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Morphological Changes Of Extracellular Matrix In Different Histopathological Grades Of Oral Squamous Cell Carcinoma

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ABSTRACT

Objective: To study the morphological changes in the extracellular matrix of oral squamous cell carcinoma in the various histopathological grades.

Methods: A total of 30 cases of different histological grades of OSCC were selected and graded by both Broder's & Anneroth's grading system. Three sections were made from each paraffin blocks and were stained by H & E stain, Verhoeff's van gieson stain and Masson's trichrome stain. ECM changes were studied as, distribution of elastic, collagen and lymphocytic infiltration.

Result: Statistical analysis of 30 cases of OSCC showed maximum i.e. 17 (56.67%) cases at buccal mucosa. Significant difference between the two histological grading systems of OSCC, Broder's and Anneroth's grading was observed. It was observed that, initial host immune response to tumorigenesis was seen as lymphocytic infiltration, which later lead to collagenolysis in the ECM making elastic fibres more readily visible.

Conclusion: Histochemical studies on ECM of OSCC especially on collagen and elastic using special stains such as Masson's trichrome and Verhoeff's van gieson respectively, are of great value in predicting tumor behaviour. The study indicated that, increased distribution of elastic fibres and collagenolysis preceded by lymphocytic infiltration are important predictive factors to assess the clinical outcome.

Keywords:
Oral squamous cell carcinoma, invasion, metastasis, special stains, histological grading, elastic fibres, extracellular matrix

1. Introduction

Oral squamous cell carcinoma (OSCC) constitutes approximately 94% of all malignant lesions in the oral cavity,(Neville et al.,2002) and because of this great dominance, the term oral cancer is almost synonymous with OSCC(Lihua Liu et al.,2008). Oral squamous cell carcinoma is primarily a disease of epithelial origin and sixth most common intraoral malignancy worldwide. Oral cancer represents the third most common form of malignancy in the developing countries, whilst in the developed countries it is the eighth most common form of cancer. Oral cancer is the commonest cancer in India, which accounts for 50-70% of total cancer mortality (Doshi et al.,2011).

Solid tumors like oral squamous cell carcinoma are composed of two discrete independent compartments, the malignant epithelial cells and the stroma in which they are dispersed. It has also been reported that the ECM (Extra cellular matrix) produced by the transformed cells differs from normal cells. Also there can be an increased production of elastic fibres in OSCC, which can limit the invasion.(Agrawal et al.,2011).The tumor microenvironment (TME) is a pivotal factor in tumorigenesis and especially in tumor progression and the pathogenesis of cancer is largely dependent on its interactions with microenvironmental components. Certain tumor-microenvironment interactions may initiate and drive circular chains of tumor progression–

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enhancing events known as vicious cycles. In a typical vicious cycle the tumor manipulates non-tumor cells in the microenvironment and harnesses them to support its progression.(Isaac P.Witz, 2009). Factors in the tumor microenvironment, such as extracellular matrix(ECM) proteins, growth factors and host immune response, play a role in extension, invasion and metastasis of the tumor. Agrawal et al.,2011 reported that alterations of ECM may play a role in the recurrence and in facilitating the invasion of tumor cells. The matrix acts as a selective macromolecular filter and plays a role in mitogenesis and differentiation. Interactions between normal cells and the matrix may be altered in neoplasia, and this may influence tumor proliferation and invasion.(Lance A. Liotta,1986). Most studies in oncology focus on the tumor grade and stage, lymph node metastases and therapeutic response. Extracellular matrix changes which indicate the propensity of tumor cells to infiltrate and metastasize can be used as one of the prognostic indicators.(Agrawal et al.,2011). Significance of these ECM changes can be studied using the two grading systems –Broder's (most commonly followed) and Anneroth's new system(tumor cell features in addition to the relationship between the tumor and underlying connective tissue are graded).

2. Methodology

Study included 30 cases of oral squamous cell carcinoma(OSCC) reported to the Department of Oral Pathology and Microbiology, Rural Dental College, Loni. Cases were obtained from the archival and also included the cases reported during the study period. Detailed case history of 30 cases of OSCC was noted in relation to age, sex, location of the lesion and the histopathological diagnosis. Specimens with adequate connective tissue thickness were taken for the study. Thirty Formalin fixed paraffin embedded tissue blocks of histopathologically diagnosed as OSCC were selected. Three sections were made from each of the paraffin embedded tissue block. Each of the three section was stained for Hematoxylin and Eosin, Masson's Trichrome and Verhoeff's Van Geison stain respectively as described by Bancroft & Gamble,2002.

1) Staining with Hematoxylin and Eosin (H & E) using routine method given by (Bancroft 2006) was followed. H & E staining was performed to confirm the previous histopathological diagnosis and the grading for OSCC was done by Broder's Grading and Anneroth's grading system for malignancy.

