Molecular Diagnosis of Surgical Margins and Local Recurrence in Head and Neck Cancer Patients: A Prospective Study

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ABSTRACT

Purpose: Approximately 10–30% of surgically treated head and neck cancer patients develop local recurrences while the resection margins are histologically tumor free. These recurrences may arise from cancer cells left behind but not detected by the pathologist, or they may develop from precursor lesions adjacent to the tumor that were not completely resected. We have investigated whether TP53-mutated DNA in the surgical margins is suitable to identify patients with head and neck squamous cell carcinoma at risk for local and locoregional recurrence.

Experimental Design: In a prospective cohort study of 76 patients with histologically tumor-free margins, the presence of TP53-mutated DNA was determined in the surgical margins using the phage plaque assay and correlated to clinical outcome. Immunostaining of the molecular-positive margins for mutated p53 protein was used to identify whether unresected precursor lesions or residual tumor cells were left behind.

Results: The absence of TP53-mutated DNA in surgical margins was significantly associated with remaining free of local and locoregional recurrence (P = 0.027 and P = 0.028, respectively). Moreover, the presence of TP53-mutated DNA in the surgical margins was an independent prognosticator for locoregional recurrence (relative risk = 7.1; P = 0.021; 95% confidence interval, 0.9–56). In 20% of the cases, the presence of TP53-mutated DNA in the surgical margins was found to be related to the presence of tumor-related precursor lesions.

Conclusions: This study shows the value of TP53-mutated DNA as a molecular marker to predict locally recurrent head and neck squamous cell carcinoma. The observation that all patients who were negative for TP53-mutated DNA in the surgical margins remained free of local recurrence raises hope that molecular analysis of histologically tumor-free surgical margins can be exploited to decide on postoperative radiotherapy. Furthermore, our data provide evidence that local recurrences originate mainly from tumor cells left behind but also originate, in part, from unresected precursor lesions.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) comprises approximately 5% of all newly diagnosed cancer cases in Europe and the United States (1). Despite significant advances in surgery and radiotherapy over the last decades, the 5-year survival rates of HNSCC patients have been improved only moderately in part due to the relatively high local recurrence rate. Even when the surgical margins are diagnosed as tumor free by histopathological examination, the local recurrence rate is still 10–30% (2). Theoretically, there are two explanations for this phenomenon. Residual cancer cells might remain undetected in the surgical margins by the pathologist [minimal residual cancer (3)]. Alternatively, previous research from our group and others suggests that tumor-related mucosal precursor lesions, “fields” of genetically altered cells, may be left behind, and these might give rise to new invasive carcinomas (3–6). Such fields preceede the tumor and can be detected in the mucosal epithelium surrounding the tumor (4). A subgroup of these fields can be recognized clinically as leukoplakia or erythroplakia, but the majority can only be diagnosed by conventional histology, immunostaining, or genetic methods.

To allow detection and distinction of minimal residual cancer and precursor lesions, we used a two-step approach using TP53-mutated DNA as marker. Mutations in the TP53 tumor suppressor gene are present in 50–60% of head and neck cancers (7), and we have demonstrated that these are reliable clonal markers for HNSCC (8). In addition, there are sensitive and reliable molecular methods for detection of TP53-mutated DNA (9, 10). DNA can be isolated from the surgical margins, and the TP53 gene can be amplified by PCR and cloned into bacteriophages. The phages can be plaque lifted and screened by differential hybridization with mutation-specific and wild-type-specific oligonucleotides as probes, allowing analysis of the amount of tumor DNA in a background of wild-type DNA. Although this so-called plaque assay is laborious, it is sensitive, quantitative, and very robust, and it has been used previously to assess the potential value of TP53-mutated DNA as marker for molecular analysis of histopathologically staged surgical margins (11, 12).

It is known from previous studies that TP53 mutations are early genetic alterations in HNSCC that are already present in (dysplastic) mucosal precursor lesions (3–5). Therefore, the presence of TP53-mutated DNA in the margins may reflect both (dysplastic) precursor lesions and residual cancer cells. Because
a mutation in the TP53 gene often causes protein overexpression, the surgical margins can be immunostained to identify the source of the mutated DNA (cancer cells or precursor lesions).

We performed a large prospective trial with long-term follow-up to assess the value of molecular diagnosis using TP53-mutated DNA for identification of HNSCC patients at high risk for local recurrence. In addition, the pathobiological mechanism of recurrence at the primary site was investigated by immunostaining.

