PATHOLOGIC EVALUATION OF SENTINEL LYMPH NODES IN ORAL SQUAMOUS CELL CARCINOMA

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Abstract: Background. The objective of this study was to determine the relative efficacy of different methods of pathologic evaluation of sentinel lymph nodes.

Methods. In this prospective study, sentinel nodes were evaluated for occult metastasis using frozen section, imprint cytology, hematoxylin-eosin staining, serial step sectioning (SSS) with hematoxylin-eosin, and immunohistochemistry (IHC). Metastases were classified into macrometastasis (>2.0 mm), micrometastasis (0.2 mm–2.0 mm), isolated tumor cells (<0.2 mm).

Results. Occult metastasis was detected in 20 of 80 patients. Frozen section and imprint cytology identified metastasis in 10 of 20 patients, hematoxylin-eosin stain in 13 patients; SSS upstaged the disease in a further 7 patients (9%). Frozen section detected macrometastasis in 7 of 8 cases but failed to detect smaller metastases (missed micrometastasis in 4 of 7 and isolated tumor cells in 5 of 5). SSS upstaged the disease by 10%, and sensitivity and negative predictive value of SSS with hematoxylin-eosin stain were 90% and 97%, respectively.

Conclusion. Frozen section and imprint cytology are not effective in identifying occult metastasis. IHC and SSS are required to identify micrometastasis and isolated tumor cells. VV C 2010 Wiley Periodicals, Inc. Head Neck 00: 000–000, 2010

Keywords: Sentinel lymph node; lymphoscintigraphy; pathology; occult metastasis; micrometastasis; isolated tumor cells; immunohistochemistry; lymph node metastasis; oral squamous cell carcinoma; tongue cancer

Lymphoscintigraphy-guided sentinel node biopsy is now emerging as an effective tool for the evaluation of occult metastasis in oral cavity squamous cell carcinoma. The concept of sentinel lymph node (SLN) biopsy is based on the fact that in head and neck squamous cell carcinoma, the lymphatic metastasis generally follows an orderly and predictable pattern of progression, beginning with the SLN and then progressing to the lymph nodes in the nodal basin.1 It has been demonstrated that the status of the sentinel node predicts the presence of metastasis in the remainder of the nodal basin. The technique to identify sentinel node biopsy...
using radiolabeled sulfur colloid and gamma probe is now well standardized in head and neck squamous cell carcinoma.2–4

The identified SLNs can be potentially subjected to different pathologic investigations with varying stringency, sensitivity, and clinical utility. This includes frozen section, imprint cytology, standard histopathology, serial step sectioning (SSS), and standard histopathology and SSS, and immunohistochemistry (IHC). Efficacy of these investigations to detect occult metastasis in head and neck cancer has not yet been systematically evaluated. The objective of this study was to investigate the relative efficacy of different pathologic evaluations to identify occult metastasis in oral tongue squamous cell carcinoma.

MATERIALS AND METHODS

The SLNs evaluated in this study were obtained from a prospective randomized clinical trial, comparing the efficacy of SLN biopsy and selective neck dissection in patients with stage I (T1 and T2, N0, M0) squamous cell carcinoma of the tongue. The patients were considered N0 if there were no significant lymph nodes determined by clinical examination and imaging studies of either contrast-enhanced CT scan or ultrasound scan. The study was initiated after obtaining Institutional Review Board approval. The study schema is summarized in Figure 1. After providing informed consent, the patients were randomized into 2 arms. All patients in both arms initially underwent lymphoscintigraphy and SLN biopsy. The lymphoscintigraphy and SLN biopsy were carried out by the previously described technique.2–4 A planned interim analysis of 80 consecutive patients of the total estimated 120 required for the clinical trial formed the study group of the present report.

SLNs were analyzed intraoperatively by frozen section and imprint cytology. Postoperatively, these lymph nodes were subjected to standard hematoxylin-eosin staining. Patients in arm 2 underwent further selective neck dissection (level I–IV) only if the frozen section or standard hematoxylin-eosin staining detected occult metastasis. All patients in arm 1 underwent selective neck dissection (level I–IV) irrespective of the SLN biopsy report. Further evaluation of all SLNs were carried out by SSS with hematoxylin-eosin stain and IHC. Patients who were identified to have occult metastasis (micrometastasis and isolated tumor cells) by SSS and IHC were observed, without subjecting them to selective neck dissection. The schema for tissue processing is summarized in Figure 2.

