Immunohistochemical detection of micrometastases in 580 cervical lymph nodes from patients with head and neck malignancies

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ABSTRACT

Aims: Our study was designed to find out the incidence of micrometastases in 580 cervical lymph nodes using serial sectioning and by using immunohistochemical stains CK(cytokeratin) and EMA(epithelial membrane antigen).

Settings and Design: study was conducted on 30 patients who were diagnosed with head and neck carcinomas and subsequently underwent neck dissections. Patients with head and neck lymphomas, sarcomas and other soft tissue tumors were not included in the study.

Materials and Method: From 30 RND(radical neck dissection) specimens, 580 lymph nodes negative for metastases on H & E( Hematoxylin and eosin) stain were examined. Serial sectioning and IHC stain was performed using pancytokeratin AE1/AE3 and EMA.

Results: On serial sectioning occult metastases was detected in one out of 580 lymph nodes. By immunohistochemistry using CK+EMA micrometastases were detected in 11 of 580 lymph nodes.

Conclusion: The use of serial sectioning with H &E and immunohistochemical analysis using CK+EMA increases the detection of micrometastases that are often elusive by routine processing in patients with head and neck malignancies. Improved methods of detecting micrometastases may provide a basis for improved planning of postoperative therapy for patients already at risk of tumor recurrence.

Key words: micrometastases, head and neck malignancies, cytokeratin, epithelial membrane antigen

INTRODUCTION

The presence or absence of cervical lymph node metastases is the most important prognostic factor for patients with squamous cell carcinoma(SCC) of the head and neck. [1] Nodal metastases are associated with a high rate of regional recurrence and disease survival. In fact, single ipsilateral lymph node( LN) metastases decreases survival by 50% and the contralateral
or bilateral LN metastases give rise to additional 50% reduction of survival rate.\textsuperscript{[2,3]}

In diagnostic procedures, ultrasonography (USG), computed tomography (CT) or MRI have been applied to increase the detection rate of cervical lymph node metastases, still the incidence of clinically and radiographically occult micrometastases remains significant.\textsuperscript{[1,3]}

The sensitivity of current routine histopathological methods in assessing regional LN involvement is imperfect in that it like clinical staging, can rarely detect the presence of micrometastases within the nodes. Also greater sampling of lymph nodes can lead to greater probability of metastases detection. It is important to realize that the term micrometastases is not synonymous with occult disease. The term occult describes the nodal metastatic disease that has not been detected by clinical staging investigations whereas micrometastases is a single deposit or multiple deposits of tumor within the lymph node sinuses with minimal replacement of nodal architecture and measuring in total no more than 3mm or 5 tumor cells at any level of sectioning.\textsuperscript{[4]}

Till date, many studies have been performed on breast, endometrial and colon cancers, the immunohistochemical method was reported to be more effective in detecting the occult micrometastases than routine H & E but studies conducted on cervical lymph nodes from RND specimens are few.

In the current study, incidence of micrometastases in lymph nodes of RND specimens were examined by serial sectioning and by using cytokeratin immunohistochemical methods.

Additional clarification of equivocal slides was performed by IHC staining for EMA.

A more rigorous approach for the detection of micrometastases should result in a more accurate staging of tumors to determine the most optimal treatment option for patients with head and neck malignancies.

**Subjects and methods**

Present study was conducted on 30 patients who were diagnosed with head and neck carcinomas and subsequently underwent neck dissections. Patients with head and neck lymphomas, sarcomas and other soft tissue tumors were not included in the study. None of the patients had previous irradiation or surgery to the neck.

After extirpation the specimen was fixed in 10% formalin. All the palpated lymph nodes were meticulously dissected. Nodes smaller than 5mm were totally embedded whereas lymph nodes 5mm or larger were sectioned every 3-4mm and totally embedded of all paraffin blocks. One five micrometer section was obtained and stained with H & E.

The blocks containing lymph nodes which came out negative on routine processing were subjected to serial sectioning and scrutinized for metastatic deposits. A section from each such block was stained with monoclonal antibodies to CK(AE1/AE3).

