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Stromal Desmoplasia as a Predictor of Regional Lymph Node Metastasis in Histological Grades of Oral Squamous Cell Carcinoma- A Polarized Microscopic Study

1. Dr. Snehal Diwate

PG student, Department of Oral Pathology and Microbiology, MGV's KBH Dental College and Hospital, Nashik, India

2. Dr. Aarti Mahajan

Professor and HOD, Department of Oral Pathology and Microbiology, MGV's KBH Dental College and Hospital, Nashik, India

3. Dr Nilima Prakash

Professor, Department of Oral Pathology and Micrology , MGV'S KBH Dental College and Hospital, Nasik, India

4. Dr Pradeep G L

Professor, Department of Oral Pathology and Microbiology, MGV'S KBH Dental College and Hospital, Nasik, India

5. Dr Gauri Deshmukh

PG Student Department of Oral Pathology and Microbiology, MGV'S KBH Dental College and Hospital, Nasik, India

6.Dr. Vaibhavi Salunkhe

Lecturer, Department of Oral Pathology and Microbiology, MGV'S KBH Dental College and Hospital, Nasik, India

Corresponding author-. Dr. Aarti Mahajan

Professor and HOD, Department of Oral Pathology and Microbiology, MGV's KBH Dental College and Hospital, Nashik, India

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KEYWORDS

oral cancer, stromal desmoplasia, Picrosirius red, nodal metastasis, polarized microscopy

ABSTRACT:

Background: The tumour stroma, including ECM and stromal cells, contributes to OSCC progression through desmoplasia, marked by increased collagen deposition. Picrosirius red staining under polarized light distinguishes collagen maturity, offering insights into stromal remodeling and its link to tumour invasiveness and lymph node metastasis.

Methods

A cross-sectional analytical study of 31 OSCC cases used Picrosirius red staining to assess stromal desmoplasia. Collagen birefringence patterns were analysed in relation to histological grade and lymph node involvement.

Results

Collagen birefringence colour was significantly associated with histological grade ($p = 0.05$). Green-yellow was observed in 50% of node-positive cases, while red-orange (42%) and yellow-orange (38%) predominated in node-negative cases ($p = 0.08$)

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INTRODUCTION

Oral squamous cell carcinoma is the world's sixth most prevalent cancer and has a 5-year survival rate of 50%-60%.[1]The number of new cases of oral cancer in India in 2020 was 135,929, establishing it second in the world.[2] The treatment of OSCC includes surgery with or without radiotherapy or chemoradiotherapy.

The connection of the stroma and the tumour cells and the stroma's properties to the invasive malignant epithelial cells are important. Desmoplasia, angiogenesis, and inflammatory cell infiltration are cancer-related stromal changes. The increased production of collagen and extracellular matrix proteins by stromal cells is known as desmoplasia. It is thought to be the host tissue's reaction and defence against invading cancer cells. The collagenous tissue, the primary component of the stroma, plays an important role in the evolution and progression of cancer.[3]

Malignant epithelial cells have received the majority of attention in cancer research; nevertheless, stromal cells and other elements of the tumour microenvironment have been shown to play a significant role in the invasion and metastasis of numerous cancers.

Picrosirius red (PSR) is a strong anionic dye that stains collagen in connective tissue. The sulfonic acid groups of PSR react with the primary groups of collagen molecules. The elongated PSR molecules adhere to the collagen fibres with lengthy axes organized parallel to them. This parallel association between dye and collagen results in an enhanced birefringence.[4]Picrosirius red can detect thin fibres and differentiate mature and immature fibres under polarized microscopy. Although this special stain is frequently used, it is seldom utilized to the fullest extent.[5]

The prognosis of OSCC will be improved by early detection and prevention of lymph node metastases, thus it is clinically important to identify histopathologic criteria that can accurately evaluate immune response. A clear prognostic, informative biomarker is required since it will serve as the foundation for specific treatment.

Materials and Method

This cross-sectional analytical study, approved by the institutional ethics committee (MGV/KBH/DC/OP/41/22) was conducted in the Department of Oral Pathology and Microbiology. Thirty-one randomly selected OSCC cases of varying histological grades, with and without lymph node metastasis, were included. Age, sex, site, tumour size, and stage according to TNM AJCC were among the clinical data that were noted. A soft-tissue microtomy was performed on tissue blocks embedded in formalin-fixed paraffin to yield tissue specimens that were 5 µm thick and stained with H&E and Picrosirius Red (Sirius red F3B in saturated picric acid).[5] Stromal desmoplasia was assessed by evaluating collagen birefringence under polarized light in five fields per case.

Collagen was categorized based on the predominant birefringence colour:

Category 1 (reddish/reddish-orange),

Category 2 (yellowish/orange/yellowish-orange), and

Category 3 (greenish-yellow/greenish).