Frozen Section and Imprint Cytology. The lymph nodes were bisected vertically along the hilum and imprint cytology was obtained. Both halves of the node were embedded in optimum cold temperature medium and a single section from each of the cut sides was obtained for the frozen section analysis. After the frozen section results were obtained, the nodes were fixed in formalin and were processed for paraffin embedding and hematoxylin-eosin stain examination was carried out using 2 sections. The remaining paraffin block was subjected to up to 20 SSSs at 4-μm thickness with a gap of 150 μm. Hematoxylin-eosin staining and IHC were carried out on 2 adjacent sections at 150 μm intervals to

FIGURE 1. Study schema. LSG, lymphoscintigraphy; SLB, sentinel lymph node; H&E, hematoxylin and eosin; IHC, immunohistochemistry; SSS, serial step sectioning. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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correlate findings of these 2 studies. The presence or absence of light microscopic evidence of metastatic tumor in the lymph nodes was determined. In addition, the number and levels of nodal metastases and evidence of extracapsular extension of tumor was also noted.

The immunohistochemical assay was carried out using a broad-spectrum anti-cytokeratin antibody cocktail of AE-1/AE-3 (Dako, Carpinteria, CA). The sections obtained for IHC were processed on poly-l-lysine coated slides. The antigen retrieval was carried out with protein digestion with trypsin for 5 minutes at room temperature. The primary antibody was applied for 60 minutes at room temperature at a concentration of 1:1200 using anti-cytokeratin antibody cocktail of AE-1/AE-3. Detection was performed using secondary antibody system (peroxidase/di-ami-no-benzene, Dako, Real EnVision). Known positive and negative controls were included with each batch. The slides were analyzed for the presence or absence of the immunostain.

The occult metastasis was classified by histopathologic criteria. This included macrometastasis where the metastases foci are larger than 2 mm (Figure 3). Micrometastasis are those with metastatic foci between 0.2 mm and 2 mm in size (Figure 4), isolated tumors cells (ITC) are...
those with clusters of ITCs less than 0.2 mm in size (Figure 5) and single tumor cells (STC) where there are single cytokeratin-positive cells within lymph nodes (Figure 6). Sensitivity of various histopathologic investigations to identify different types of metastasis was determined.

RESULTS

Lymphoscintigraphy technique was effective in identifying SLNs in all the 80 patients accrued in this study. There were a total of 192 SLNs identified from the 80 patients. The number of SLNs per patient ranged from 1 to 5 (median, 2.2).

Overall, 20 of the 80 patients had occult metastasis (25%). Intraoperative detection of occult metastasis by frozen section and imprint cytology was positive in 10 of the 80 patients (12.5%). Routine pathologic evaluation with hematoxylin-eosin staining detected occult metastasis in 13 patients (16.25%). SSS with hematoxylin-eosin stain and IHC identified occult metastasis in 7 additional patients (25%; Table 1).

The type of occult metastasis observed included macrometastasis (Figure 3) in 8 patients, micrometastasis (Figure 4) in 7 patients, isolated tumor cells in 5 patients (Figure 5), and single tumor cells (Figure 6) in 2 patients. Three patients with macrometastasis and 2 patients with micrometastasis demonstrated extracapsular spread. Relative efficacy of various methods in detecting occult metastasis is shown in Table 2. Intraoperative analysis with frozen section and imprint cytology was able to identify macrometastasis in most patients (7 of 8), but failed to identify micrometastasis (4 of 7), and isolated tumor cells (5 of 5) in the majority of the patients. Micrometastasis was effectively identified by routine hematoxylin-eosin staining (7 of 7), but ITC were detected only by SSS. Detection of single tumor cells required SSS and IHC staining.

Overall efficacy of various methods is listed in Table 3. Detection of occult metastasis by IHC is considered the gold standard and relative efficacy of other methods are tabulated in this table. Sensitivity of frozen section and imprint cytology was very low (50%), whereas the negative predictive value was 86%. SSS with hematoxylin-eosin stain had a sensitivity of 90% (missed only single cell deposits) and negative predictive value of 97%.

DISCUSSION

The objective of sentinel node biopsy is to identify occult metastasis with low morbidity and high sensitivity. In comparison to selective neck dissection where the lymph node yield is around 25 nodes, as reported in this study, with sentinel

Table 1. Detection of occult metastasis with various pathologic methods.

