Intraoperative Molecular Margin Analysis in Head and Neck Cancer

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Background: Tumor-specific molecular alterations in surgical margins have been shown to predict risk of local recurrence. However, assays used for these analyses are time-consuming and therefore cannot be used in the intraoperative setting.

Objective: To detect and quantify tumor-specific methylated promoter sequences in surgical margins in a time frame suitable for intraoperative use.

Design: A novel quantitative methylation-specific polymerase chain reaction (QMSP) protocol.

Methods: A total of 13 patients with head and neck squamous cell carcinoma (HNSCC) were initially characterized for molecular alterations in their tumor at the time of biopsy. Six primary tumors were found to harbor promoter hypermethylation for p16 and O6–methylguanine-DNA-methyltransferase (MGMT) genes. Rapid QMSP was then used to identify promoter hypermethylation of these genes in the surgical margins. Results were compared with standard intraoperative histologic frozen section analysis and with conventional QMSP.

Results: Using our rapid QMSP assay, we found that 3 patients had methylation-positive margins. Tumor margins from 2 patients were methylated for p16 alone, and margins from 1 patient were methylated for p16 and MGMT simultaneously. Molecular margin analysis was completed in less than 5 hours, a time frame appropriate for selected major HNSCC resections that require combined primary tumor resection, cervical lymphadenectomy, and complex reconstruction. This technique was comparable in sensitivity to conventional QMSP.

Conclusion: Rapid molecular margin analysis using QMSP is feasible and may be performed intraoperatively in selected patients with HNSCC that requires extensive resection.


Head and neck squamous cell carcinoma (HNSCC) is a devastating and life-threatening disease with a high incidence of morbidity and mortality in advanced disease. More than 40,000 new cases of HNSCC are diagnosed in the United States and more than 500,000 worldwide each year. Despite aggressive surgical and other therapies, more than 50% of all patients have advanced disease at the time of diagnosis, and many of these ultimately die of local or regional recurrence. The adequacy of surgical resection of the primary tumor is conventionally determined intraoperatively by histologic hematoxylin-eosin diagnosis using frozen section margins.\(^1\) The presence of microscopic tumor at the surgical margins is associated with increased local recurrence and reduced survival.\(^2\) However, despite apparent complete gross and microscopic excisions of primary HNSCC, there is a significant rate of local recurrence in patients with histologically negative margins.\(^3\)

Attempts to improve detection of tumor cells in histologically negative surgical margins have been based on improved understanding of the biology of HNSCC. Genetic and epigenetic alterations are hallmarks of human cancers, including the proto-oncogene activation and tumor suppressor gene inactivation via mutation, loss, and promoter hypermethylation.\(^4\) Since genetic alterations precede phenotypic changes, histologic assessment alone may be inadequate to accurately detect the presence of transformed cells in surgical margins. It is becoming evident that molecular assays detect occult malignant cells previously missed by physical examination and standard histopathologic techniques.

Brennan et al\(^5\) used a mutation-specific plaque hybridization assay to as-
tumors for methylation of p16 and MGMT genes using methylation-specific polymerase chain reaction (MSP). In this study, 56% of the primary tumors were found to have aberrant methylation of at least 1 of these genes.

Sanchez-Cespedes et al.25 found that for those patients with available paired serum samples, the same methylation pattern was detected in 42% of cases. In another study,26 oral rinse and swab samples were analyzed for p16 and MGMT methylation. For 63% of cases with methylated primary tumors, abnormal methylated DNA was detected in matched oral rinse and swab samples. None of the nonmethylated primary HNSCC displayed methylation in the corresponding oral rinse and swab. Of 30 saliva samples from healthy control subjects (15 smokers and 15 nonsmokers), only 1 sample from a smoking patient was positive for DNA methylation at 2 target genes. These findings demonstrate that tumor suppressor gene (TSG) hypermethylation can be used for the detection of minimal quantities of tumor-specific DNA in a background of normal DNA from nontumor tissues. Detection of TSG promoter hypermethylation, therefore, is a promising means to detect small amounts of residual tumor in surgical margins.

