

DIAGNOSTIC ACCURACY OF VARIOUS METHODS TO DETECT LYMPH NODE METASTASES IN ORAL SQUAMOUS CELL CARCINOMA

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HOW TO CITE THIS ARTICLE:

Priyanka Agarwal, Minal Chaudhary, Swati Patil, Alka Hande, Madhuri Gawande, Puneet Agarwal. "Diagnostic Accuracy of Various Methods to Detect Lymph Node Metastases in Oral Squamous Cell Carcinoma". Journal of Evolution of Medical and Dental Sciences 2014; Vol. 3, Issue 22, June 02; Page: 6003-6010, DOI: 10.14260/jemds/2014/2696

ABSTRACT: The present study was undertaken with the purpose to compare the sensitivity of various methods for detection of lymph node metastases: Intra-operative frozen sections H & E staining, Conventional H & E staining on formalin fixed tissue, Serial –step sectioning by conventional H&E staining & Immunohistochemical staining by Pancytokeratin antibody. **METHOD:** The study included 80 consecutive cases of oral squamous cell carcinoma, who underwent radical neck dissection. The various level of lymph nodes in these cases were checked for metastases by 4 techniques i.e. intra-operative frozen sections H&E staining, conventional H&E staining on formalin fixed tissue, serial –step sectioning by conventional H & E staining & immunohistochemical staining by Pancytokeratin antibody. **RESULTS:** Considering IHC as a gold standard, we observed highest sensitivity & specificity for serial sectioning at 53.7%, & 98.9% when compared to intraoperative frozen section and conventional H&E which were 32.5%, 97.1% & 44.7%, 98.2% respectively. **CONCLUSION:** Thus we conclude that the most sensitive method to detect lymph node metastasis in case of Oral Squamous cell carcinoma is step serial section when considering IHC as a gold standard. **KEYWORDS:** intra-operative frozen sections H & E staining, conventional H & E staining, serial –step sectioning by conventional H & E staining, immunohistochemical staining.

INTRODUCTION: Oral cancer is the sixth most common cancer worldwide In India, it account one fourth of male cancers and one tenth of female cancers (Yeole 2001). Preoperative clinical methods and newer radiographic techniques are suboptimal and misdiagnose the presence or absence of nodal metastasis in many patients.¹ When compared to node-negative disease, the presence of even a single micrometastasis in a lymph node is associated with a significant difference in recurrence and survival.²

The deleterious effect of cervical metastases on prognosis is so great that even a 20% chance of metastases in an otherwise clinically and radiographically negative neck pushes most clinicians toward its treatment. By definition, treatment subjects, 80% of patients to therapy-related morbidity despite the fact that these patients have no neck disease.³

The current management of the clinically node negative (cN0) neck commonly includes routine elective neck dissection (END) with pathologic examination of the removed lymph nodes. END or cervical lymphadenectomy done at the time of primary surgery for squamous cell carcinoma of head and neck (SCCHN) for a cN0 neck, is associated with a significantly improved regional recurrence-free survival and lower incidence of distant metastases.¹

But on the other hand, the patient who undergoes lymphadenectomy inappropriately suffers the needless postoperative complications like pain, numbness, motor dysfunction and lymphedema.

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Treatment is always indicated for clinically N-positive disease, but clinically N0 disease presents a therapeutic dilemma.

Intraoperative frozen sections (FS) of sentinel lymph nodes (SLNs) can be used to detect metastatic disease, allowing immediate cervical lymph node dissection in oral squamous cell carcinoma (OSCC).⁴ A pathologically negative sentinel lymph node predicts the absence of metastasis to the remainder of the regional lymph node basin with about a 98% degree of certainty.⁵ However, pathological inconsistency in the SLNs diagnosis is sometimes encountered when the results of FS and permanent sections are compared.⁴

Reports indicate that, approximately 5-20% of head & neck squamous cell carcinoma (HNSCC) patients' harbored occult metastasis not identified by routine histopathological examination but detected by other methods such as step serial sectioning or immunohistochemistry.⁶

This paper aims to compare the diagnostic accuracy of frozen sections, conventional H&E staining and serial step sectioning as compared to IHC in detection of lymph node metastases.

