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Identification of Micrometastasis using Papanicolaou Stain in Nodal Tissues of Oral Squamous Cell Carcinoma - A Retrospective Study

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Abstract

Metastasis is innate tendency of malignancy. Lymphatic spread plays a crucial role in the progression of Head and Neck Squamous Cell Carcinoma and significantly influences tumor staging. The presence of regional lymph node involvement reduces survival rates by nearly half. The presence of single micrometastasis affects the prognosis, recurrence and overall survival rate of the patient. Macrometastasis is the nodal deposit examined by surgeon on clinical examination or by radiologist in the imaging studies and can be routinely diagnosed in Hematoxylin and Eosin staining. But, micrometastasis cannot be determined easily by clinical and radiographic examination. Even in Hematoxylin and Eosin staining, micrometastasis is often obscure. The current research focuses on identifying the efficacy of special stain Papanicolaou to detect the micrometastasis in lymphnode, which was undetected in Hematoxylin and Eosin staining. The study included 432 lymph nodes obtained from 40 patients diagnosed with Oral Squamous Cell Carcinoma. Hematoxylin and Eosin staining was done and 100 lymph nodes were proven non-metastatic. Modified Papanicolaou stain was used for identification of micrometastatic deposit. Out of 100 Hematoxylin and Eosin stained non-metastatic lymph nodes, Papanicolaou stain was useful in detecting micrometastasis in 9 lymph nodes. This accounted to 9% of non-metastatic lymph node sections used in our study. Papanicolaou staining has shown greater effectiveness than routine Hematoxylin and Eosin in detecting micrometastasis, while also being cost-effective, widely accessible, and less technique-sensitive.

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Introduction

Oral squamous cell carcinoma (OSCC) is eleventh most common cancer worldwide, with increased incidence reported in Indian sub-continent. Approximately 94% of all oral malignancies are squamous cell carcinoma.[1] In India, oral cancer is the most common cancer among men and the third most frequent among women in several regions.[2] Despite progress in treatment methods and understanding of the molecular basis of the disease, survival rates remain largely unchanged and have remained in range of 50 to 59% over last several decades. Therefore, early diagnosis and prevention are essential for improving patient outcomes.[1]

Predicting the prognosis of Oral Squamous Cell Carcinoma (OSCC) remains challenging, despite advances in diagnostic techniques and oncological therapies. Nearly half of the affected patients succumb within the first two years following diagnosis. Prognostic outcomes are influenced by several variables, including patient-related factors such as age and gender, as well as tumor-related characteristics like size, location, histopathological grade, and metastatic potential. Among these, lymphatic metastasis is regarded as the most critical determinant of patient outcome in OSCC. The development of cervical nodal metastases reduces the five-year survival rate of individuals with squamous cell carcinoma of the upper aerodigestive tract by approximately 50%.[3]

Metastasis is an inherent characteristic of malignant tumors.[5] In Head and Neck Squamous Cell Carcinoma, lymphatic spread represents the most significant pathway of dissemination and serves as a key determinant of tumor staging.[4] The likelihood of nodal involvement is largely influenced by the size and anatomical location of the primary lesion. The presence of regional lymphadenopathy is associated with nearly a 50% reduction in survival rates.[4]

Macrometastasis refers to nodal deposits that can be identified either through clinical examination by the surgeon or via imaging studies conducted by the radiologist. However, Micrometastasis cannot be determined easily by clinical and radiographic examination. In Hematoxylin and eosin staining, Micrometastasis is often obscure.[5] The presence of single Micrometastasis affects the prognosis, recurrence and overall survival rate of the patient. Approximately, 10% of patients with histologically proved non-metastatic lymph nodes showed recurrence suggesting the importance of Micrometastasis in prognosis.[6]

The present study aims at identifying the significance of special stain Papanicolaou to detect the Micrometastases in lymphnode, which were undetected in Hematoxylin and Eosin staining.

Materials and Methods

A total of 432 lymph nodes from 40 patients with Oral Squamous Cell Carcinoma (OSCC) were examined. Hematoxylin and Eosin (H&E) staining confirmed 100 non-metastatic lymph nodes. For comparison, 200 lymph node sections were analyzed—100 stained with H&E and 100 with modified Papanicolaou (PAP) stain.

The study utilized paraffin-embedded tissue blocks of 100 reactive, non-metastatic lymph nodes obtained from OSCC patients who underwent elective or radical neck dissection. Lymph nodes from Levels I, II, and III were included. All 100 H&E-stained sections showed no evidence of metastasis. These sections were then subjected to modified PAP staining to evaluate micrometastasis.

Hematoxylin and Eosin (H&E) Staining Protocol

1. Fix tissues in 10% formalin, process, and embed in paraffin.
2. Cut sections at 4 µm thickness.
3. Deparaffinize in xylene and hydrate through graded alcohols (100%, 90%, 80%, 70%).
4. Stain with Hematoxylin for 3–5 minutes.
5. Wash in running tap water until sections appear blue (≤5 minutes).
6. Differentiate in 1% acid alcohol (5 minutes).
7. Wash, then blue in alkaline solution (e.g., ammonia water), followed by tap water rinse.
8. Counterstain with 1% Eosin Y (10 minutes).
9. Wash in tap water (1–5 minutes).
10. Dehydrate through ascending alcohol grades, clear in xylene, and mount with DPX.

Modified Papanicolaou (PAP) Staining Protocol

1. Dewax sections and hydrate to water.
2. Stain with Harris Hematoxylin (6 minutes).
3. Rinse twice in tap water.
4. Differentiate in 1% acid alcohol.