PATIENTS AND METHODS

Patients and Tissue Specimens. The study protocol was approved by the Institutional Review Board of the Vrije Universiteit Medical Center, and written informed consent was obtained from all patients. Clinicopathological data were derived from patient records and pathology reports. The enrollment started in September 1997 and ended in September 2000. In total, 179 HNSCC patients who were scheduled for surgical treatment consented to enrollment in the study. The criteria for inclusion in the trial were as follows: (a) tumor-free surgical margins as assessed by routine histopathological examination; and (b) a mutation in the TP53 gene. Nucleic acids of the tumors were analyzed for TP53 mutations in exons 4–10 as described previously (3, 10). In total, 76 patients met both inclusion criteria (Table 1).

At the time of surgery, four to five margin samples were taken from the surgical defect after tumor excision (three or four superficial mucosal margin samples designated M1–M3/M4 and one deep connective/muscle tissue margin sample designated M5) and used for molecular analysis. To prevent tumor cell contamination, the operating field was rinsed extensively with Dakin’s solution [0.5% sodium hypochlorite (pH 11.5) and 0.2% sodium carbonate], and the instruments and gloves were changed before sampling. Immediately after surgery and before routine (histological) processing of the surgical specimen, a sample of the primary tumor was obtained. These samples and margin samples M1–M5 were immediately snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Molecular Assay for TP53-Mutated DNA and RNA. Margins samples M1–M5 were homogenized in RNAzol, and RNA and DNA were isolated by RNAzol/DNAsat (Campro Scientific, Veenendaal, the Netherlands). Plaque assays for TP53-mutated DNA were performed according to the method of Sidransky et al. (9), with a few minor modifications as described previously (10). RNA was converted into cDNA by reverse transcriptase before PCR amplification. Proper positive (primary tumor RNA/DNA) and negative (wild-type RNA/DNA) controls were included. On average, 5000 plaques for each margin were screened, and the ratio of mutant:wild-type plaques was calculated. A case was designated positive when TP53-mutated DNA was detected in one or more surgical margins. Molecular data were not used for clinical management of the enrolled patients.

Identification of Tumor or Precursor Lesions in Surgical Margins by Immunohistochemistry. All H&E-stained paraffin-embedded sections from the surgical specimens were reviewed by an experienced pathologist (J. A. K.) without previous knowledge of the results of the molecular analyses. The following histological parameters were scored: presence of invasive tumor; presence of perineural tumor growth; and the presence and grade of dysplasia. In addition, the distance between tumor and deep surgical margin was estimated.

For cases with TP53-mutated DNA-positive margins, parallel sections were analyzed by immunohistochemistry using monoclonal anti-p53 antibody DO7 (DAKO, Glostrup, Denmark) and murine IgG as negative control to detect residual tumor or precursor lesions. First, paraffin-embedded sections of the corresponding tumors were used to check p53 overexpression. Subsequently, all margins were screened by p53 immunostaining. The presence of residual tumor was confirmed by histological review of a parallel H&E-stained section. Margins that demonstrated a positively stained para- or suprabasal cell cluster in the mucosal epithelium suggesting a precursor lesion were further investigated. The stained areas (always >20% of a margin or >4 mm) were microdissected from parallel sections and analyzed by plaque assay or sequencing to confirm that the mutation was identical to that in the corresponding tumor. These cases were assigned as tumor-related precursor lesion (“field”). It was further noted that TP53-mutated DNA immunopositive fields were always classified histologically as dysplastic. In cases in which the tumors did not show TP53-mutated DNA overexpression (mostly in the case of frameshift mutations), dysplastic regions in the surgical margins, when present, were microdissected, and the DNA was sequenced to confirm the mutation identified in the corresponding tumor. Based on these results, TP53-mutated DNA-positive margins were classified as