All microscopic slides were examined by at least two examiners. The presence and size of metastases inside and outside the lymph nodes was recorded.

Micrometastases were defined as the presence in lymph node of either a single cell or a cluster of 5 or fewer cells that are...
immunohistochemically (IHC) positive for cytokeratin. A cluster of >5 tumor cells is not regarded as micrometastases because such an aggregate usually can be recognized as a metastatic deposit by conventional histological examination. According to study conducted by van den brekel et al in 1992 metastases measuring 3mm or small were defined as micrometastases. Although some reticular cells and plasma cells may show positivity with cytokeratin but the staining pattern of reticular cells would be strikingly different from that of neoplastic cells. Neoplastic cells exhibit strong and globoid cytoplasmic reactivity whereas reticulum cells mostly show a delicate decoration of their cytoplasmic processes.

RESULTS
Out of 30 neck dissection specimens, 20 were RND, 9 modified RND one was of extended neck dissection. A total of 636 lymph nodes were isolated from 30 specimens ranging from 8 to 43 lymph nodes per specimen, average 21.2 lymph nodes per neck dissection specimen. Out of 636 lymph nodes, 56 were positive for metastases on routine processing and 580 lymph nodes were negative and showed reactive hyperplasia. In our study of 30 cases maximum number of patients were in age group of 41 to 50 years that is 13 cases and 27 were males and only 3 were females. This shows higher incidence of head and neck malignancies in older males.

In 5 of 30 cases, primary site of tumor was not known. Among remaining 25 cases, in majority of patients i.e 19(63.3%) the site of primary tumor was oral cavity. The primary tumor was SCC in 26(86.7%) cases.

The stage of primary sites and cervical lymph nodes were reclassified by staging of the American joint committee on cancer(1997). Out of 25 cases with known primary site 12(48%) were at T2 stage, 10(40%) at T1 and 3(12%) at T3 stage. Out of 30 cases 14(46.6%) cases were at N0 stage followed by 9(30%) at N1 and 7(23.4%) at N2 stage. None of our case was at N3 stage.

On serial sectioning only one of the 30 cases was positive for micrometastases and out of 580 lymphnodes which were negative on routine H and E, one lymph node showed metastatic tumor deposit in the subcapsular sinus and subsequently was also positive on cytokeratin and EMA (P=0.09).

On IHC staining with CK, 6(20%) of 30 cases were positive for MMS, 5(16.7) were doubtful (whether CK positive cells were dendritic reticulum cells, macrophages or tumor cells) [figure-2] and 19(63.3%) were negative. Out of 580 LN 8(1.4%) were positive, 564(97.2%) were negative and 8(1.4%) showed doubtful positivity.

EMA confirmed MMS in 2 of 5 cases which showed equivocal results on CK, all cases those were negative on CK were also negative on EMA. Out of 580 LN, 7(1.2%) showed micrometastases on EMA. Out of 8 lymph nodes(5cases) which showed equivocal results on CK, EMA confirmed MMS in 5(2cases) of them.
On combing both the IHC stains CK+EMA 11 lymph nodes (8 cases) were positive out of 580 lymph nodes.[Figure-4] [table-1]

Out of total 8 cases which were positive for MMS in 3 cases the primary tumor was tonsil followed by buccal mucosa in 2 cases (p=0.663). In 7 cases primary tumor was SCC and in one of the case primary tumor was non viable (p=0.458). Eleven lymph nodes positive for MMS, 8 were <1cm and 3 were greater than 1cm in diameter (p=0.02).

On the basis of differentiation of primary tumor, MMS was higher in poorly differentiated type (p=0.01). out of 11 LN, 5 were at level II, 3 at level V, 2 at level III and one at level IV (p=0.224).