Statistical Analysis

Analysis using SPSS version 22.0 were conducted. Chi-square tests were used to assess the significance of the study's components. ANOVA and t-tests was used to compare averages.

All statistical tests were performed at 95% confidence intervals; keeping p value of less than 0.05 or equal to as statistically significant.

Results

Among the 31 cases analysed, 90% of patients were over 45 years old, with a male predominance of 84%**.** The site distribution indicated that 84% of cases involved the buccal mucosa, while the remaining cases affected the lip (3%) and tongue (13%)(**Pie Chart 1**).According to histological grades, 16% were well-differentiated, 68% moderately differentiated, and 16% poorly differentiated(**Pie Chart 2)** . Nodal metastasis revealed 39% of cases were node-positive, while 61% were node-negative. Chi-square tests showed a significant association between polarizing colours and histological grades (p=0.05), but no significant association between fibre arrangement and histological grades (p=1)(**Table 1)**. In terms of nodal metastasis, the distribution of polarizing colours for node-positive cases was 10% red-orange, 40% yellow-orange, and 50% green-yellow. Node-negative cases showed 42% red-orange, 38% yellow-orange, and 20% green-yellow, with no

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statistically significant association (p=0.08)**(Graph 1).** For fibre arrangement, node-positive cases had 3 with parallel and 7 with unparalleled fibres, while node-negative cases had 11 parallel and 10 unparalleled. This analysis yielded no significant association. (p=0.24) **(Graph 2)** Finally, polarizing colour distributions varied by nodal status, with different frequencies observed across categories (N0 to N3). No significant association was found (p=0.7). **(Table 2)**

Discussion

Carcinogenesis involves changes not just to the tumour cells but also to the surrounding tissue.[3]The extracellular matrix of macromolecules gives tissues and cells structural stability. The most prevalent protein in the extracellular matrix is collagen and any modifications to any element of this matrix may cause the physiochemical characteristics of tissues to be disrupted, which could modify the interactions between cells and the matrix as well as the cellular phenotype. Critical interactions between invasive tumour cells and the host's extracellular matrix produce an environment that supports the growth and spread of tumour cells. Malignant tumour cells produce receptors on their surface that are involved in the creation of extracellular matrix molecules.[6]It has been suggested that stromal desmoplasia is a remodelling process in which cancer cells demolish the pre-existing extracellular matrix and causes new matrix creation by stimulating fibroblasts.[7]By altering the microenvironment near the tumours' invasive edge and stimulating development, fibroblasts are thought to contribute to the tumour growth and the spread of disease.[8]In histopathological investigations, Picrosirius red staining is useful in conjunction with polarizing microscopy because it significantly increases the birefringence of birefringent structures.[9]Anisotropic structures can be more easily seen when using polarizing microscopy, as they seem shiny or bright against a dark background. The polarization colours of collagen fibres in the fibrotic process have demonstrated that as the fibres mature, their proteoglycan content changes and they become more dehydrated, which increases the amount of cross-links and stainable side groups and causes the collagen fibres diameter to expand noticeably.[9]

In OSCC, metastases to the cervical lymph nodes are a significant risk factor for both disease-specific and overall mortality as well as increased recurrence.[10]There is a 50% reduction in overall survival associated with the presence of a single metastatic lymph node.[11]Therefore, the proper care of the disease depends on the diagnosis of the existence and degree of lymph node metastases.[12]Therefore, the purpose of our study was to assess stromal characteristics such as desmoplasia using polarized microscopy and correlated with lymph node metastasis which would allow for a better prognostic insight into the biological behaviour rather than looking at tumour cells alone.

In present study, we compared the stromal desmoplasia in histological grades of oral squamous cell carcinoma, by comparing the birefringence colours of collagen. In WDSCC cases, predominantly reddish-orange birefringence there were red-orange (60%) and yellow-orange (40%) cases and the arrangement of fibres was parallel (40%) cases and in most cases it was unparallel(60%) cases. In the MDSCC category, yellow-orange colours were most predominantly observed (42%) and the distribution of the arrangement of fibres was both parallel and unparallel equally in all (50%) cases. PDSCC cases had more cases (80%) with predominant greenish-yellow birefringence and equal cases (50%) showed parallel and unparallel fibre arrangements. Therefore, polarizing colours were significantly associated with the histological grades of OSCC (p=0.05) although there was no statistically significant association of grades and fibre arrangement (p=1) **(Table 1)** The present study showed a difference in the colours of collagen fibres from Red Orange to Green Yellow, mainly surrounding the tumour islands using PSR in the stroma of varying grades of OSCC. Studies by **Aparna et al(2010)[13], Kalele et al(2014)[5], Gopinathan et al(2015)[14], Zainab H et al(2019)[3]** observed a similar change in the colour of collagen fibres in the stroma of various grades of OSCC. Contrary to the results of our study, **Kardam P et al (2016)**[15] found a statistically significant difference in the orientation and packing of collagen fibres in different grades of OSCC.A study done by **Nayak et at (2019)**[16]observed that the fibre arrangements change gradually from WDSCC to PDSCC.

