

ORIGINAL ARTICLE

Detection of micrometastasis in lymph nodes of oral squamous cell carcinoma: A comparative study

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ABSTRACT

Background: The annual mortality rate from head and neck squamous cell carcinoma (HNSCC) is over 11,000 worldwide. Squamous cell carcinoma of the head and neck (SCCHN) frequently metastasizes to the regional lymph nodes which are the first site of arrest of tumor cells that have invaded the peritumoral lymphatics, hence the strongest predictor of disease prognosis and outcome. **Aim:** The present study aims to compare the efficacy of frozen sections (cryosection), step-serial sectioning conventional H and E staining, immunohistochemistry (IHC) and RT-PCR analysis in detection of lymph node micrometastasis. **Materials and Methods:** A prospective series of 30 patients who were diagnosed with primary squamous cell carcinoma of the oral cavity and underwent surgical treatment including unilateral or bilateral selective neck dissection were considered for the study. **Result:** Metastatic carcinomatous cells were observed in H and E staining of frozen section in 18 lymph nodes (54%) and in 19 lymph nodes (57%) in step-serial sectioned H and E-stained sections of the 78 lymph nodes from 30 patients. Carcinomatous cells were immunolabeled with pancytokeratin in 18 lymphnodes (54%). CK19 mRNA was detected in 33 lymph nodes of 16 patients. RT-PCR gave positive signals for 24% and 23% of lymph nodes positive by histopathology and immunohistochemistry. **Conclusion:** Our study demonstrated that RT-PCR is far more sensitive in detection of micrometastasis than any other technique used in routine procedures and immunohistochemistry. Fifty-three percent patients with micrometastasis detected by RT-PCR had large T3/T4 tumors. Prognosis was poor for patients who were positive for micrometastasis detected only by RT-PCR, among which two patients died within a period of 6 months. **Key words:** Cryosection, immunohistochemistry, lymph node, micrometastasis, oral squamous cell carcinoma, RT-PCR, step-serial sectioning

INTRODUCTION

Oral cancer is the sixth most common cancer worldwide, with a high prevalence in South Asia. The annual estimated incidence is around 2,75,000 for oral and 1,30,000 for the pharyngeal cancers excluding nasopharynx, two-third of these cases occur in developing countries of South and Southeast Asia, parts of Western France, the Eastern Europe,

Latin America, Carribean and in Pacific regions. Among these Bhopal (India) tops the list with highest age-adjusted incidence rate (AAR) for cancers of both the tongue (10.9/10,000) and mouth (9.6/100,000) in the world. The annual mortality rate from HNSCC is over 11,000, which corresponds to 2% of all cancer deaths.^[1]

Squamous cell carcinoma of the head and neck (SCCHN) frequently metastasizes to the regional lymph nodes which are the first sites of arrest for tumor cells that have invaded the peritumoral lymphatics; hence, the strongest predictor of disease prognosis and outcome.^[2,3]

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. The deleterious effect of cervical metastases on prognosis is so

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10.4103/0973-029X.125202

great that even a 20% chance of metastases in an otherwise clinically and radiographically negative neck pushes most clinicians towards extensive treatment. Regional recurrence rates of approximately 10% are reported in patients with histologically negative neck dissection specimens, suggesting that carcinomatous cells were present but not detected in the resected nodes. In a recent report, approximately 5-20% of HNSCC patients harbored occult metastases not identified by routine histopathological examination but detected by other methods such as immunohistochemistry (IHC) or molecular biology, that is, reverse transcriptase- polymerase chain reaction (RT-PCR).^[2,4,5]

The present study aims to compare the efficacy of frozen sections (cryosection), step-serial sectioning conventional H and E staining, immunohistochemistry (IHC) and RT-PCR analysis in detection of lymph node micrometastasis. It also aims to assess the correlation between presence of micrometastasis and the tumor size along with histopathological grading of the tumor.

MATERIAL AND METHODS

Patients and neck dissection samples

This study, conducted according to the institutional and ethical rules concerning research on tissue specimens, was exempted from informed consent from the patients. The study was approved by the Institutional Ethical Committee.

A prospective series of 30 patients diagnosed with primary squamous cell carcinoma of the oral cavity by the Department of Oral and Maxillofacial Pathology were selected for the study. Patients underwent surgical treatment including unilateral or bilateral selective neck dissection in the year 2009 between the months of February and August at the Department of Oral and Maxillofacial Surgery. The study included 30 patients, 23 males and 7 females. The details regarding site and age are shown in Table 1. An inclusion criteria of primarily diagnosed cases of squamous cell carcinoma was followed while recurrent cases were excluded from the study.