Based on the above studies, we explored the feasibility of detection of tumor-specific promoter hypermethylation in surgical margins from patients with primary HNSCC that demonstrates promoter hypermethylation. The present study was designed so that we could evaluate the possibility of performing this assay in a real-time intraoperative manner.

METHODS

SPECIMEN COLLECTION

We enrolled patients diagnosed with HNSCC who would undergo surgical resection. Informed consent was obtained from each individual prior to tissue collection per institutional review board protocol.

At the time of biopsy, tumor and lymphocyte specimens were provided for initial methylation status evaluation. This tissue was divided and used for tumor microdissection, sequencing, histologic examination, and DNA extraction.

Several surgical margin samples were taken directly from the operating room of the Johns Hopkins Hospital at the time of definitive surgery. For each case identified, hematoxylin-eosin–stained slides of the tumor and margins were prepared and reviewed by one of us (W.H.W.), a head-and-neck pathologist with extensive experience in tumor grading. In addition, margins from the immediately adjacent areas were harvested for molecular studies. We chose cases in which the extent of disease would require extensive resection and thus provide us with maximum intraoperative time to perform this assay.

INITIAL DETERMINATION OF METHYLATION STATUS

At the time of preliminary diagnostic biopsy, tumor taken from the operating room at the Johns Hopkins Hospital underwent DNA extraction followed by conventional bisulfite treatment and quantitative MSP (QMSP). If aberrant promoter hypermethylation of tumor suppressor gene p16 or the DNA repair gene MGMT were detected, rapid digestion, bisulfite treatment, and QMSP were then performed in a rapid fashion at the


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time of definitive surgery to determine the status of the tumor margins.

CONVENTIONAL DNA EXTRACTION
Specimens were microdissected on a cryostat and harvested. They were then digested in sodium dodecyl sulfate (1%) and proteinase K (1 mg/mL), incubated at 48°C overnight, extracted with phenol chloroform, and precipitated with ethanol.6

RAPID DNA EXTRACTION
Tumor and margin specimens were “snap frozen” in liquid nitrogen. Half of the specimen was pulverized; DNA was extracted using a rapid digestion kit and protocol (DNeasy tissue kit; Qiagen, Valencia, Calif), which was altered by doubling the volume concentration (40 µL) of proteinase K to facilitate rapid digestion.

CONVENTIONAL BISULFITE TREATMENT
A solution was prepared using 3M sodium bisulfite, 1M hydroquinone, and 2M sodium hydroxide. Tumor and margin DNA samples were added to this mixture and allowed to incubate for 3 hours at 70°C. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, Wis) and rinsed twice in 2 mL of 90% isopropanol and once in 3M sodium hydroxide. To deaminate the samples, 7.5M ammonium acetate was added. The samples were precipitated for an additional hour with 100% ethanol and resuspended in water. Normal lymphocyte DNA from control individuals and from the patients were extracted and treated identically for use as positive controls.

RAPID BISULFITE TREATMENT
Two micrograms of DNA was denatured using 2M sodium hydroxide and modified as stated above by using 3M sodium bisulfite for 1 hour at 95°C. DNA samples were then purified using Wizard DNA purification resin (Promega), treated with 3M sodium hydroxide, precipitated immediately with 100% ethanol and resuspended in 60 µL of water. Normal lymphocyte DNA from control individuals and from the patients was extracted and treated identically for use as positive controls.

METHYLATION-SPECIFIC POLYMERASE CHAIN REACTION
Methylation-specific polymerase chain reaction involves chemical modification of genomic DNA using sodium bisulfite (described previously), which converts unmethylated, but not methylated, cytosine to uracil. Subsequent polymerase chain reaction (PCR) using promoters specific for either methylated or modified DNA provides determination of the presence of methylated DNA in a given sample. Methylation-specific PCR uses specific primers for each PCR reaction, allowing the assessment of methylation patterns for a given gene. The analysis of p16 and MGMT had sensitivity, without modification, in the 1:10000 to 1:100000 ranges. That is, the assay distinguished samples having no promoter region methylation of p16 or MGMT from those where only 0.001 of the input DNA was derived from a hypermethylated source.