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>No. of positive patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen section</td>
<td>10</td>
<td>12.5</td>
</tr>
<tr>
<td>Imprint cytology</td>
<td>10</td>
<td>12.5</td>
</tr>
<tr>
<td>Routine hematoxylin-eosin</td>
<td>13</td>
<td>16.25</td>
</tr>
<tr>
<td>Step sectioning hematoxylin-eosin</td>
<td>18</td>
<td>22.5</td>
</tr>
<tr>
<td>Step sectioning IHC</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>

Abbreviation: IHC, immunohistochemistry.
Intraoperative detection of metastatic deposits in sentinel node biopsy is of paramount importance to make the sentinel node biopsy procedure patient friendly. As observed in this study, and as reported by others, intraoperative frozen section analysis was shown to have high false-negative rates. On the contrary, recent studies have shown reasonable negative predictive value with frozen section analysis (83%). In the present study, sensitivity and negative predictive value of frozen section was 50% and 85.7%, respectively. The difference in result reported by our study and that of previous authors may be because of the size of occult metastasis that is to be determined by the method. Intraoperative evaluation with frozen section was accurate in detecting macrometastasis (7 of 8), but was not effective in detecting micrometastasis and isolated tumor cell deposits. Moreover, if smaller deposits (mainly isolated tumor cells) are located within the tissue used for frozen section analysis, it would be missed in eventual analysis for micrometastasis. It has been argued that frozen section analysis prolongs the duration of the procedure. This was, however, not our experience, as the sentinel node biopsy is carried out as the initial procedure. By coordinating with the histopathology service, the frozen section result can be made available during the resection of primary tumor. This sequence could not be followed in 2 patients, where the sentinel nodes were localized to the level 1 region, and radioactivity of the primary tumor deterred detection of the SLN. It was necessary to undertake excision of the primary tumor before isolating the SLN.

Imprint cytology as an alternative to frozen section analysis of lymph nodes was evaluated by other authors. In the present study, the detection rates of imprint cytology and frozen section were identical. As in frozen section, imprint cytology failed to detect micrometastasis and isolated tumor cells. For better utilization of SLN biopsy, it is essential to develop a novel technology that can detect smaller deposits intraoperatively in SLNs. Intraoperative ultra-rapid IHC and intraoperative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) evaluation may aid in the future to improve the sensitivity of intraoperative detection of occult metastasis.

In this study, the occult metastasis was stratified into macrometastasis, micrometastasis, and isolated tumor cells. The term micrometastasis is erroneously used for any metastasis detected by histologic analysis of clinically-negative (N0) neck. However, histologically detected metastases are correctly termed as occult metastases.

Table 2. Relative efficacy of various methods in detecting different type of occult metastasis.

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Type of occult metastasis</th>
<th>No. of macrometastasis</th>
<th>No. of micrometastasis</th>
<th>No. of isolated tumor cell</th>
<th>No. of single cell deposits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen section</td>
<td></td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Imprint cytology</td>
<td></td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Routine hematoxylin-eosin</td>
<td></td>
<td>8</td>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Step sectioning hematoxylin-eosin</td>
<td></td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Step sectioning immunohistochemistry</td>
<td></td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3. Overall relative efficacy of different methods (Immunohistochemistry is considered as “gold standard”).

<table>
<thead>
<tr>
<th>Method/test</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Positive predictive value, %</th>
<th>Negative predictive value, %</th>
<th>Accuracy, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen/imprint</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>85.7</td>
<td>87.5</td>
</tr>
<tr>
<td>Routine hematoxylin-eosin</td>
<td>65</td>
<td>100</td>
<td>100</td>
<td>89.6</td>
<td>91.3</td>
</tr>
<tr>
<td>Step sectioning hematoxylin-eosin</td>
<td>90</td>
<td>100</td>
<td>100</td>
<td>96.8</td>
<td>97.5</td>
</tr>
</tbody>
</table>
metastases, which can be further stratified based on histopathologic criteria. Hermanek et al.\textsuperscript{11} proposed histopathologic classification of occult metastasis for breast cancer into macrometastasis, micrometastasis, and isolated tumor cells. Widely used staging scheme for breast cancer formally defines macrometastasis as those metastatic deposits more than 2 mm in diameter. The micrometastasis as metastatic deposit between 2 mm and 0.2 mm in diameter. Deposits less than 0.2 mm are defined as isolated tumor cells.\textsuperscript{12} This can be either single cell deposits or a cluster of tumor cells.