2) Staining of Collagen Fibres was done using Masson's Trichrome method (Masson 1929) & changes in the collagen fibres were graded according to their staining intensity as:

a. Thick & in bundles b. Thin & Separate

3) Staining of Elastic Fibres was done using Verhoeff's Van Gieson method (1908)- Elastic fibers were stained black and were grouped by their distribution and staining intensity as, Minimal (Grade 1), Moderate (Grade 2) Marked/Severe (Grade 3)

Elastic fibres were also categorized depending on their nature into two groups: Fragmented & Bundled. Grading systems used to grade OSCC in this study were as follows: Slides were examined and diagnosed as oral squamous cell carcinoma (OSCC) using Broder's grading system 1927 (Descriptive system):

Grade- I	Well differentiated	= <25% undifferentiated cells
Grade- II	Moderately differentiated	= <50% undifferentiated cells
Grade III	Poorly differentiated	= <75% undifferentiated cells
Grade IV	Anaplastic / Pleomorphic	= > undifferentiated cells

Anneroth et al. (1984) histologic grading system: Cases of OSCC were also graded and scored as per the modification of a recent malignancy grading system recommended given by Anneroth et

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al. in 1984. Grading of malignancy of tumor-host relationship was done using parameters like pattern of invasion, stage of invasion and Lympho-plasmacytic infiltrate. Each of these features were scored from 1-4 according to definitions given by Anneroth. The scores for each morphologic feature were summarized into a total malignancy score. Prognosis was determined from the total malignancy score as higher the score, poorer the prognosis.
Malignancy score and prognosis were correlated as follows:

Score	**Prognosis**
3-6	Grade I- Good
7-10	Grade II- Moderate
11-14	Grade III- Poor

Morphologic parameters	Score 1	2	3	4
Pattern of invasion	Pushing, well delineated infiltrating borders	Infiltrating, solid cords, bands and or strands	Small groups or cords of infiltrating cells (n>15)	Marked and widespread cellular dissociation in small groups of cells (n<15) and/or in single cells
Stage of invasion (Depth)	Carcinoma in situ /or Questionable invasion	Distinct invasion, involving lamina propria only	Invasion below lamina propria adjacent to muscles, salivary gland tissues and periosteum	Extensive and deep invasion replacing most of the stromal tissue and infiltrating jaw bone
Lympho-plasmacytic infiltrate	Marked	Moderate	Slight	None

3. Results

The data obtained was analyzed by applying Z test of difference between two proportions (($p<0.05$) and Chi-square test of significance ($p<0.05$). Out of 30 cases of OSCC, maximum i.e. 17 (56.67%) cases were found at buccal mucosa. 12(70.58%) cases were Well differentiated OSCC and this was statistically significant (Z test of difference, $p<0.05$). Comparison between Broders Grading and Anneroth's Histologic Grading Systems showed that, Anneroth's grading had 19 (63.33%) cases of OSCC as Grade II and 11(36.66%) cases Grade I. As per Broder's grading, out of 30 cases of OSCC, maximum 20 cases were Grade I and was statistically significant ($P<0.05$). 3 cases of Grade III OSCC as per Broder's grading were scored as Grade II by Anneroth's grading. This shows the significant difference between the two histological grading systems of OSCC, Broder's and Anneroth's grading. Broders Grading showed 12(40%) cases with moderate degree of distribution of Elastic Fibres in different grades of OSCC.(Table 1) Minimum degree of deposition of elastic fibres was seen in 8(40%) cases of Grade I category.**(Fig 1)** Distribution of Elastic Fibres in different grades of OSCC(Anneroth's Histologic Grading) showed moderate degree of distribution of elastic fibres in maximum i.e. 9 (47. 36%)

cases in Grade II category(Table 2). Severe degree of distribution of elastic fibres was seen in 6 (31.57%) cases of Grade II category. Minimum degree of deposition of elastic fibres was seen in 5(45.45%) cases of Grade I category. Distribution of Collagen Fibres in different grades of OSCC(Broders Grading 18 (60%) cases showed thin and separate collagen fibres, while 12(40%) showed collagen fibres thick in bundles and this was statistically significant by Chi-square test,($p<0.05$) (Table 3). Distribution of Collagen Fibres in different grades of OSCC (Anneroth's Histologic Grading): Out of 30 cases of OSCC, 18 (60%) showed thin and separate collagen fibres, while 12(40%) showed collagen fibres thick in bundles(Table 4). Thin and separate collagen fibres were seen maximum in 11(57.89%) cases of Grade II category.(Fig 7,8) Thick bundles of collagen fibres were seen maximum in 8 (42.10%) cases of Grade II category of OSCC(Fig 6).

