ISSN: 2581-5989
PubMed - National Library of Medicine - ID: 101738774

International Journal of Dental Science and Innovative Research (IJDSIR)

IJDSIR : Dental Publication Service

Available Online at: www.ijdsir.com

Volume – 4, Issue – 4, August - 2021, Page No. : 277 - 284

Detection of micro metastasis in histopathologically negative nodes of Oral Squamous Cell Carcinoma using pan cytokeratin

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Citation of this Article: Mulla A F, Shah A A, Ingle Y , Vibhute N A, Srivastava S R, Bussari S, "Detection of micro metastasis in histopathologically negative nodes of Oral Squamous Cell Carcinoma using pan cytokeratin", IJDSIR- August - 2021, Vol. – 4, Issue - 4, P. No. 277 – 284.

Type of Publication: Original Research Article

Conflicts of Interest: Nil

Abstract

Introduction: Cervical lymph node metastasis is of the most important prognostic factors in patients with OSCC, particularly with N0 neck. Cancer staging tends to be underestimated if this micrometastasis remains undetected by H & E staining which may be associated with recurrence or distant metastasis resulting in a poor prognosis.

Objective: To detect occult metastasis in histopathologically negative lymph nodes of oral squamous cell carcinoma (OSCC) patients using Pan-cytokeratin (pan-CK) antibody.

Material & Method: Formalin fixed paraffin embedded tissues of histopathologically negative lymph nodes (195) were retrieved from 35 patients of OSCC. All the slides were subjected to immunohistochemistry using pan-CK antibody with appropriate controls.

Results: Out of total 369 LNs, metastasis was observed in 174 (47.2%) lymph nodes. Occult metastasis was observed in 12 (6.2%) lymph nodes from 5 (14.3%) cases resulting in upstaging of these cases.

Conclusion: pan-CK helps in detecting occult metastasis in histopathologically negative nodes of OSCC cases. Inclusion of pan-CK assessment of LNs in day-to-day practice is warranted.

Keywords: Squamous Cell Carcinoma, Metastasis, Immunohistochemistry, Lymph Node, Pan-cyokeratin

Introduction

Cervical lymph node metastasis is considered as one of the most important prognostic factors in patients with oral squamous cell carcinoma (OSCC). Clinical lymph node

metastasis or macrometastasis is considered as one or more palpable and fixed cervical nodes in patients with HNSCC which can be identified either by the surgeon on physical examination or by the radiologist via imaging studies.[1]

Micrometastases, are deposits which are tinier than the established occult metastases therefore, which are likely to be missed in the initial examination of routine hematoxylin and eosin (H & E) – stained slides. To overcome such limitations and to detect lymph node micrometastasis, several methods have been investigated which include immunohistochemistry (IHC) and molecular biology techniques like polymerase chain reaction etc.[1-3]

Cytokeratins forms a vital constituent of the cytoskeleton of both normal and malignant epithelial cells. Since it is usually absent in any types of cells constituting the lymph nodes except during development of a tumor metastasis. Therefore, positive expression of cytokeratin in a lymph node is considered to be metastasis of a tumor. [4]

The pan-cytokeratin AE1/AE3 (PanCK) is a combinationl of antibodies that includes a wide range of cytokeratins of different molecular weights and is shown to be very useful in detecting micro metastases. Thus, Immunohistochemical staining using PanCK can be a more effective method in identifying nodal micro metastasis even in lymph nodes which fail to demonstrate histological evidence by conventional methods. Search of literature revealed only few researches in this direction and therefore the need of the study was identified. [5]

Material & Methods

Thirty five patients clinically diagnosed and histopathologically confirmed as primary OSCC were considered for the study. Based on the TNM staging, patients with only T1N0M0 and T2N0M0 were selected and those who had undergone any form of treatment for OSCC (chemotherapy, radiotherapy etc) were excluded. As part of the surgical protocol, the patients underwent supra-omohyoid neck dissection involving the cervical lymph nodes (CLN) of levels I, II, and III.

For routine examination, the specimens were fixed in 10% neutral buffered formalin. All the lymph nodes were cut in half and were then processed for routine histologic examination. The diagnosis was based on microscopic examination of 2 – 3 sections of 4μm thickness from each node stained with H & E. Subsequently, to detect micro metastasis the nodes which were diagnosed as histopathologically negative on routine H&E staining were further subjected to Immunohistochemical staining using pan-cytokeratin antibody (AE1/AE3) (DAKO Corporation, CA, USA).