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics of the 76 patients included in this prospective study in relation to the presence or absence of TP53-mutated DNA in the margins</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TP53-mutated DNA in margin</td>
</tr>
<tr>
<td>N</td>
<td>50</td>
</tr>
<tr>
<td>Median age (yrs)</td>
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<td>Age range (yrs)</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
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<tr>
<td>Tumor site</td>
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<td>Oral cavity</td>
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</tr>
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<td>Oropharynx</td>
<td>14</td>
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<tr>
<td>Larynx</td>
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<td>Hypopharynx</td>
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<td>Tumor stage</td>
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<tr>
<td>T1/T0</td>
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<tr>
<td>T3/T4</td>
<td>10</td>
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<tr>
<td>Recurrent tumor</td>
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<tr>
<td>Lymph node metastases</td>
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<tr>
<td>N0</td>
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<tr>
<td>N+</td>
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<td>Delayed N+</td>
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</tr>
<tr>
<td>Recurrent tumor</td>
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<td>Stage (UICC 1998)</td>
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<td>Recurrent tumor</td>
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<td>Therapy</td>
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<td>Surgery</td>
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<td>Surgery and radiotherapy</td>
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</tbody>
</table>

* One patient presented with a local recurrent tumor.
* UICC, Union International Contre Cancer.
either containing tumor, field, or no histological substrate (NHS).

Statistical Analysis. The major statistical end point of the prognostic study was the number of patients remaining free of local and locoregional recurrence. Local recurrence was defined as tumor regrowth < 2 cm away from the index tumor and within 3 years. Time to recurrence or death was measured from the date of histological diagnosis. Patients who developed other primary tumors were censored for all outcomes at the incidence date of the second tumor. Delayed lymph node metastases that developed in an untreated neck during follow-up were not regarded as recurrence. Kaplan-Meier estimates and log-rank tests were computed with BMDP software (13). To investigate the influence of other parameters on the association between TP53-mutated DNA in the margin and disease, the Cox proportional hazards model for covariates was used. When P values were <0.05, associations were considered significant.

RESULTS

Molecular Analysis of Surgical Margins in a Prospective Cohort Study. Margin samples M1–M5 of the 76 patients who fulfilled the inclusion criteria were analyzed using the TP53 plaque hybridization assay. A representative example of the assay is shown in Fig. 1. The molecular data and the main clinical characteristics of the 76 patients are summarized in Table 1. In total, 50 of 76 (66%) patients showed one or more TP53-mutated DNA-positive margin samples. The percentage of TP53-mutated DNA/positive margin ranged from 0.01% to 15%.

Subsequently, the association of the presence of TP53-mutated DNA in the margin samples with oncologic outcome was determined. Of the 50 cases with TP53-mutated DNA-positive margin samples, 9 developed a local recurrence, and 4 developed a regional recurrence, whereas all patients with TP53-mutated DNA-negative margins remained local recurrence free, and only 1 developed a regional recurrence. Kaplan-Meier estimates and the associated log-rank tests showed that the number of patients remaining free of local and locoregional recurrence (P = 0.027 and 0.028, respectively) was significantly higher in the group with TP53-mutated DNA-negative margins (Fig. 2). The median follow-up interval was 28.1 months (range, 3.5–57.3 months). Patient, tumor, and other margin characteristics were comparable in the groups with TP53-mutated DNA-positive and -negative margins. Clinical covariates that were significantly associated with remaining free of local or locoregional recurrence were all related to the presence of lymph node metastases in the neck (N stage, stage, and number of lymph node metastases). No other clinical parameters, including age,
specimens that had been used for routine histological examination or death. Patients who developed distant metastases or second primary tumors were censored at the date of recurrence or death, respectively. The patients at risk are indicated in the graphs. The numbers are identical because, in the upper graph, the patients with regional recurrence were censored at the incidence date of recurrence, whereas in the lower graph these were counted as an event.

The influence of all parameters on the association between $TP53$-mutated DNA margin status and development of locoregional recurrence was investigated with the Cox proportional hazards model. It could not be determined for development of local recurrence because no events occurred in the group with $TP53$-mutated DNA-negative margins. The multivariate relative risk of the presence of a $TP53$-mutated DNA-positive margin was 7.1 ($P = 0.021; 95\%$ confidence interval = 0.9–56) for developing locoregional recurrence. Neither N stage, number of lymph node metastases, nor stage significantly influenced the development of locoregional recurrence, indicating that the presence of $TP53$-mutated DNA in the surgical margins is an independent prognosticator.