Unlike in breast cancer, there is no uniform histopathologic staging available for occult metastases in head and neck cancer. Some studies have included 3 mm as the upper limit of size of micrometastasis,\textsuperscript{13} but these studies do not always mention lower limit for micrometastasis. Most studies though use 2 mm as the upper limit and 0.2 mm as the lower limit for micrometastasis.\textsuperscript{14} Micrometastasis as metastatic deposits less than 0.2 mm are generally defined as isolated tumor cells.\textsuperscript{11,12,14,15} For uniformity of reporting, in head and neck cancer also, it is advisable to follow the standard criteria established in other tumor types. This will allow us to determine the prognostic significance of varying levels of metastasis.

In our study, routine pathologic evaluation detected occult metastasis in 13 cases (16.2\%) but missed metastasis in 7 cases. SSS with hematoxylin-eosin stain and IHC identified the metastasis in 20 cases (25\%) and hence further upstaged the neck by about 9\%. This finding is similar to other published series in the literature.\textsuperscript{16–18} SSS was necessary to detect micrometastatic deposits. The routine pathologic evaluation was not sufficient to detect these metastasis. IHC was needed only to identify isolated tumor cells. The clinical significance of these smaller foci of metastasis is not established.

Routine pathologic evaluation of a neck node consists of identifying each individual node, bisecting the node at its center and then staining 1 or 2 sections to find light microscopic evidence of metastatic deposits.\textsuperscript{19} This, in reality, is an incomplete examination, where central sections serve as a proxy for the whole node. If deposits were small and present in other regions of the node, they would be missed. Studies have shown that routine evaluation misses up to 21\% of disease nodes in breast cancer.\textsuperscript{20} It has been shown that SSS with hematoxylin-eosin stain and IHC and molecular methods identify smaller metastasis more accurately. Nelson\textsuperscript{21} has reported that hematoxylin-eosin staining with step sections identifies 1 cancer cell among 10,000 normal cells. IHC identifies 1 tumor cell among 100,000 normal cells. RT-PCR is the most sensitive of all. It identifies 1 cell among 1 million normal cells. In clinical practice, SSS with hematoxylin-eosin staining upstages the tumor in 10\%, whereas IHC further upstages it to 10\% more.\textsuperscript{16–18} Based on the findings of this interim analysis of the prospective trial, the protocol was revised to incorporate the results of SSS and standard hematoxylin-eosin staining as an indicator to plan neck dissection, rather than standard hematoxylin-eosin alone.

Although the focus of this study was not to determine clinical significance of various degrees of metastasis, with the limited follow-up data available in this study it seems that isolated tumor cells may have clinical significance with 30\% of the patients developing neck recurrence during the follow-up period (Table 2). The significance of micrometastasis and isolated tumor cells are better delineated in breast cancer.\textsuperscript{11,12,22,23} Based on these findings, the pathologic N0 nodal staging of breast cancer is now modified to include the following subcategories. The pN0 denotes no regional lymph node metastasis histologically and no evidence of isolated tumor cells. The pN0 subcategories are: pN0(i-) = no regional lymph node metastasis both histologically and by IHC; and pN0(i+) = no regional lymph node metastasis histologically, positive IHC, however, no IHC cluster was greater than 0.2 mm. In addition, based on molecular evidence of metastasis detected by RT-PCR, 2 other pN0 subcategories are also included – pN0(mol-) and pN0(mol+). The pN1 was further subclassified to include pN1mi, which include micrometastasis (0.2 mm to 2.0 mm tumor deposits). It is necessary that the nodal metastasis in head and neck squamous cell carcinoma (HNSCC) is also reported using the system adapted in breast cancer, so that the clinical significance of these type of metastasis can be better delineated. Further larger studies with long-term follow-up are required to address this issue.

CONCLUSION

The relative efficacy of different methods of pathologic evaluation of SLNs was evaluated in this study. It was demonstrated that detection rates of frozen section and imprint cytology are
comparable; however, these methods are ineffective to detect micrometastasis. SSS and hematoxylin-eosin staining are effective in detecting all metastasis but isolated tumor cells. For the detection of isolated tumor cells, it is necessary to employ SSS and IHC. Larger studies with long-term follow-up are essential to determine the clinical significance of various grades of occult metastasis, in particular that of micrometastasis and isolated tumor cells.

REFERENCES