QUANTITATIVE MSP
This PCR system uses 2 amplification primers and an additional amplicon-specific fluorogenic hybridization probe with

the target sequence within the amplicon.27 The probe is labeled with 2 fluorescent dyes: one serves as reporter on the 5′ end (FAM), and its emission spectrum is quenched by a second fluorescent dye on the 3′ end (TAMRA). During the extension phase of the PCR, the 5′ to 3′ exonuclease activity of the Taq DNA polymerase cleaves the reporter from the probe, thus releasing it from the quencher.28 If amplification occurs, this reaction results in an increase in the fluorescent emission of the reporter dye and is monitored during the PCR process. The fluorogenic PCR reaction was carried out in a reaction volume of 20 µL. Fluorogenic probes were custom designed and then synthesized by Qiagen-Operon (Alameda, Calif). The PCR primers were designed and then synthesized by Invitrogen (Carlsbad, Calif). Each PCR reaction mixture consisted of 66nM of each primer; 200nM of probe; 5 µL of platinum Taq polymerase, 200µM deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 400µM deoxyuridine triphosphate; 16.6mM ammonium sulfate; 67mM Trizma; 6.7mM magnesium chloride (2.5mM for p16), 10mM mercaptoethanol; and 0.1% dimethyl sulfoxide. Treated DNA (2.4 µL) was used in each real-time MSP reaction. Thermal cycling began with a denaturation step of 95°C for 2 minutes. The thermal profile for the PCR was 95°C for 15 seconds and 60°C for 60 seconds. Fifty cycles of amplification were run. Amplifications were carried out in a 384-well reaction plate format in a PE Applied Biosystems 7900 Sequence detector (Applied Biosystems, Foster City, Calif). Primers and probes were designed for p16 and MGMT.

During the gene characterization phase, primers and a probe were designed to amplify an internal reference gene, β-actin. These were located in areas without CpG nucleotides, thus amplifying the modified β-actin gene independently of the methylation status of CpG nucleotides. To determine the relative levels of methylated promoter DNA in each sample, the values of the gene of interest were compared with the values of the internal reference (β-actin) gene to obtain a ratio that was then multiplied by 100 to give a percentage value ([gene of interest/ internal reference] × 100).

During the rapid QMSP, serial 10-fold dilutions of tumor were mixed into normal template from tumor only down to a 1:10000 dilution. Threshold values for the margin amplification curves were quantitated relative to the thresholds for the corresponding serial dilution of tumor DNA into normal DNA. All samples were run in triplicate. All margins and the serial tumor–normal dilutions were run concurrently on the same plate. Leukocyte DNA for a healthy individual was previously methylated in vitro with excess SssI methyltransferase (New England Biolabs Inc, Beverly, Mass) to generate completely methylated DNA at all CpG sites and was used as a positive control. Each plate included negative controls (bisulfite-treated lymphocyte and normal tissue DNA from the patient) and multiple water blanks.

RESULTS

We used QMSP to determine the presence of promoter methylation of 2 genes in 13 patients with HNSCC. Six of those patients’ tumors were found to harbor a promoter hypermethylation of either p16 or MGMT. Three tumors were found to harbor p16 promoter hypermethylation, 2 tumors were found to harbor MGMT promoter hypermethylation, and 1 tumor was found to harbor both p16 and MGMT promoter hypermethylation simultaneously. Methylation levels in these nonmicrodissected tumors were expressed as ratios of the gene of interest to β-actin ([gene of interest/β-actin] × 100) and
Findings of Rapid Quantitative Methylation-Specific Polymerase Chain Reaction Evaluation of 22 Surgical Margins