Identification of Occult Metastasis

Two observers evaluated the immunostained slides. The possibility of false positive results was eliminated as inter-observer agreement regarding the presence of occult metastasis was 100%. On detection of one or more strongly immunoreactive epithelial cells either in the capsule, sub capsular sinus or in the cortex of the lymph node, cases were scored as positive for occult lymph node metastasis (LNM). The identification of occult metastasis was done in accordance to the criteria given by the College of American Pathologists, which suggested that IHC positive cells should be both visualized and confirmed on the H&E–stained section to definitely qualify as micrometastasis.[6] All the IHC positively stained cells were further confirmed to be cytologically atypical epithelial cells (neoplastic cells) showing enlarged nuclear size and apparent increased nuclear:cytoplasmic ratio, therafter they were finally labelled as occult LNM. Further, the largest diameter of the occult metastasis foci was microscopically measured with a standardized measuring ocular device by two

observers individually in order to further categorize occult LNM as isolated tumour cells (ITC) and micrometastasis according to the criteria given by Hermanek[7]. **[Table 1; Fig.1 a & b].**[7]

Category	Size of Tumour Deposit
Isolated Tumour Cells (ITC)	< 0.2 mm
Micrometastasis	>0.2mm but < 2mm

Table 1: Categorization of Occult Metastasis

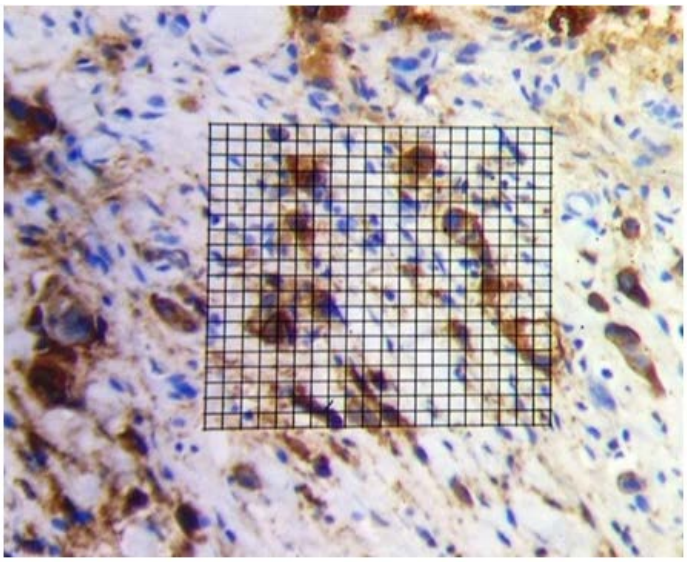

Fig. 1 a

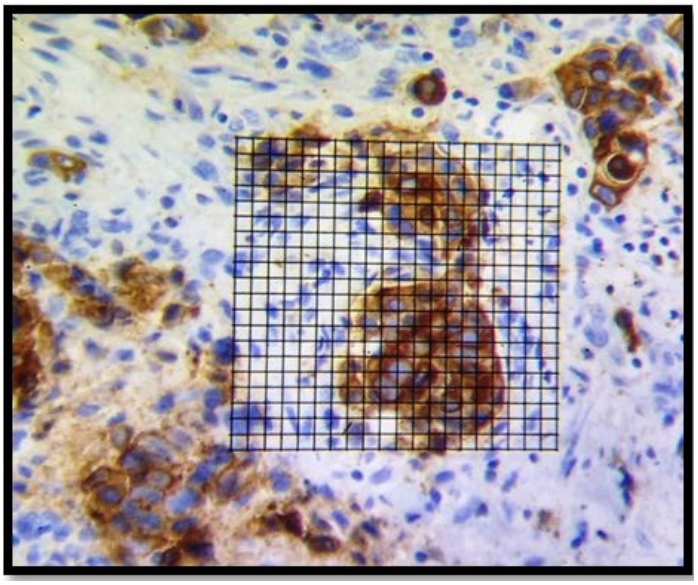

Fig. 1 b

Results

The clinic-pathological distribution of all the cases is summarized in Table 2. SOHND was done for all the cases and a total of 369 CLNs were obtained with 104 (28.2%) LNs being from Level I, 152 (41.2%) LNs from Level II and 113 (30.6%) LNs from Level III. All the LNs were subjected to routine H & E staining and a total of 174 (47.2%) LNs were positive for metastasis.

The remaining 195 (52.8%) LNs which were negative for metastasis on routine staining were subjected to IHC staining using PanCK antibody.(Fig.2) Occult metastasis was observed in 12 (6.2%) LNs from 5(14.3%) patients which were further categorized as 1 (8.3%) from Level I, 9 (75%) from Level II and 2 (16.7%) from Level III. Further,off the 12 lymph nodes which were IHC positive, isolated tumour cells were found in 3 lymph nodes (25%) whereas micrometastasis was detected in 9 cases (75%).