MATERIALS AND METHOD: The present study was conducted in the Department of Oral Pathology and Microbiology, at Sharad Pawar Dental College and Hospital from June 2010 to August 2012, after obtaining approval from the Institutional Ethical Committee of Datta Meghe Institute of Medical Sciences, Sawangi (M), Wardha, Maharashtra. Eighty patients consecutively diagnosed as primary squamous cell carcinoma of the oral cavity after incisional biopsy who underwent surgical resection along with neck dissection were included in the study. An informed consent was obtained from all the participants included in the study.

The sentinel and various non-sentinel lymph nodes were identified and accessed sequentially during radical neck dissection (RND). First, the lymph nodes at the time of surgery, were stained by frozen section hematoxylin and eosin method. The remaining tissue of the lymph node was processed for paraffin- embedded sections. The paraffin- embedded lymph node was again sectioned by step serial sectioning method (50µm sections apart) and stained for conventional H&E.

A node was labeled as positive when it shows loss of architecture and epithelial cells infiltrating the lymph node, usually in the form of small islands and groups.

Those lymph node which were negative for malignancy on serial sectioned evaluation, were then evaluated for IHC. Every 19th and 29th section was taken for IHC staining with pancytokeratin.

Positive and Negative Controls: Tissues from squamous cell carcinoma samples were used as positive control, the epithelial cells and islands stain positively with cytokeratin antibody in these samples. One positive control was included for each immunohistochemical cohort. One section from each positive control was used as the negative control by omitting the primary antibody and by incubating with phosphate buffer saline (PBS).

The positive control was examined for the presence of a colored end product at the site of the target antigen (DAB chromogen brown end product). The presence of this colored product was interpreted as positive staining result, indicating proper performance of kit reagents. The absence of nonspecific staining in the negative control confirmed the specificity of primary antibody.

Sections were considered positive if cells showed positive cytoplasmic Diamino Benzidine (DAB) staining (brown color) for the CK antigen when viewed under 40×.

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Statistical analysis was carried out to evaluate the sensitivity and specificity of the three methods used for detection of lymph node metastasis detection i.e. Intraoperative frozen sectioning, Conventional H & E, and Step serial sectioning, using SPSS for analysis.

RESULTS: Eighty patients were enrolled during the study period. The baseline characteristics of the study participants are shown in table 1.

Variable		
Mean age (SD) in years	52.3 (10.7)	
Sex M:F	7:3	
Location	Frequency	Percent
Buccal mucosa	39	48.75
Floor of mouth	1	1.25
Gingivo-buccal sulcus	19	23.75
Labial mucosa	12	15
Lip & labio-gingival sulcus	2	2.5
Retromolar area	2	2.5
Tongue	5	6.25
Total	80	100.0

Table 1: Baseline characteristics of the study participants (N=80)

Considering IHC as a gold standard, we observed a highest sensitivity & specificity for serial sectioning.

Technique		Frequency	Sensitivity	Specificity	Positive predictive value	Negative predictive value	LR
Frozen section	+	12.2	32.5%	97.1%	12.15	87.85	11.21
	-	87.8					
Conventional H & E	+	15.2	44.7%	98.2%	15.19	84.81	24.83
	-	84.8					
Step	+	17.5	53.7%	98.9%	17.47	82.53	48.82
	-	82.5					