In 7 of 25 cases positive for MMS in 3 cases the primary tumor was of <2cm (t1) and in 4 cases between 2-4cm (T2) stage (p=0.461)

In the classification based on the lymph node stage, out of 14N0 cases three were upstaged to N1 with one MMS positive per case and three others were upstaged to N2 with two lymph node MMS each. Out of 8N1 cases, 2 (25%) were upstaged to N2.[table-II]

Statistically cytokeratin was 100% sensitive and 88% specific whereas EMA was 62.5% sensitive and 100% specific for detection of MMS.[table-III]

Statistically significant correlation was found between MMS and differentiation of primary tumor and size of lymph node, more common in poorly differentiated tumor and in LN <1cm in size. No significant correlation was found between MMS and level of lymph node, site, type and size of primary tumor.

Table-1 Detection of micrometastases on immunohistochemical staining(CK+EMA)

<table>
<thead>
<tr>
<th>IHC</th>
<th>Number of cases (%)</th>
<th>Number of lymph nodes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>8 (26.7%)</td>
<td>11 (1.9%)</td>
</tr>
<tr>
<td>Negative</td>
<td>22 (73.3%)</td>
<td>569 (98.1%)</td>
</tr>
<tr>
<td>total</td>
<td>30 (100%)</td>
<td>580 (100%)</td>
</tr>
</tbody>
</table>
Table-II Upstaging of cases by using immunohistochemistry(CK+EMA) for detection of micrometastases

<table>
<thead>
<tr>
<th>N Stage(n= no. of cases)</th>
<th>Upstaged to N1</th>
<th>Upstaged to N2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0(n=14)</td>
<td>3(21.4%)</td>
<td>3(21.4%)</td>
<td>6(42.8%)</td>
</tr>
<tr>
<td>N1(n=8)</td>
<td></td>
<td>2(25%)</td>
<td>2(25%)</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Table-III
Correlation between IHC(CK+EMA) staining and serial sectioning for detection of micrometastases

<table>
<thead>
<tr>
<th>IHC</th>
<th>Cases positive on s/s</th>
<th>Cases negative on s/s</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>29</td>
<td>30</td>
</tr>
</tbody>
</table>
DISCUSSION

Worldwide HNSCC constitute 15% of male cancers and approximately 600000 cases in female.\(^6\)

The single most important factor affecting prognosis for patients with SCC of upper aerodigestive tract is the stage of the disease at time of initial diagnosis and treatment. Patients who present with tumors localized to the primary site without dissemination to regional lymph nodes enjoy an excellent prognosis. On the other hand when disseminated to regional lymph nodes

![Figure-1](photomicrograph showing cluster of malignant cells in subcapsular sinus after serial sectioning[H &E X200])

*Figure-1* photomicrograph showing cluster of malignant cells in subcapsular sinus after serial sectioning[H &E X200]

*Figure-2* photomicrograph showing dendritic cells positive for cytokeratin-false positivity[CK X200]

*Figure-3* Photomicrograph showing cells which show equivocal results on cytokeratin staining, required further confirmation by staining with EMA[CK X200]

*Figure-4* photomicrograph revealing cluster of malignant cells positive for cytokeratin[CK x200]
take place, the probability of 5 year survival regardless of treatment rendered, reduces to nearly one half of that seen in early stage patients. Therefore the single most important prognostic factor in the treatment of patients with SCC of the head and neck is the status of cervical lymph nodes.[9]

The clinically negative neck is defined by the presence of palpable or radiologically suspicious lymph nodes. However patients staged CN0 can harbour lymphatic metastases that are too small to be detected by imaging or palpation. These subclinical or occult metastases are detected on pathological examination of cervical LNs following neck dissection. Thus there can be discordance between clinical and pathological nodal stage.[10]

The sensitivity of current routine histopathological methods in assessing regional lymph node involvement is imperfect in that it, like clinical staging can rarely detect the presence of micrometastases with in the nodes. The detection of micrometastases by the pathologist depends very much on the efforts put into the histopathological examination. The number of lymph nodes examined, the number of sections examined per lymph node and the precision of the microscopic examination to determine to a great extent the number of metastases found. Now, it has been proven that additional deeper sectioning and immunostaining can help to find additional micrometastases. It seems clear that no routinely applicable technique is able to detect all micrometastases.[11]