Discussion

The status of regional lymph nodes (RLNs) is of paramount importance in determining prognosis in patients with HNSCC and the presence of regional metastasis worsens the prognosis and greatly reduces the rate of their disease – free survival. For assessing the extent of loco – regional disease, clinicians depend on both physical examination and imaging methods pre – operatively. It is also observed that clinical evaluation in the assessment of lymph node status is only partially reliable. The low sensitivity associated with neck palpation is largely dependent on various factors like size and site of the primary tumour, and patient factors like short, obese neck.[8] However, even the most sophisticated techniques for imaging fail to detect occult metastasis.[9]

It has been shown that there is a risk of occult metastasis in patients with primary HNSCC even with cN0 stage. It has been suggested that most of these micrometastases may eventually become apparent if left untreated. Wei et

al and Spiro et al recommended elective SONHD (level I–III) for the treatment of patients of OSCC even with a N0 neck.[10,11] Also, for inclusion of patients in our study with advanced neck disease, it was believed that this could bias the results as these patients already had advanced disease and were more likely to harbor occult metastases. Hence, to avoid heterogenecity in the study population and minimize the bias, we considered patients with only Stage I & II OSCC only.(i.e T1/T2, N0, M0)

The sectioning protocol for assessment of the entire node is extremely laborious and not very cost effective, further it is also essential to strike a balance between a vigorous technique and manageable workload so that it is practically feasible. Studies have shown that there is a little advantage of serial sectioning over the one section method because of the limited positivity and identical results obtained by both techniques.[12] Also, the number of sections to be studied for detection of LNM from each lymph node, is not specified by the UICC.[13] Thus, it is evident that an optimum balance is needed between detection of sensitivity & specificity, histopathological workload and cost factor involved[14]. Thus, considering the lack of consensus and practical limitations, after splitting the lymph node into 2 halves, sections were made and stained for both H & E and IHC staining to detect LNM.

A total of 369 LNs were harvested from these patients and on routine H&E examination, metastasis was observed in 47.2% LNs. 195 LNs which were found negative on routine H&E examination were further subjected to IHC staining using pancytokeratin antibody (AE1/AE3). IHC staining revealed micrometastasis and ITC's in 6.2% LNs from 5/35 cases (14.3%), which was in accordance with the previous studies.[15-18]

When the occurrence of occult metastasis was correlated with the clinico - pathological features of the primary tumour (Table 3), it was observed that occult meatstasis occurred mostly in males (80%) and the average age of the cases with micrometastasis/ITC was 50.4 years which was slightly less as compared to the average age group of the cases with established metastasis. This was similar to the finding by **Woolgar (1999).**[19] The possible explanation for the decrease in the average age for these cases could be that micrometastasis must have been present for a limited period of time and additionally strong immune system of younger patients favours dormancy or cell destruction.[20]

It was observed that in our study the predominant site of the primary tumour in cases of occult metastasis was the tongue and buccal mucosa. The primary tumour showed a predominantly infiltrating pattern. Our study also showed that 60% of the cases showing pathologic occult metastasis were T2N0M0 (Stage II).

Off the 12 lymph nodes showing occult metastasis on IHC staining, micrometastasis was found in 9 (75%) and ITC's were seen in 3 (25%) LNs. The foci of micrometastasis were 1 – 1.5 mm in diameter and were mostly multifocal whereas the ITCs were found in clusters no larger than 0.2 mm in largest diameter, showing a predominantly dispersed pattern within the LNs. (Table 3)

Within the LNs, occult metastases was located in the subcapsular sinus in 25% LNs, within the superficial cortex in 33.33% LNs and in the deeper cortex in the remaining 41.67% LNs, which was in accordance with the findings of Woolgar J A (1999)[19] (Table 3). Anatomy, prolonged intracapsular course and the abundance of valves in the juxtacapsular channels of the afferent lymphatic channels possibly explains the location of micrometastases / ITCs within sinuses at the periphery of the node. Thus, growth of a metastatic focus lodged within a capsular/ juxtacapsular channel or within the peripheral sinus may lead to early extracapsular spread.[21]

The distribution of occult metastasis across the different clinical levels of lymph nodes in this study was shown to

be statistically significant. (p-value < 0.001) with 75% of micrometastasis occurring in the Level II group of lymph nodes which was in accordance with the findings of Ambrosch et al. (1995)[21] and Ambrosch and Brinck (1996)[22] and Yoshida K.et al. (2005).[23]