**Immunohistochemistry.** All margins of the 76 surgical specimens that had been used for routine histological examination were analyzed by histopathological review. The margins of the 50 cases positive for $TP53$-mutated DNA were investigated by immunohistochemistry to identify the source. In 40 of 50 primary tumors, the mutated p53 protein was overexpressed, which allowed margin screening with anti-p53 DO7 to detect either tumor or unresected precursor lesions. In two cases, a small cluster of p53-positive carcinoma cells was observed that was already present in the routine H&E staining but was missed by routine histology (Fig. 3). In eight additional cases, a precursor lesion was visualized in the surgical margins by p53 immunostaining (Fig. 3). The mutation was, in all cases, identical to that of the tumor, as determined by sequencing or plaque assay of the microdissected immunostained fields, indicating that the precursor lesions and tumors were clonally related. In 10 tumors with a p53 mutation, there was no overexpression of p53 observed, and the margins could therefore not be screened by p53 immunostaining. In these 10 cases, histological review of H&E-stained and cytokeratin-immunostained sections was used to identify tumor, but cancer cells were not detected. In addition, dysplastic changes that were observed by the pathologist were used to identify putative tumor-related fields. All dysplastic mucosal regions in the margins surrounding these tumors were microdissected and sequenced for $TP53$ mutations. In two additional cases, a tumor-related field could be identified by this approach because the $TP53$ mutations found were identical to those in the primary tumor. Hence, in 10 of 50 (20\%) cases, a p53-positive, tumor-related field was identified in one or more margins of the surgical specimen. The fields were always $>20\%$ of a margin section and larger than 4 mm.

In 38 cases, no histological substrate could be detected in the margins that would have explained the positive plaque assay. These were classified as NHS. Within this group, seven local recurrences occurred. All data are summarized in Fig. 3.

**RNA Plaque Assays.** Based on the outcome data (50 of 76 cases were $TP53$-mutated DNA positive in one or more margins, whereas only 9 of 50 cases developed a local recurrence), we reasoned that tumor cells or tumor DNA might have contaminated the surgical margins, leading to false positive results. To distinguish between tumor cells and tumor DNA, we performed RNA plaque assays. Case 97-50, which was negative in the DNA plaque assay, was used as negative control. Cases 98-95, 98-63, and 98-62 were used as positive control. The selected margins of these cases contained a tumor-related precursor lesion (98-95 and 98-63) or tumor (98-62) as determined by immunohistological staining and molecular analysis of the stained regions. As can be seen in Table 2, all positive and negative controls revealed the expected values. However, of the four margins that were positive in the DNA plaque assay, only one (25\%) was also positive in the RNA plaque assay. The data from margin 99-12 should be interpreted with caution because only 420 plaques with insert were screened.

**DISCUSSION**

In the present study, we have demonstrated that the presence of $TP53$-mutated DNA in histologically tumor-free surgical margins is an independent prognostic factor for local and locoregional recurrence in HNSCC patients. Of the 50 patients with $TP53$-mutated DNA in the surgical margins, 9 developed a
local recurrence, and 4 developed a regional recurrence. No other histological parameters determined on the surgical margins (including the presence of invasive or perineural tumor growth, the presence and grade of dysplasia, and distance between tumor and deep surgical margin) predicted the development of local or locoregional recurrence. It is noteworthy that all 26 patients with TP53-mutated DNA-negative margins remained local recurrence free, including 12 cases who were not treated with postoperative radiotherapy [median follow-up period for these 12 patients was 31.4 months (range, 4.4–55.1 months)]. Only 1 case in the group of 26 patients developed a regional recurrence. These data are thus in concordance with the previously published studies (11, 12).

It could be argued that small clusters of tumor cells were present in the surgical margins that escaped detection by the pathologist. Indeed, in two cases with TP53-mutated DNA-positive margins, small tumor clusters were seen after p53 immunostaining. In one case, it appeared as a tumor embolus, although it could not be distinguished whether this was tumor in a lymphatic or blood vessel. The chance, however, of finding these tumor emboli or other microscopic clusters of neoplastic cells when evaluating few sections is very small, and this may explain why we could not find any histological substrate in the TP53-mutated DNA-positive margins of 38 patients, whereas 7 of these 38 patients developed a local recurrence. We previously performed additional stepwise histological analysis of TP53-mutated DNA positive margins in which we failed to find a histological substrate, but we never found tumor, despite the fact that these patients had also developed a local recurrence (3). Molecular analysis therefore seems superior over immunohistochemical analysis for the detection of residual cancer cells in surgical margins.