It was observed that the birefringence hue of collagen in node-positive cases was predominantly green-yellow whereas in node-negative cases, red orange and yellow orange colours of fibres were observed (p=0.08) **(Graph**

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1). In the present study, node-negative cases had parallel fibre arrangement whereas node positive displayed mostly unparallel fibre arrangement, (p=0.24). **(Graph 2)** Similarly, **Devendra et al** found no correlation between both the polarizing colours and organization of collagen fibres with nodal metastasis and TNM stages in OSCC.[17]

Contrary to the results of our study, **Manjunatha et al (2015)**[18] in OSCC found a significant change in the arrangement of collagen fibres between early stage and the advanced stage. A strong correlation between collagen fibre abundance in OSCC tissue and tumour development, recurrence, and nodal metastases status has been found reflecting its significant involvement in the carcinogenic process a study by **Li et al (2013)**[19] **Junquiera et al**[20] and **Szendroi et al**[21] have stated that thick fibres exhibit orange to red polarization colour, whereas thin fibres typically display a green to a yellowish hue, suggesting that the thickness of the fibre may be the cause of the colour spectrum. Also a collagen fibre's diameter and packing determine its birefringence colour.[22]A changed internal packing of collagen fibre could be the cause of the colour change as long as the diameter remained unchanged. **Sharf et al**[23] commented nuclear resonance investigations on the physical aggregation of collagen fibres also showed a colour profile of orange to red, which indicated well packed fibres, and green to greenish-yellow, which indicated poorly packed fibres. Although, qualitative collagen analysis can be performed using special stains such Van Gieson's, Masson's Trichrome, and Picrosirius Red, PSR stains thin fibres without fading, unlike other stains.[24] Under a polarized microscope, collagen shows birefringence and distinguishes between procollagens, intermediates, and pathologic collagen fibres.[25] PSR can assess both neo collagen generation and collagen content.[24] Therefore, collagen fibre thickness, maturity, and molecular packing are all determininants of the colour profile and intensity of birefringence. Collagen Type I fibres are dense and show birefringence with longer wavelength polarization colours, such as red orange or yellow, when mature and tightly packed whereas collagen that is thin fibrillar, such as Type III or immature Type I, has a greenish birefringence.[22] In the tumour microenvironment, cancer-associated fibroblasts (CAFs) are one of the most critical cellular elements. These are fibroblasts which are phenotypically altered and actively participate in the process of tumourigenesis, promoting growth and metastasis. They have an “active phenotype” since they produce a variety of contractile proteins with accumulation of α-smooth muscle actin characteristic of myofibroblastic differentiation.[26,27]The CAFs in OSCC with myofibroblastic differentiation are associated with dense collagen deposition and stromal desmoplasia.[28,29]The study we performed analysed the stroma's response in various grades of OSCC and found that predominant polarizing colours were statistically associated with the histological grades of OSCC and node-negative cases had parallel fibre arrangement whereas node-positive displayed mostly unparalleled fibre arrangement. According to Gopinathan et al, the early fibroproliferative response may have caused the shift in fibre thickness with OSCC differentiation.[14]On comparing the polarizing colours with Nodal Status i.e. Intergroup comparison, no significant association was observed (p=0.07).**(Table 2).**This novel, cross-sectional analytical study involved histopathologically diagnosed cases of OSCC cases wherein stromal desmoplasia assessed using Picrosirius red staining under polarized microscopy was correlated with Lymph node status. The use of Picrosirius red and polarized light in this study allowed us to assess the stromal reactions in progressive grades and is a simple, quick, yet a valuable method. The limitations of the study included a small sample size and unequal number of cases in different grades of OSCC. This study is retrospective and was based on a single-institution cohort.

SUMMARY AND CONCLUSION

One important indicator of disease-specific survival in OSCC patients is cervical lymph node metastases. The existence or lack of metastasis in the cervical lymph nodes may have a significant impact on the eventual outcome of oral cancer. A poor prognosis has been attributed to advanced-stage disease and the presence of metastatic lymph nodes.

We found that 60% cases of WDSCC showed predominantly reddish-orange birefringence, 42% cases of MDSCC showed predominant yellow-orange birefringence and 80% cases of PDSCC showed Greenish-yellow birefringence**.** There was a statistically significant difference in the polarizing colours among the histological grades of OSCC. It was observed that the polarizing colour of collagen in half of the node-positive

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cases was green-yellow whereas in node-negative cases, there was a predominance of red-orange and showed yellow-orange colours.