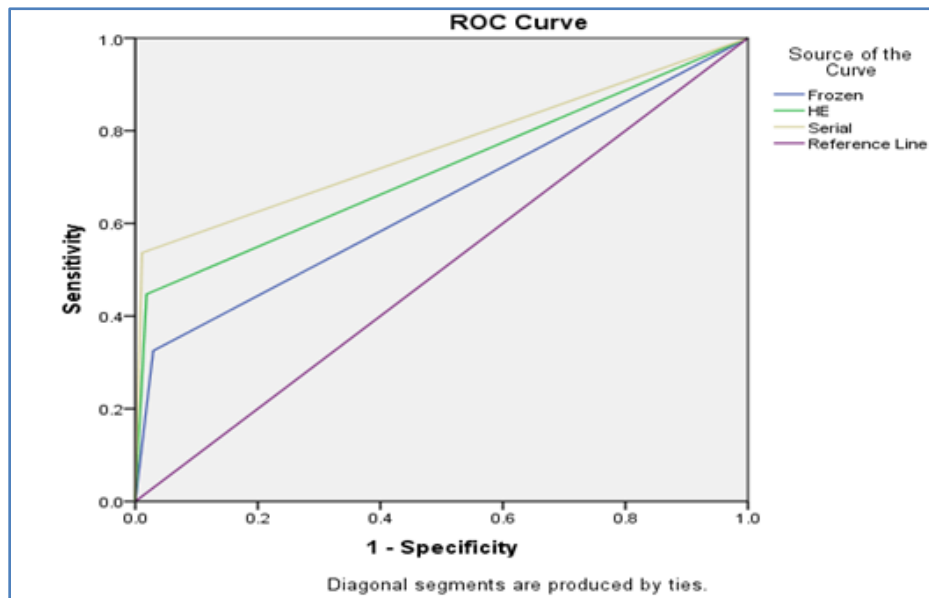
Table 2

IHC	Percent	Frequency
Absent=0	68.9	272
Present=1	31.1	123
Total	100	395

Table 3: Showing the frequency distribution of positive cases with malignancy by IHC

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ROC Curve:



ROC Curve

Test Result Variable(s)	Area	Std. Error ^a	Asymptotic Sig. ^b	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Frozen	.648	.032	.000	.585	.711
HE	.714	.031	.000	.653	.776
Serial	.763	.030	.000	.704	.821

Table 4: Area Under the Curve

Maximum area under the curve is seen in step serial section, which is followed by conventional H&E and then frozen section. This is indicative of higher sensitivity of step serial section as compared to frozen section and conventional H&E staining.

The detection of lymph node metastasis was significantly higher with Step serial sectioning as compared to conventional H&E (17.5% Vs 15.2%; $p < 0.001$).

DISCUSSION: Various methods have been devised to detect micrometastasis including intraoperative histological analysis by frozen sections; conventional H&E stained sections, step serial sectioning, IHC and highly sensitive techniques like Reverse Transcriptase -PCR and Real time PCR. The present study evaluated metastasis in sentinel and non-sentinel lymph nodes by 4 methods so that chances of missing even the smallest foci of metastasis were minimized.

The intraoperative examination (frozen section analysis) and the routine pathological assessment of the node underestimate the true incidence of metastasis. False negative nodes may contain micrometastasis, which may not be obvious in the histologic slides examined. Serial Sections, IHC & other molecular techniques increases the detection rate of node metastasis and could be useful

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for better assessing the status of the lymph nodes. These sensitive and specific methods have been shown to upstage patients previously referred to as "lymph node negative".⁷

The advantage of this technique over the other technique is that it can be performed simultaneously saving time and money and benefiting both patients and clinicians.⁷ This is one of the important advantages of frozen section analysis i.e. detection of positive SLN and other positive lymph nodes and thereby avoiding the need for reoperation.^{8,4}

But this technique suffers the disadvantage that the sections may undergo artefactual changes before going for permanent H&E sections.⁸ One more disadvantage is that intra-operative diagnosis using frozen section is slightly more difficult compared with post-operative diagnosis using paraffin embedded sections.

Also most lymph nodes are surrounded by thick adipose tissue and hence frozen specimens are frequently twisted, broken or wrinkled, and difficult to observe. Adipose tissue should therefore be removed as far as possible and several sections should be prepared to allow accurate observation of all sections of the lymph nodes.⁹

In this study the sensitivity of frozen section is around 32.5% with a specificity of 97.1%. This is in accordance with the study by Rassekh CH et al on cervical lymph nodes for metastasis from aero digestive tract neoplasm and in which accuracy of 92.3% was determined.¹⁰ Wada et al & Kurosumi et al who have reported intraoperative sensitivity for lymph nodes in breast cancer in the range of 47% to 74% whereas in melanoma the sensitivity ranges from 38% to 47% [Ferris].^{9,4}

Many patients have to undergo second surgeries to complete lymph node dissection in cases in which definitive pathologic assessment identifies metastasis that were missed on the frozen section examination. The limitation of frozen section is that it is most accurate at detecting the foci of nodal metastasis that are > 2mm in size, therefore deposits less than 2mm are missed.⁴

So considering that frozen section is limited by technical parameters and therefore often fails to detect metastases, we carried out conventional H&E staining of the paraffin embedded sections. This method helps in minimizing the chances of missing metastatic deposits during frozen section analysis.