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Tumor Stage</th>
<th>p16 Methylation</th>
<th>MGMT Methylation</th>
<th>Margin History</th>
<th>Rapid Assay Time, h</th>
<th>Margin 1</th>
<th>Margin 2</th>
<th>Margin 3</th>
<th>Margin 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tongue, T2 N1 M0</td>
<td>+ (57%)</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Tongue, T2 N1 M0</td>
<td>+ (17%)</td>
<td>+</td>
<td>–</td>
<td>5</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Hypopharynx, T2 N1 M0</td>
<td>+ (30%)</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Tongue, T2 N2 M0</td>
<td>+ (16%)</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>Floor of mouth, T4 N0 M0</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>4.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>Larynx, T2 N1 M0</td>
<td>– (12%)</td>
<td>–</td>
<td>–</td>
<td>4.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: MGMT, O6–methylguanine-DNA-methyltransferase gene; NA, not applicable; +, positive finding; –, negative finding.

Rapid quantitative methylation-specific polymerase chain reaction amplification plot. The log plot of fluorescence (y-axis) plotted against the polymerase chain reaction (PCR) cycle (x-axis) shows T:N serial dilution curves (1:100-1:10000). Two positive margins for promoter hypermethylation of p16 are seen: margin 4 (M4), between tumor and 1:10 dilution; and margin 3 (M3), overlapping 1:100 dilution. Margin 1 (M1) is negative, below 1:10000 dilution, while margin 2 (M2) and the normal control curves are below the threshold and cannot be seen.

Ranged from 16% to 57% for p16 and 12% to 22% for MGMT.

Rapid QMSP analysis of tumor and surgical margins was undertaken at the time of definitive surgery. The tumor and margin samples were snap frozen and pulverized and underwent rapid digestion and DNA extraction (DNeasy; Qiagen). This procedure routinely yielded DNA concentrations between 25 and 90 µg/µL, which was sufficient to perform QMSP. We then performed rapid bisulfite treatment and subsequent QMSP.

Each patient had multiple surgical margins harvested for molecular analysis (mean, 3.6; range, 3-4). A total of 22 margins were examined using rapid QMSP (Table). For 3 patients, we found no promoter hypermethylation in any of the surgical margins. For 1 patient with 4 margins, we found p16 methylation in 1 margin above the 1:10 dilution level and another margin at the level of 1:100 dilution. A rapid QMSP amplification curve showing 2 margins positive for promoter hypermethylation of p16 is shown in the Figure. For a second patient with 3 surgical margins, we found methylation of 2 margins between the 1:1000 and 1:10000 dilution levels.

Finally, we examined 4 margins for a third patient in whom the primary tumor was methylated for both p16 and MGMT. In this case, 2 margins (2/4) were methylated for p16: one margin between the 1:1000 and 1:10000 dilution levels, and the second margin above the 1:10 dilution level. This identical second margin (1/4) was found to be methylated for MGMT at a level above the 1:10 dilution as well.

In total, 7 (32%) of 22 of the surgical margins harbored cells with promoter hypermethylation. Of note, all adjacent surgical margins harvested for routine frozen histologic examination during surgery were deemed negative. Each laboratory procedure of rapid QMSP was performed by 2 of us (D.G. and B.G.M.) and required less than 5 hours (mean, 4.8 hours; range, 4.5-5 hours) to complete from the time the tissue specimens were received from the operating room until the time of MSP. Assays were repeated using conventional methods as outlined earlier and were completely reproducible using conventional QMSP. At the time of this writing, no recurrences have been reported in any of the 6 patients whose margins were analyzed.