Lymph nodes, macroscopically isolated from the patients at the time of surgery, were dissected and cut into 3- μ m slices vertically. The even-numbered slices were stained with frozen section Hematoxylin and Eosin (H and E) method and the odd-numbered slices were immediately stored in sterile 1.5-ml tubes containing RNA Later (Ambion, USA) solution, as per the manufacturer's protocol.^[5,15]

The remaining node was then fixed and sectioning was done from the paraffin-embedded tissue. The paraffin-embedded lymph node was then subjected to step-serial sectioning with a difference of 100- μ m sections apart and stained by conventional H and E and IHC method for pancytokeration (MNF clone 116, Dako, Denmark) alternatively. Then sections of lymph

nodes positive by RT-PCR were again stained for CK-19. The same procedures were followed for six lymph nodes of patients diagnosed as ameloblastoma (negative control) in the same study period in our department.^[5,15]

Positive and negative control

MCF-7 (Human mammary carcinoma cell line) was considered as a positive control for CK19 mRNA expression and six lymph nodes from patient diagnosed for ameloblastoma and undergone resection for the same were considered as a negative control. Control lymph nodes were processed for RNA extraction.

RT-PCR assay

Total RNA isolation was performed from the pooled odd number sections by using TRIZOL (Invitrogen USA) method as per manufacturer's instruction. Briefly, 1000 μ l of the TRIZOL was added as two splits in to microsectioned tissues and lysed with the help of hand held pestle and mortar (Sigma,

Table 1: Characteristics of patients with HNSCC

Age	Sex	TNM staging clinical (cTNM)	Location
56	M	T2N1M0	Upper left gingivo-alveolar region
55	M	T1N0M0	Lower right alveolar mucosa
45	F	T2N2aM0	Lower labial mucosa
29	M	T3N1M0	right buccal mucosa
29	M	T3N2bM0	Left buccal mucosa
65	M	T2N2bM0	Maxilla
60	M	T3N2bM0	Labial mucosa
45	M	T3N1M0	Left buccal mucosa
70	M	T2N2cM0	Labial vestibule
55	M	T2N2bM0	gingivo-buccal sulcus
62	M	T2N2bM0	lower left vestibular mucosa
60	M	T3N2aM0	Right Buccal mucosa
65	M	T2N2cM0	Lower anterior Labial mucosa
40	F	T2N2cM0	Mucosa from the submental region
32	M	T2N2aM0	Left buccal mucosa
65	M	T2N2bM0	Left maxilla and involved mucosa
50	M	T2N2bM0	Gingival sulcus
60	M	T2N2bM0	Labial mucosa
40	M	T3N1M0	Left buccal mucosa
60	M	T3N2bM0	Buccal-gingiva and alveolar mucosa
55	M	T3N2cM0	Lower left alveolar mucosa
30	M	T2N2bM0	Right metastatic lymph node
61	F	T3N2cM0	Right buccal mucosa
49	M	T3N2cM0	Labial and buccal mucosa on both sides
61	F	T2N2aM0	Gingivo-buccal sulcus
40	M	T2N2aM0	Left buccal vestibule
48	M	T2N1M0	Buccal mucosa
60	F	T2N1M0	Buccal and alveolar mucosa
65	F	T2N1M0	Buccal vestibule

cTNM: Clinical tumor size lymph node and metastasis staging; HNSCC: Head and neck squamous cell carcinoma

USA) and further procedure was followed according to the TRIZOL manufacturer's instruction. The air-dried RNA pellet was dissolved into 30- μ l sterile nuclease-free water and stored in -80°C till its use. The quantity and quality of the isolated RNA was estimated by Nanodrop (Thermo, USA) followed by in 1% agarose gel, respectively. Approximately 1.5 μg of total RNA was subjected to DNase (Ambion, USA) treatment and the complete removal was further carried out by using PCR using RPII primers. The resultant DNA-free RNA was converted to cDNA by using high capacity cDNA Reverse Transcription Kit (ABI, USA).