In this study 15% nodes were given positive following conventional H&E method which was first termed negative in frozen section. Based on the study the clinician should realize the diagnostic limitation of this procedure. The sensitivity of H&E was 44.7% with a specificity of 98.2%. This is in accordance with the study of Khanna et al who have reported sensitivity of H&E to be 40% for sentinel lymph node evaluation. H&E therefore results in a significant increase in identification of low volume metastasis and consequently to stage migration as many of the former node negative cases containing occult metastasis were now placed into the node positive micrometastasis group.

The reason for this increase in detection could be because it is easy to diagnose the metastatic foci by conventional H&E as the tissue is neither twisted nor wrinkled and also the observer is more comfortable with this viewing modality.

The American Joint Committee on Cancer (AJCC) has modified the PTNM classification by splitting the micrometastasis category into two. Micrometastasis between 0.2-2.0 mm are staged as pN1mi and metastasis less than 0.2mm are called isolated tumor cells and staged as nN0(i+). Surgeons treat pN1mi lesions as true metastasis and recommend axillary dissection and systemic therapy. Isolated tumor cells or pNo (i+) are considered as node negative for further treatment decisions.¹¹

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Since only one or two sections are evaluated by the histopathologist, there are chances of missing the foci of metastasis, specially isolated tumor cells present in another region of lymph node. This in reality is an incomplete examination, in which the central section serves as a proxy for whole node.¹² Studies have shown that routine evaluation misses upto 21% of diseased nodes.¹³

So in order to identify small deposits that may be present in another region of lymph node, we carried out step serial sectioning of the same paraffin embedded tissues. In this study all the lymph nodes (395) from 80 patients were serially sectioned, the sections were 5µm thick and every 9th section was stained for H&E and examined while every 10th section was taken on a silane coated slide for IHC. A total of 20, 000 sections were made, out of them 5, 000 were stained with H&E and examined.

A single 5µm section from 1cm lymph node samples only 1/2000th of the node.¹⁴ So smaller metastasis present at the time of primary diagnosis may therefore be missed by conventional method. To overcome this limitation, it has been stated that in practice, multiple sections of node which are negative or contained only micrometastasis on initial assessment¹⁵ leads to additional positive lymph nodes detection. Serial or step sectioning can detect smaller metastasis by more detailed observation thereby increasing the lymph node positive group and upstaging the tumor.⁷

In our study 9 nodes were given positive following step serial sectioning method which was first termed negative in conventional H&E method for single central section. The sensitivity of step serial sectioning was computed as 53.7% and 44.7% by conventional H&E analysis, which was approx. 9% more compared to conventional H and E (44.7%). This was found to be statistically significant. The specificities of both the technique were relatively similar/equally good. (98.9% vs 98.2%)

After observing the result of frozen section, conventional H&E, & step serial sectioning, we carried out immunohistochemistry of the same with pancytokeratin antibody (Monoclonal mouse anti-human cytokeratin clone AE/AE3) to further increase the accuracy in detection of metastasis in sentinel and non-sentinel nodes.

In this study IHC detected additional 54 positive nodes. This validates our claim that IHC is a much more sensitive technique and can be used as gold standard technique for the diagnosis of metastasis. This study is in agreement with study results of Yokomaya whose comparative analysis also showed immunohistochemistry to be more sensitive than routine histology in detection of metastases.

Studies observe that H&E staining with step serial identifies one cancer cell among 10, 000 normal cells while IHC identifies one tumor cell amongst 1, 00, 000 normal cells, giving proof to our belief that IHC can be used as gold standard. So in clinical practice, step serial sectioning with H&E staining upstages the tumor in 10%, whereas IHC upstages it by a further 10% of cases.¹²

CONCLUSION: IHC is the gold standard in detecting lymph node metastasis. But especially in resource-constrained setting where IHC is not available step sectioning should be practiced routinely, as it has high sensitivity & specificity as compared to conventional H&E, in detection of micro-metastasis.

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Date of Submission: 07/05/2014.
Date of Peer Review: 08/05/2014.
Date of Acceptance: 16/05/2014.
Date of Publishing: 29/05/2014.