5. Rinse thoroughly in tap water.
6. Dip in ammonia water, then wash in running water (10 minutes).
7. Rinse in distilled water (5 minutes).
8. Stain with Eosin Y, rinse in distilled water, and dehydrate.
9. Stain with Orange G6 (4 minutes), rinse in 95% alcohol.
10. Stain with Eosin Azure (EA) (4 minutes).
11. Rinse in 95% alcohol (two changes).
12. Dehydrate in absolute alcohol, clear, and mount.

Results

Out of 100 Hematoxylin and Eosin stained non-metastatic lymph nodes, Papanicolaou stain was useful in detecting micrometastasis in 9 lymph nodes. This represented 9% of the lymph node sections without metastasis that were included in our study.

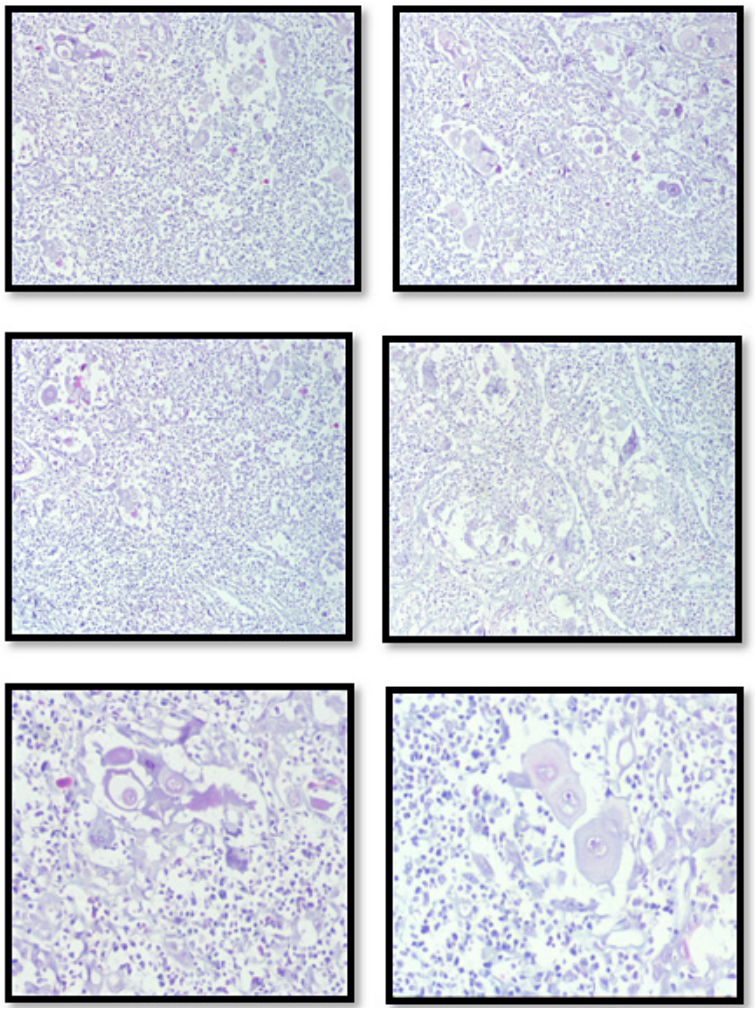

Fig. 1: Micrometastasis in Lymph Nodes

Discussion

According to the Union for International Cancer Control (UICC), metastatic deposits measuring between 0.2 mm and 0.2 cm within the sectioned area are classified as micrometastasis.[7] Black *et al.* further described micrometastasis as tumor deposits involving less than 20% of the sectioned area.[8] Only a limited number of studies have investigated the presence of micrometastasis in cervical lymph nodes associated with oral squamous cell carcinoma.[9]

The addition of phloxine B on papanicolaou stain to demonstrate keratin in tissue sections was repoted by Elzay RP.[11] In the study of comparing the efficacy of Haematoxylin and Eosin stained tissue sections with Papanicolaou stained tissue sections by Preethi S *et al.*, Papanicolaou staining has been shown to highlight the morphological characteristics of keratinization with greater clarity.[12,13]

Micrometastasis was detected effectively by Papnicolaou stain compared to Hematoxylin and Eosin staining. This study highlights that Papanicolaou staining is a cost-effective, easily accessible, and routinely applicable method that demonstrates higher sensitivity in detecting micrometastatic deposits.[14] It may be effectively utilized as a complementary technique to the widely used immunohistochemical marker, Pancytokeratin, for the evaluation of lymph nodes in oral squamous cell carcinoma.[15] Employing Papanicolaou stain for all lymph node sections, rather than conventional Hematoxylin and Eosin, facilitates more reliable identification of micrometastasis. Subsequently, only the suspected sections need confirmation with Pancytokeratin, eliminating the need for immunohistochemical analysis of every node. This strategy allows for the detection of even isolated micrometastatic foci in a cost-efficient manner, thereby offering additional prognostic insights by identifying tumors with a greater propensity for regional lymph node involvement.

Conclusion

Although, 9% of the cases proved to be positive for micrometastasis, the study population is small to come to a conclusive diagnosis. Future studies with an expanded population base are essential and comparing PAP stain with pan cytokeratin marker should be done, so that, in near future, PAP stain could be considered as important adjunct to IHC for detection of Micrometastasis in regional lymph nodes.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

Ethical approval was obtained by GITAM Dental College and Hospital Ethical committe.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Author Contributions

- **Hyandavi Balla:** Conceptualization, Methodology, Writing – Original Draft.
- **Divya Uppala:** Supervision.
- **Sreekanth Kotina:** Data Collection, Validation, Analysis.
- **Sahithi Pamidimukkala:** Visualization, Writing – Review & Editing.
- **Tejaswi Kodem:** Writing – Review & Editing.

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