The prepared cDNA was used as a template to detect the occurrence of micrometastasis. Metastases was detected by presence of expression of cytokeratin 19 in lymph nodes by using gene-specific primers.^[6] RNA polymerase II was used as a calibrator housekeeping gene.^[7] The same PCR technique was followed for both the genes with initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 15 sec, the annealing at 56°C for 15 sec, extension at 72°C for 30 sec and a final extension at 72°C for 1 min. The amplicons 267 bp and 80 bp of RP II and CK 19 respectively, were resolved in 1.8% agarose gel with ethidium bromide gel.

Immunohistochemistry

Immunohistochemistry was performed on 4- μm sections of formalin-fixed, paraffin-embedded samples for 78 lymph nodes with pancytokeratin (MNF clone 116, Dako, Denmark) and IHC was repeated with CK 19 for nodes in which CK19 mRNA was detected by RT-PCR assay. Positive and negative control was applied every time. Nodes positive by H and E staining were taken as positive control and nodes removed during surgery for ameloblastoma (benign tumor) were considered as negative control. Immunostaining was carried out using the standard avidin-biotin technique with the specifications.

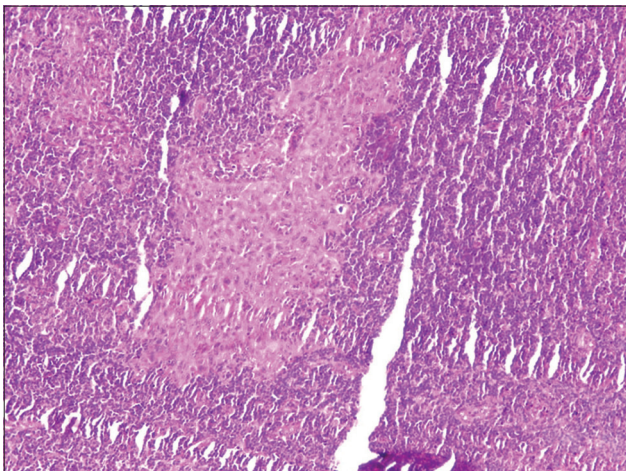


Figure 1: Tumor islands can be seen in frozen section of lymphnodes (H&E stain, $\times 100$)

Statistical analysis

The χ^2 -test was applied to find the comparative statistical significance of each method. The same test was applied to find the correlation of positive findings of this test with tumor size and histological grading of the tumor. For the correlation of histological grading with the positive results, χ^2 -test for goodness of fit was applied. Differences were considered significant for $P < 0.05$. These statistical analyses were performed using SPSS version 11.0 software.

RESULTS

Histopathological examination

Metastatic carcinomatous cells were observed in H and E staining of frozen section in 18 (54%) and in 19 (57%) in step-serial sectioned H and E-stained sections of the 78 lymph nodes of 30 patients. In Figure 1 tumor islands can be seen in the H and E-stained frozen section ($\times 100$) and Figure 2 shows dysplastic cells with mitotic figures, evident under higher magnification of frozen section ($\times 400$).

RT-PCR

CK19 mRNA was detected in 33 lymph nodes of 16 patients. RT-PCR gave positive signals for 24% and 23% of lymph nodes positive by histopathology and immunohistochemistry. RT-PCR gave additional signals for 17% of nodes. Detailed results of CK19 mRNA detection by real-time RT-PCR according to tumor site are shown in Table 2 and detection along with the level of lymph node involved along with the clinical and pathological status of the lymph node is shown in Table 3.

Immunohistochemistry

In the 78 lymph nodes of 30 patients, carcinomatous cells were immunolabeled with pancytokeratin in 18 (54%) nodes

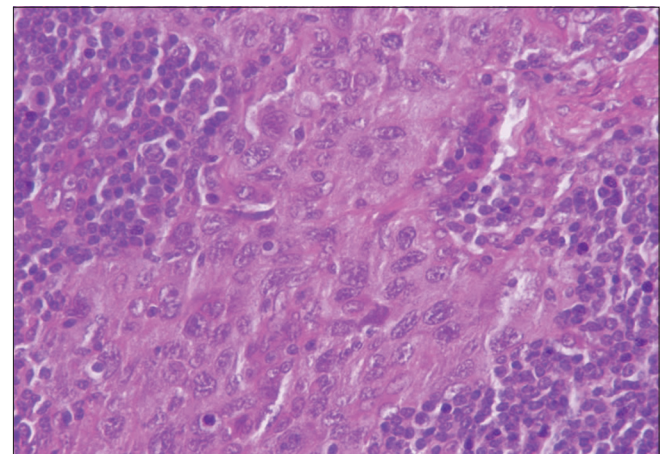


Figure 2: Dysplastic cells with mitotic figures are evident under higher magnification (H&E stain, $\times 400$) of frozen section

positive by H and E staining of frozen section and step-serial sectioning conventional H and E staining but IHC was negative for the additional node showing micrometastasis by step-serial sectioning H and E staining. The nodes positive for CK19 mRNA were again stained for CK 19 antibody but no additional carcinomatous cell was immunodetected by IHC. Figure 3 shows carcinomatous cells in lymph nodes when incubated with pancytokeratin antibody (magnification ×100).