The identification of micrometastasis by more sensitive techniques like IHC, needs a review for the current staging classifications of the UICC in HNSCC. Although the micrometastases must be less than 0.2 mm in greatest dimensions to be classified as pN1mi rather than N1, it is the upstaging of micrometastasis from N0 to N1mi that needs to be answeresd.[23] Prognosis may be different for these patients than with established metastasis, which is classified as N1 in the current UICC classification. In case of breast cancer and malignant melanoma, detection of micrometastases resulted in revised upstaging of nodal status to N1(mi). However, adoption of this nomenclature by the UICC for the revised upstaging of HNSCC remains awaited.[22, 24]

In our study results revealed an upstaging of 14.3 % cases to pN1(mi) which was statistically significant (p-value <0.001) and was in accordance with studies done by Hamakawa et.al (2000)[13] and Barrera et.al (2003)[16] where pN upstaging was done in 12.3% and 29% patients respectively.

The independent prognostic significance of the isolated tumor cells remains to be proven.[7] It is generally agreed that micrometastases/ITC's do not necessarily form metastatic foci, and most of the occult metastases at the single-cell level are eliminated by the immune defense mechanism.[25] However, some investigators reported that they do have proliferative potency and observed the formation of metastatic foci in their experiments.[26] Therefore, it may be imperative to detect actual micrometastases and to clarify their clinical significance. However, it has been suggested that HNSCC patients with detectable micrometastasis represent a subset of patients who should be followed up meticulously for possible recurrence or distant metastasis.[8]

Thus, although, the results for IHC staining in the present study were not statistically significant, the detection of occult metastasis cannot be ignored as there is a possibility that these foci of micrometastasis may progress to overt metastasis eventually leading to recurrence and poor prognosis.

Lymph node ratio (LNR) is one of the most valuable prognostic factor for risk stratification in patients with oral squamous cell carcinoma (OSCC). It represents the fraction of metastatic nodes among all harvested nodes thus, actually determining the degree of cancer spread and clearance. In the present study, 3 patients had LNR ≥ 0.25 (> 2.5%). These patients were lost for follow-up.A study done by Ebrahimi A et.al (2010)[27] showed that node positive patients with LNR < 2.5% had similar outcomes to patients shown to be node negative and were thus grouped into the low-risk category and patients with an LNR of 2.5% to 7.5%, 7.5% to 20%, and >20% had 2.6, 3.7, and 4.4 times the risk of death from OSCC, respectively, when compared with patients with an LNR <2.5%. [28] It has been strongly suggested that LNR should be considered as an independent prognostic factor in OSCC and could be used in conjunction with the current TNM staging to determine the prognosis and selection for adjuvant therapy.[27]The association between increasing LNR and both regional and distant metastasis has definite implications for adjuvant therapy that merits further study.[28]

Some most commonly encountered problems with IHC staining are cross-reactivity with other antigens and false positivity. Hence, application of highly sensitive molecular assays such as for comparing and better detection of occult metastasis can be considered.

In future studies, the relation between clinico–pathological parameters of primary tumour like depth of invasion, lymphatic vessel invasion, neural invasion and lymph node micrometastases should be considered and the risks of micrometastases should be investigated in a larger sample size, with better clinico – pathological co – relation and longer follow up in order to establish an appropriate treatment plan. Also, the lymph node ratio estimation should be evaluated further to be considered as an important independent prognostic indicator to determine survival rate and loco – regional recurrence in OSCC patients. Further research is also necessary to establish means to distinguish true micrometastasis which are capable of seeding tumour deposits at a new site from those with the genotypic and phenotypic characteristics that will not support progression. Besides, a larger sample size coupled with highly sensitive molecular assays like PCR based assays, reverse transcriptase-(RT) PCR may give us better insight in identifying occult metastasis.

Conclusions

The subject of micrometastasis in HNSCC is intriguing. The introduction of more sensitive techniques like immunohistochemistry to identify occult metastases in the LNs can be used to refine the staging system, resulting in upstaging of a significant percentage of cases. Since metastatic status of the LNs is a decisive factor for the necessity of post – surgical adjuvant therapy, detection of micrometastasis may provide a basis for the improved planning of post-operative therapy, as these patients could have a higher risk of tumour recurrence.

It may be practically impossible to do these studies on each and every lymph node. However, considering the impact that upstaging may have on the treatment and prognosis, this technique may be justified. Therefore, it would be advisable to use the immunohistochemical staining method as a routine diagnostic tool for early metastatic cancers specifically in which the LNs are negative in routine H&E staining and also for determination of the lymph node ratio, which in recent times has been shown to be an important independent prognostic indicator in OSCC patients.

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