In 10 of 50 cases (20%), we established that the presence of TP53-mutated DNA in the surgical margins could be explained by the presence of mucosal precursor lesions. In 1 of these 10 cases, a local recurrence developed. Additional genetic profiling indicated that this recurrence was derived from the precursor lesion because the same early genetic changes were found, whereas the late genetic changes differed (data not shown). From this prospective study using TP53-mutated DNA as molecular marker, it cannot be reliably deduced in what frequency local recurrences originate from residual tumor cells or unresected tumor-related precursor lesions. First, mutations in the TP53 gene do not always occur in precursor lesions, and this may lead to bias (5). Second, a number of patients with an incompletely resected field were lost to follow-up by non-disease-related deaths, causing a bias in outcome. We therefore decided to investi-
gate the role of fields in more detail in a retrospective study using allelic loss as molecular marker.\(^4\)

Despite these encouraging results, we were surprised to note the high percentage of cases with TP53-mutated DNA in the margins (50 of 76, 66%), whereas only 9 of 50 cases (20%) developed a local recurrence. The median follow-up interval was 28.1 months (range, 3.5–57.3 months), which is long enough for the development of local recurrences. The high frequency of positive cases, only a fraction of which developed local recurrences, could be explained by the fact that a large proportion of patients received postoperative radiotherapy that eradicated residual cancer cells. However, not all patients received postoperative radiotherapy. As described, in 12 of 50 patients with TP53-mutated DNA in the margin samples, a histopathological substrate could be found in the surgical margins (a precursor lesion in 10 cases and tumor in 2 cases), and these were therefore correctly classified as TP53-mutated DNA positive. In the remaining 38 cases, classified as NHS, 7 local recurrences occurred. In the remaining 31 cases, residual cancer cells might have been present in the margins, but these could then have been eradicated successfully by postoperative radiotherapy. However, of these 31 patients, 11 were treated with surgery only, and 0 of these 11 developed a local recurrence (median follow-up period, 34.0 months; range, 9.7–47.7 months). Theoretically, the possibility that small numbers of tumor cells were present in the margins of these 11 patients but did not give rise to local tumor outgrowth can never be ruled out. However, this possibility does not seem very likely because minimal residual cancer in lymph nodes is of clinical relevance (14), indicating that small numbers of squamous tumor cells can develop into recurrent tumor, at least in the neck.

The explanation for the high frequency of cases with molecular-positive margins thus seems more related to the experimental approach. Due to stringent control measures during processing of margin samples M1–M5, we consider amplimer contamination unlikely. However, despite our precautions during sampling in the operation room (extensive rinsing of the operation field, new gloves, and new instruments), the possibility that either tumor cells or tumor DNA contaminated margin samples M1–M5 cannot be ruled out. We tried to distinguish between these two possibilities by performing plaque assays on RNA isolated from margin samples. RNA is less stable in body fluids, and negative RNA plaque assays on margin samples that are positive in the DNA plaque assay would suggest that tumor DNA and not tumor cells contaminated the surgical margins. The data indeed indicated that tumor DNA appears to contaminate the margins in some cases. Tumor DNA might leak to the saliva and contaminate the margins or leak to the lymph and enter the circulation. This latter possibility is not unlikely because tumor DNA can be detected in other body fluids such as blood (15, 16). Obviously, these results will decrease the specificity of the assay and hamper the specificity of DNA-based assays in general. This specificity problem might be circumvented by using RNA plaque assays, but these were very difficult to perform. We also investigated whether the specificity could be increased in the DNA plaque assay by using different thresholds to call a margin positive, but this had a large negative effect on the sensitivity.

Taken together, the data presented indicate that molecular assessment of the margins might be of value in the decision-making process for postoperative radiotherapy, although this observation should first be confirmed in a larger series of patients or in a pooled analysis. In most institutions at present, all patients with T3 and T4 tumors are treated with postoperative radiotherapy to the primary tumor and the neck, regardless of the histopathological assessment of the surgical margins. It seems that patients without histologically detectable tumor and without TP53-mutated DNA in the surgical margins could be spared postoperative radiotherapy. This hypothesis would necessitate further investigation in a clinical trial in which the decision for postoperative radiotherapy would depend on the molecular margin status. A limitation that will hamper implementation of molecular staging of surgical margins to investigate this hypothesis is the laboriousness of the TP53-mutated DNA plaque assay. Other methods for sensitive detection of point mutations are available, but these need to be validated for the large assortment of mutations that can occur in the TP53 gene (10).

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