Table 2: Comparison between the results of intraoperative frozen section analysis (F), step-serial sectioning H and E staining (C), immunohistochemistry and RT-PCR analysis

Analysis by	Positive	Negative	Total
H and E ^F	18	60	78
H and E ^C	19	59	78
IHC	18	60	78
RT-PCR	33	45	78

RT-PCR: Reverse transcriptase-polymerase chain reaction

Table 3: Results of histopathological examination and CK14 mRNA real-time RT-PCR in the various cervical lymph node levels expressed as the number positive levels/total number of levels investigated

Age	Sex	Clinical node cN	Pathologic pN	Position	H&E	IHC	PCR-CK19
56	M	N1	N2a	Level I	Pov	Pov	Pov
				Level II	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
55	M	N0	N0	level II	Neg	Neg	Neg
				level II	Neg	Neg	Neg
45	F	N2a	N2a	Level I	Neg	Neg	Neg
				level II	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
29	M	N1	N1	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
				Level III	Neg	Neg	Neg
29	M	N2b	N2b	level II	Pov	Pov	Pov
				Level III	Neg	Neg	Neg
				Level I	Pov	Pov	Pov
65	M	N2b	N2b	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
50	F	N2b	N2b	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
60	M	N2b	N2b	Level I	Neg	Neg	Neg
				Jugular	Neg	Neg	Neg
45	M	N1	N2b	Level II	Pov	Pov	Pov
				Level II	Pov	Pov	Pov
70	M	N2c	N2c	Level II	Neg	Neg	Pov

Contd...

Table 3: Contd....

55	M	N2b	N2b	Level II	Neg	Neg	Pov
				Level II	Pov	Pov	Pov
				Level II	Pov	Pov	Pov
62	M	N2b	N2b	Level I	Neg	Neg	Neg
				Level I	Pov	Pov	Pov
				Level I	Neg	Neg	Neg
60	M	N2a	N2a	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
65	M	N2c	N2c	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
40	F	N2c	N2c	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
32	M	N2a	N2a	Level I	Pov	Pov	Pov
				Level II	Neg	Neg	Pov
				Level II	Neg	Neg	Neg
65	M	N2b	N2b	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
50	M	N2b	N2b	Level III	Neg	Neg	Pov
				Level II	Pov	Pov	Pov
60	M	N2b	N2b	Level I	Neg	Neg	Pov
				Level IV	Neg	Neg	Neg
40	M	N1	N1	Level I	Neg	Neg	Pov
				Level II	Neg	Neg	Pov
				Level III	Neg	Neg	Pov
60	M	N2b	N2b	Level I	Neg	Neg	Neg
				Level I	Neg	Neg	Neg
				Level I	Neg	Neg	Neg
55	M	N2c	N2c	Level II	Pov	Pov	Pov
				Level II	Pov	Pov	Pov
				Level III	Pov	Pov	Pov
30	M	N2b	N2b	Level II	Pov	Pov	Pov
				Level II	Pov	Pov	Pov
61	F	N2c	N2c	Level I	Pov	Pov	Pov
				Level II	Neg	Neg	Pov
49	M	N2c	N2c	Level I	Neg	Neg	Pov
				Level I	Neg	Neg	Pov

Contd...

Table 3: Contd....