In the present study we use two techniques for detection of micrometastases i.e serial sectioning and immunostaining using antibodies to cytokeratin and EMA. On serial sectioning only single lymph node of one case(3.3%) was positive for micrometastases. On combining both IHC stains (CK+EMA) 8 of 30 cases and 11 of 580 lymph nodes(1.9%) were positive for micrometastases. This increase in the detection rate of occult metastases in neck dissection specimens is seen in accordance with following studies in literature.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Method used</th>
<th>Detection rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CK</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK</td>
<td>0</td>
</tr>
</tbody>
</table>
Small discrepancies may be attributed to differences in methodology of IHC including the type of antibodies used, cohort sizes, patient characteristics and definition of micrometastases. Kwon et al \cite{3} claimed IHC method to be more sensitive in detecting micrometastases than serial sectioning, which was also ascertained in our study.

According to Brekel et al \cite{1} it is hard to compare IHC to additional sectioning to detect MMS. The techniques are complementary, as immunostaining will detect metastases that might be overlooked in the original slides, whereas deeper sectioning detects metastases that are located at another level in the lymph node. The major advantage of IHC is that this technique facilitates the detection of MMS by enhancing the contrast between metastatic and lymphatic tissue.

Barrera et al \cite{2} concluded the use of serial sectioning and IHC analysis increase the detection rate of MMS but serial sectioning is too time consuming and expensive for routine practice.

Only a few studies are there which revealed prognostic significance of MMS. Brekel et al \cite{1} and Ambrosh et al \cite{11} found that the detection of MMS had not influenced the post operative treatment decisions, so they did not recommend the use of IHC and s/s for routine use. Whereas Rhee et al \cite{14} found that 3 of 5 patients with occult metastases developed recurrence in the neck, while 1 of 5 patients with no evidence of MMS had regional recurrence. Thups they recommended the detection of MMS in routine practice.

We studied the incidence of MMS with various clinicopathological parameters like tumor size (T-stage), tumor grade, site of primary tumor, histological type and differentiation of primary tumor as well as with the size and level of lymph node involved.

Our studies are comparable to other studies in the past revealing no significant correlation between T stage of primary tumor and MMS. In the present study, out of 14 N0 cases 3 were upstaged to N1 and 3 were upstaged to N2. out of 8 N1 cases, 2 were upstaged to N2.

As MMS increases the nodal stage in low staged cancers, which can lead to increase in the rate of local recurrence, additional RT or CT can be taken into account after the neck dissection. So IHC method can play a crucial role in diagnosis and treatment of early metastases of cancer.

No statistically significant correlation was found between site and histological subtype of primary
tumor. However incidence of MMS was higher in the group with poorly differentiated tumor cells; this may be because of increased chance of misdiagnosis of these poorly differentiated cells as lymphocytes or histiocytes on H & E. The incidence of MMS increases as the differentiation of primary tumor became poor. Thus, a statistically significant correlation was present between differentiation of primary tumor and MMS.

The higher incidence of MMS in lymph nodes with a smaller diameter is thought to be caused by rapid growth, poor differentiation and an increased number of relatively small sized tumor cells in early metastatic cancers, all of which decrease the detection rate in routine H & E. Statistically significant correlation was found between size of lymph node and MMS (P=0.02)

Our study revealed that CK was 100% sensitive and 88% specific and EMA was 62.5% sensitive and 100% specific for detection of MMS. So, the subject of MMS in head and neck cancer is fascinating. The newer technologies including IHC, molecular markers and s/s have been used to identify microscopic metastatic disease which remain unidentifiable but routine H& E stains. This may be the reason why even after appropriate neck dissection and post operative RT, occasionally we see recurrent disease in a small percentage of patients. The MMS increases the nodal stage in low staged cancers, which leads to an increase in rate of local recurrence. Untreated MMS are also expected to proliferate gradually and be detected sooner or later as overt metastases. Therefore it would be advisable to use the IHC stain method as a diagnostic tool for early metastatic cancers in which lymph nodes are negative in routine & E, since these methods may provide a basis for improved planning of postoperative therapy.

References