**COMMENT**

The importance of tumor-free surgical margins has been well documented, and the status of the surgical margins in HNSCC is a predictor of recurrence.\(^7\) The adequacy of surgical resection in cancer surgery is determined intraoperatively by histologic examination of those margins by an expert pathologist. Unfortunately, local recurrences occur in up to 50% of these patients even with histologically negative margins.\(^29\) It is also known that genetic alterations precede phenotypic changes, which suggests that histologic assessment alone may be inadequate to predict recurrence.\(^8\) Therefore, after a seemingly adequate resection, epithelial cells that have undergone transformation at the molecular level but appear phenotypically normal may be left behind. In addition, occult tumor cells, undetectable by conventional microscopic analysis, may be present in normal-appearing tissue.\(^30\)
Molecular markers have shown promise in identification of occult cells that may undergo malignant transformation. Using a mutation-specific plaque hybridization assay for the molecular analysis of surgical margins and lymph nodes from patients with HNSCC, Brennan et al. have shown that patients with molecularly positive margins have increased risk of recurrence. These findings were confirmed in subsequent studies. The main limitation of conventional molecular analysis of surgical margins, which precludes its intraoperative use, is the extended time required to complete the assays.

Hypermethylation of the CpG islands located in the promoter regions of tumor suppressor genes is now confirmed as an important mechanism for gene inactivation. Detection of hypermethylation has been used to confirm as an important mechanism for gene inactivation. Rosas et al. found that these 2 genes contained hypermethylated promoters and yield an extensive panel of targets for this assay. However, in a recent study, Yamashita et al. were able to demonstrate that high-throughput techniques may be used to evaluate a large number of methylated promoters and provide a useful intraoperative tool for molecular assessment of surgical margin tissue.

We undertook a multiple-stage assay, which included rapid DNA tissue extraction, rapid bisulfite treatment, and standard QMSP. The conventional QMSP technique requires more than a day from time of tissue extraction to completion. Our process shortens this process so that it can be performed in a time frame suitable for intraoperative use in selected cases. However, our assay is labor intensive and must be performed by at least 2 individuals simultaneously.

There are a number of potential technical pitfalls in our technique, including inadequate DNA extraction, incomplete denaturation of DNA, and incomplete bisulfite treatment. However, these did not appear to be significant: we were able to produce identical results with both rapid and conventional QMSP margin analysis.

This rapid QMSP assay in its present form is potentially useful only for HNSCC surgical cases that require extensive resection and possible reconstruction because it requires almost 5 hours to complete. However, initial resection of the primary tumor followed by neck dissection and reconstruction in major cases would allow for this assay to be used in real time.

Novel, high-throughput automated technologies are being developed that will allow this assay and similar molecular assays to be performed by a single individual in a relatively short time. This would prove useful in short- to medium-duration head and neck surgery cases and may even allow evaluation of tumors and margins in an outpatient setting.

In our study we investigated the presence of promoter hypermethylation of only 2 genes: p16 and MGMT. Rosas et al. found that these 2 genes contained hypermethylated promoter regions in 56% of the HNSCC samples that they examined. Therefore, only a proportion of primary HNSCC may manifest alterations useful for this assay. However, in a recent study, Yamashita et al. were able to demonstrate that high-throughput techniques may be used to evaluate a large number of methylated promoters and yield an extensive panel of targets from which margin analysis may be performed. Therefore, the optimal intraoperative technique would necessitate a survey of multiple genes to accurately determine the actual methylation status of excised HNSCC tumors and subsequent margins. In the future, this might allow for our current 2-stage process (methylation determination at the time of biopsy followed by margin analysis at the time of definitive surgery) to be performed in 1 stage using an extended panel of target genes. The present study was designed to demonstrate the technical feasibility of surgical margin analysis with QMSP, but, clearly, larger prospective studies are required to document the association of methylated targets in margin specimens with tumor recurrence.

We have developed a rapid QMSP assay that we used to detect aberrant promoter hypermethylation in the surgical margins of 6 patients with HNSCC, and we found positive methylated margins in 3 patients. In this preliminary study, we have demonstrated the feasibility of this assay for intraoperative use. Future research should examine the clinical implications of surgical margin hypermethylation. This will include close clinical follow-up of patients found to harbor promoter hypermethylation in their surgical margins. It would be of interest to determine the sensitivity and specificity of this molecular margin assay in relation to mutation-specific plaque hybridization assays and immunohistochemical assays that are too time-consuming and cumbersome to be useful in a real-time setting. Ultimately, this technology could facilitate more precise and complete surgical resection of primary HNSCC.

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