				Level IV	Neg	Neg	Neg
61	F	N2a	N2a	Level I	Pov	Pov	Pov
40	M	N2a	N2a	Level I	Neg	Neg	Pov
				Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
48	M	N1	N2a	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
60	F	N1	N1	Level I	Neg	Neg	Neg
				Level II	Neg	Neg	Neg
65	F	N1	N2a	Level II	Neg	Neg	Neg
				Level II	Neg	Neg	Neg

Neg: Negative, Pov: positive

DISCUSSION

Carcinomatous cell diffusion in cervical lymph nodes is a major determinant of therapy and prognosis for patients with HNSCC^[8] as cure rates for patients with metastatic lymph nodes drop to one-half of those of patients without nodal involvement (Shah, 1990).^[5] Micrometastases represent tumor deposits measuring less than 2 mm in diameter. As histopathological analysis of neck dissection specimens is usually performed on several 3-4- μ m sections from each lymph node, micrometastases can be easily missed on routine light microscopy.^[5,9] For example, it has been reported that 21.9% of patients with cancer of the oral cavity have micrometastases with an average diameter of 1.36 mm.^[5,10] Therefore, the consequence of missing this micrometastasis directly affects the disease-free survival of a patient with carcinoma.

Extensive research of literature in English language has not revealed any studies reported exclusively on detection of micrometastasis in oral squamous cell carcinoma, neither any comparative analysis of all the four methods like cryosection, conventional H and E staining, immunohistochemistry and RT-PCR analysis in oral squamous cell carcinoma was found which adds to the uniqueness of this study.

The intraoperative histopathological diagnosis using frozen sections is done as a next to the surgical room investigation during the neck dissections to mark the extent of the tissue involvement by the neoplasm. The advantage of this technique over other techniques is that it can be performed simultaneously saving time and money and benefitting both patients and clinicians by reducing the chances of second surgery. In our study the sensitivity of frozen section detection was around 54.4% which is in accordance with the studies who have reported intraoperative sensitivity for lymph nodes micrometastases detection by frozen section in breast cancer in the range of 47-74% whereas in melanoma the

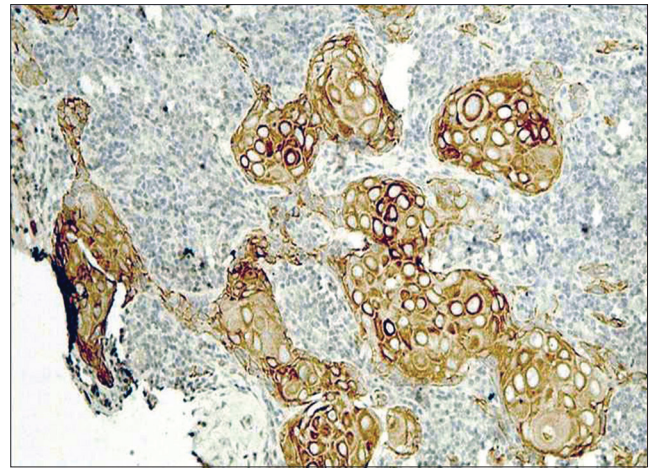


Figure 3: Carcinomatous cells in lymph nodes when incubated with pancytokeratin antibody (IHC stain, $\times 100$)

sensitivity is even worse, with reports from 38% to 47%.^[3,11] According to Ferlito *et al.* (2000), selective neck dissection in HNSCC provides important information concerning the prognosis, rendering minimum rate of recurrence and aiding in therapeutic decisions.^[12]

A single 5- μ m section from 1-cm lymph node samples only 1/2000th of that node.^[12] So, micrometastasis present at the time of primary diagnosis is, therefore, often missed by conventional method. In relation to this limitation, it has been stated that in practice, multiple sections of nodes which are negative or contained only micrometastasis on initial assessment rarely revealed further tumor deposits.^[13] In this study, a single node from one patient showed positivity following step-serial sectioning method which was first termed negative in frozen section. Thus in our study, the sensitivity of step-serial sectioning H and E staining was 57% and 54% by frozen section analysis. Immunohistochemistry has been introduced in the hope to increase the detection rate of micrometastasis in histologically involved nodes by the use of antibodies against epithelial cell (cytokeratin) like pancytokeratin (5,6,8,17,19), anticytokeratin (AE1, CAM 5.2), Ber-EP4 or tumor-associated (e.g. p53, k-ras, etc.) antigens.^[14] With this technique, lymph node micrometastasis is thought to be detected in upto 45% of node-negative patients with breast cancer, non-small-cell lung cancer and esophageal carcinoma.^[14] In this study, the sensitivity of detection of IHC was same as that for frozen section analysis and approximately equal to step-serial sectioning except for a single node. The reason for this may be that the step-serial sectioning method used in this study has a chance of detecting micrometastasis at 100 μ m, as compared to IHC. It is stated that a group of 10 average tumor cells having a diameter of about 32 μ m, will always be found with a step size of 36 μ m or every ninth section^[15] and in our study we have used serial sectioning at step size of 100 μ m i.e. 88 μ m tumor cells should be detected more efficiently at every twenty-fifth section as compared to a single section of IHC. This is well supported by authors

like Woolgar, Czerneki and Van Brekl *et al.*, who in their experiments have stated the importance of serial sectioning in detection of micrometastasis as compared to IHC.^[13,16]

