be identified to improve detection sensitivity.

In addition, we noted that two margins were positive with the UT5085 marker but not with the L17686 marker. It is possible that we detected precursor lesions with less advanced genetic alterations; alternatively, the findings may indicate that different aggressive subclones are related to the heterogeneity of tumors.

Immunochemical analysis is another encouraging method, as reported by Nathan et al. (12), who found that proto-oncogene eIF4E was overexpressed in 98% of tumor and 52% of surgical margin specimens and that the finding of eIF4E-negative stains was significantly and independently associated with disease-free survival.

In an analysis of 10 patients with LOH, Tabor et al. (24) found that 7 had biologically positive surgical margins; however, dysplasia was also found in all 7 of the cases. Because dysplasia and close margins are each associated with a significantly increased rate of local recurrence (25–27), inclusion of such patients complicates the interpretation of data.

### Table 2 Patient characteristics according to surgical margin status

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Surgical margins</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative, n = 19</td>
<td>Positive, n = 7</td>
</tr>
<tr>
<td>Age, years</td>
<td>62.5 ± 11.5</td>
<td>63.8 ± 11.4</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3 (15.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Male</td>
<td>16 (84.2)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Site, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>7 (36.8)</td>
<td>2 (29)</td>
</tr>
<tr>
<td>Larynx</td>
<td>1 (5.3)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>6 (31.6)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>5 (26.3)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Tumor stage, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (stage I or II)</td>
<td>4 (21)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Advanced (stage III or IV)</td>
<td>15 (79)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>pT stage, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1 or pT2</td>
<td>5 (26)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>pT3 or pT4</td>
<td>14 (74)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>pN stage, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0-pN2a</td>
<td>13 (68)</td>
<td>5 (71)</td>
</tr>
<tr>
<td>pN2b-pN3</td>
<td>6 (32)</td>
<td>2 (29)</td>
</tr>
<tr>
<td>Histopathologic gravity signs, b n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>13 (68)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>Yes</td>
<td>6 (32)</td>
<td>3 (42)</td>
</tr>
<tr>
<td>Postoperative radiotherapy, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4 (21)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>Yes</td>
<td>15 (79)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Local recurrence, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>17 (89)</td>
<td>2 (29)</td>
</tr>
<tr>
<td>Yes</td>
<td>2 (11)</td>
<td>5 (71)</td>
</tr>
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</tbody>
</table>

*Fisher’s exact test.

bHistopathologic gravity signs: perineural invasion, angiolymphatic invasion, or both.

Log-rank test.

In an analysis of 10 patients with LOH, Tabor et al. (24) found that 7 had biologically positive surgical margins; however, dysplasia was also found in all 7 of the cases. Because dysplasia and close margins are each associated with a significantly increased rate of local recurrence (25–27), inclusion of such patients complicates the interpretation of data.
Therefore, advantages of our study design are the extensive pathological analysis of the tumor and surgical margin specimens and the exclusion of all of the patients with either malignancy within 5 mm or findings of all grades of dysplasia. The inherent anatomical and functional limits of resection, deep soft-tissue margins, represent greater clearance problems than do the mucosal margins. The molecular assessment described herein could allow study of large specimens of this deep tissue, which would be more representative than a single 5-μm-thick section with light examination.

Because tetranucleotide MSI detection can be easily performed within 2 weeks after surgery in a routine practice, the detection of molecular alterations in surgical margins might prove useful in postoperative decision-making, such as the addition of adjuvant radiotherapy. However, our study, like previously published studies, cannot give definitive guidelines, because most of the patients had advanced disease with node involvement and clearly needed postoperative radiotherapy. Tumor stage was not associated with local recurrence, probably because we selected patients after exhaustive pathological assessment or because the sample size was small. Nevertheless, we believe that molecular assessment of the surgical margins should be done in patients with midsize tumors who have a moderate risk of local recurrence and a low risk of node recurrence. Clearly, additional studies with larger sample sizes are necessary to validate our current findings. Such analysis could also be informative for determining whether patients with recurrent disease who are undergoing salvage surgery should undergo repeat irradiation. We are currently collecting surgical margins in such patients so that we might determine whether tetranucleotide MSI detection could play a role in routine clinical decision-making.

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REFERENCES


Table 3 Univariate and multivariate Cox proportional hazards models in estimating time to local recurrence

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Parameter estimate</th>
<th>SE</th>
<th>P</th>
<th>Hazard ratio (confidence interval)</th>
</tr>
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<tr>
<td>Age</td>
<td>−0.01</td>
<td>0.03</td>
<td>0.75</td>
<td>0.99 (0.93–1.06)</td>
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<td>Stage (early versus advanced)</td>
<td>0.04</td>
<td>0.84</td>
<td>0.96</td>
<td>1.04 (0.20–5.37)</td>
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<td>pT (1 or 2 versus 3 or 4)</td>
<td>0.30</td>
<td>0.84</td>
<td>0.72</td>
<td>1.35 (0.26–6.96)</td>
</tr>
<tr>
<td>pN (0-2a versus 2b-3)</td>
<td>0.63</td>
<td>0.76</td>
<td>0.41</td>
<td>1.87 (0.42–8.40)</td>
</tr>
<tr>
<td>Histopathologic gravity signs (+ versus −)</td>
<td>0.84</td>
<td>0.76</td>
<td>0.27</td>
<td>2.32 (0.52–10.39)</td>
</tr>
<tr>
<td>Postoperative radiotherapy (yes versus no)</td>
<td>−0.78</td>
<td>0.77</td>
<td>0.31</td>
<td>0.46 (0.10–2.06)</td>
</tr>
<tr>
<td>Surgical margins (+ versus −)</td>
<td>1.87</td>
<td>0.84</td>
<td>0.01</td>
<td>6.49 (1.26–33.57)</td>
</tr>
</tbody>
</table>

*No covariate other than surgical margin status was entered in the multivariate analyses, because P > 0.10 for all other covariates.*


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