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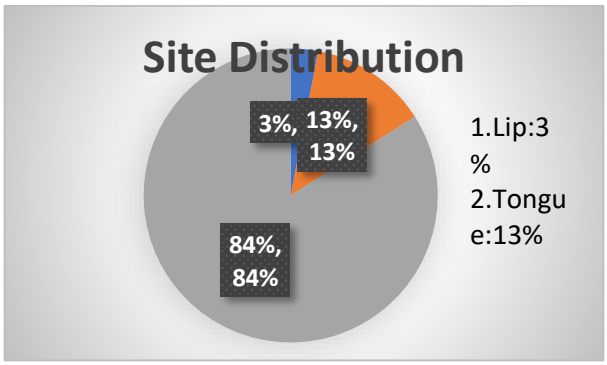
Pie Charts, Tables and Graphs

Pie chart 1: Distribution of the cases according to Site



Pie chart 2: Distribution of the cases according to Histological Grades (Bryne's Grading)

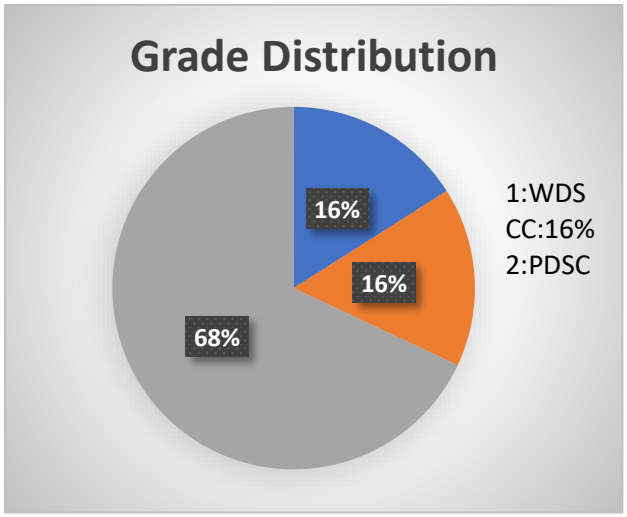


Table 1: Association of Stromal desmoplasia-most predominant polarizing colour and arrangement of fibres with histological grades of oral squamous cell carcinoma using Chi-square test

Grade	Polarizing colour			Arrangement of fibres	
	Red-Orange	Yellow Orange	Greenish Yellow	Parallel	Unparalleled
WDSCC	3	2	0	2	3
MDSCC	7	9	5	11	11
PDSCC	0	1	4	2	2
p-value	p=0.05			p=1	

Table 2: Association of polarizing colours amongst cases of varying Nodal Status using Chi-square test.

Nodal status	Polarizing Colour			Chi-square,p value
	Red Orange	Yellow Orange	Green Yellow	

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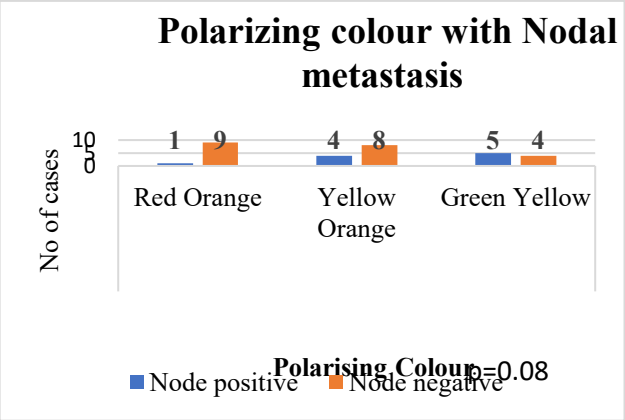
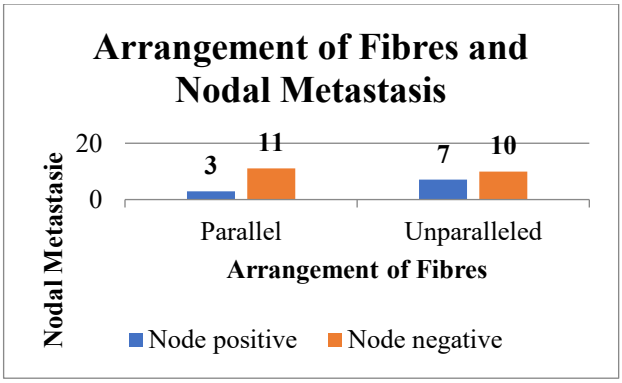
N0	9	9	3	Chi-square=8.7, p=0.7
N1	0	2	1	
N2A	0	0	2	
N2B	0	0	2	
N3	1	2	0	

Graph 1: Association of Most predominant Polarizing colour with Nodal metastasis Using Chi-square test



Graph 2: Association of Arrangement of Fibres and Nodal Metastasis using Chi-square test



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