RT-PCR analysis can detect a single cancer cell among 10^6 to 10^9 background cells while IHC can detect a single cancer cell in 10^5 background cells and H and E staining a single cancer cell among 104-105 cells in the background.^[17-19] Various molecular markers, particularly CK mRNAs, have therefore been investigated in order to detect the presence of occult tumor cells in lymph nodes.^[8] The detection rates for CK19 have been in the range of 9-67%, therefore we consider that CK19 mRNA is a reliable marker for carcinomatous cell detection in HNCC as in other carcinomas.^[5,14,20]

Lymph nodes surgically isolated from patients of non-malignant tumor such as ameloblastoma were used as a negative control for our analysis and did not find any expression of CK19 in these nodes. This proves the reliability of our primer design in detecting true expression of CK 19mRNA. This is in agreement with the studies of Nogochi *et al.*, and Schoenfeld *et al.*, who did not get any signals from the control group and normal lymph nodes.^[2,21]

All nodes detected positive by frozen section H and E staining were also positive by IHC and RT-PCR analysis and also the one node detected positive by step-serial sectioning H and E staining was positive for RT-PCR. But in addition, we detected 17% more nodes showing positive expression for CK 19mRNA by RT-PCR increasing the detection rate upto 42% by RT-PCR. This states that 17% of lymph nodes given negative by intra-operative frozen section H and E staining, step-serial sectioning H and E staining and IHC together were detected to have micrometastases by RT-PCR which was not detected by any of the other methods. This might be because of the highest sensitivity of RT-PCR as compared to the rest of the techniques. Interestingly, five patients reported back for recurrence within 6 months of surgery. Among these patients, three patients had level I, II and III lymphnodes positive by the all four methods and two patients showed positive detection for level I and II by RT-PCR only. Prognosis was poor for patients given positive only by RT-PCR, among which two patients died in a period within 6 months.

The accuracy rate of step-serial sectioning was found to be 24% as compared to 23% by intra-operative frozen section H and E staining and IHC. This is very well in range as compared to the 20% accuracy rate for serial-step sectioning H and E method in oral squamous cell carcinoma 5-20% accuracy rate for IHC in HNSCC and a bit less as compared accuracy rate for IHC in HNSCC and slightly lesser as compared to carcinoma.^[3,5,13]

Considering RT-PCR to be the gold standard, the sensitivity of intra-operative frozen section analysis and IHC was 54% and for serial section H and E staining, it was 57%, a comparable study in breast carcinoma showed sensitivity of 89.5% by

frozen sections and 85.7% by permanent serial sectioning, respectively.^[22]

In our study, 53% patients with micrometastasis detected by RT-PCR had large T3/T4 tumors while only 26% patients detected by other three methods showed the same observations. There was no discernable correlation found between the sizes of the primary tumor and histological grading of the tumor. This does not allow any firm conclusions to be drawn concerning the relationship between the clinical features of the primary tumor and the pathologic status of the metastatic nodes. The small sample size may be the reason for the same.

Our study demonstrated that RT-PCR is far more sensitive in detection of micrometastasis than any other technique used in routine procedures and immunohistochemistry. Recently, automated PCR which can serve the purpose in the cryosection room are also available. Ferris *et al.*, showed the feasibility of automated, intraoperative staging of cervical lymph nodes and the possibility that such an approach may eventually prove superior to conventional pathology.^[3] This type of method should be used in institutions to reassess lymph node status in relation to occult lymphatic spread in patients with HNSCC.

ACKNOWLEDGMENT

I am grateful to Dr. Tapan Chakrabarti, acting director, NEERI, for his wholehearted support for this study.

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How to cite this article: Thakare E, Gawande M, Chaudhary M, Seralathan M, Kannan K. Detection of micrometastasis in lymph nodes of oral squamous cell carcinoma: A comparative study. *J Oral Maxillofac Pathol* 2013;17:374-80.

Source of Support: Nil. **Conflict of Interest:** None declared.