4. Discussions

Oral cancer is an epithelial neoplasia generally beginning as a focal clonal overgrowth of altered stem cells near the basement membrane, expanding upward and laterally, replacing the normal epithelium. The neoplastic process is a beginning with normal epithelium progressing through hyperplasia to dysplasia to carcinoma in situ and invasive carcinoma. The treatment for OSCC is surgery followed by radiotherapy and chemotherapy. Report of the histopathologist guides the clinician to plan further treatment (radiotherapy or chemotherapy) and to assess prognosis according to the grade of the tumor and adequacy of resection margins. Hence, the importance of detecting changes adjacent to tumor tissue and the ECM cannot be denied. Tumor microenvironment (TME) is an evolving concept which defines the behavior of cancer not by the genetics of the tumor cells alone, but by the surrounding milieu that the tumor cells need for survival, growth, proliferation and metastasis (Astekar et al.,2013). Recently many researchers have studied the alterations in the extracellular matrix (ECM), and found that ECM may play a decisive role in extension, invasion, metastasis and recurrence of the tumor. Also the concept of epithelial-mesenchymal transition (EMT) has added knowledge about the underlying mechanisms involved in the metastasis. Characterization of these changes has mostly been based on immunohistochemical and genetic studies. Also, the ultrastructural changes in malignant transformation of oral mucosa have been reported. (Cheng & Hudson,2002) In the present study, morphological changes of Extracellular Matrix in different histopathological grades of oral squamous cell carcinoma were studied using special histochemical stains like verhoeff's van gieson and Masson's trichrome which are easily available and less expensive.

In our study comparison was done between Broders method of grading with a modified Anneroth et al., grading system which showed statistically significant difference. This may be because; the Broder's grading of SCC is based on the assessment of differentiation or maturation of the tumor cell population alone without consideration of tumor-host relationship. Bryne et al.,1989 and Anneroth et al.,1984 suggested that in contrast to Broder's grading, the Anneroth's grading system has highly significant prognostic value as it considers the tumor-host relationship which could be helpful to predict biologic activity of tumor and is therefore appropriate grading system for classification of malignancy in OSCC.

In the present study, distribution of elastic fibres in different grades of OSCC graded by Anneroth's Histologic Grading was studied and was found statistically significant ($p<0.05$). Elastic fibres in OSCC by both grading systems showed that distribution of elastic fibres increases with advancing grade of tumor. Agrawal et al.,2011 in their ultrastructural study on oxytalan, elaunin and elastic fibres found presence of all three fibres of elastic system, but the

predominant fibres were elastic fibres which were seen to be increased in the ECM around tumor cells of OSCC. Our study also suggests that alterations in the stroma of OSCC may lead to increase in elastic fibres. In present study 21(70%) of cases showed moderate to severe degree of distribution of elastic fibres (Fig 2). The response of the ECM to tumor cell and lymphocyte-induced damage may be similar to the fibrosis of healing and may result in increased production of elastic fibres over time explaining the moderate to severe degree of deposition of elastic fibres in moderately differentiated and poorly differentiated OSCC cases in the present study (Fig 3 & 4). According to Shinohara et al.,1996, in highly invasive and metastatic primary tumors, the expression of vitronectin, laminin and type IV collagen is decreased, while expression of fibronectin & tenascin increased in the tumor stroma at the invasive site when compared to non-invasive and non-metastatic primary tumors. In our study, elastic fibres were evident in all 30 cases of different OSCC grades, out of which 21 (70%) cases showed distribution of elastic fibres of moderate to severe degree. All 3 cases of poorly differentiated OSCC in the present study showed thin and separate collagen fibres corresponding to collagenolytic activity in tumor stroma, while distribution of elastic fibres was of moderate to severe degree. Reason for this could be that, stromal alterations in the form of proteolysis and collagenolysis may lead to disintegration of extracellular matrix of OSCC which could make the elastic fibres more readily visible in the advancing grades of tumor.

In present study, collagen fibres changes in different grades of OSCC were evaluated using Masson's trichrome stain. Out of 30 cases of OSCC (as per Broders Grading), 18 (60%) cases showed thin and separate collagen fibres, while 12(40%) showed collagen fibres thick in bundles(Fig 5) and this was statistically significant ($p<0.05$). Poorly differentiated OSCC cases showed maximum i.e. 3(100%) cases with thin and separate collagen fibres (Fig 8). Majority of 18 cases showing thin and separate collagen fibres, 10 cases were of well differentiated (Grade I) OSCC, 5 cases of moderately differentiated (Grade II) OSCC and 3 cases of poorly differentiated (Grade III) OSCC.

In our study we found 12 (40%) of cases showing collagen fibres in thick bundles. This was in agreement with George et al.,2012 , who found collagen was more abundant than elastic in the stroma, particularly around cell nests like a scaffold, preventing the migration of tumor cells. Above findings were in agreement with Agrawal et al.,2011, who on ultrastructural examination found decreased collagen bundles in the ECM of OSCC. This can be explained as; the tumor cells secrete collagenase which helps in the lysis of the basement membrane which may possibly lead to recruitment of lymphocytes to this area as a host immune response. These lymphocytes then release cytokines like interleukin (IL-1) which induce enzymes like MMPs, especially MMP-1 causing collagen degradation and facilitating tumor cell infiltration and metastasis. Also the proteolytic degradation of ECM leads to changes in cell-cell and cell-matrix interactions with increased invasive potential. (Agrawal et al., 2011), (Doshi et al.,2011), (Yves et al.,2004). In our study, 60% cases showed thin and separate distribution of collagen fibres. All cases of poorly differentiated OSCC showed collagen fibres which were thin and separate. With increasing grade of tumor, collagen fibres showed quantitative as well as qualitative changes. The collagen disintegration may be due to stroma undergoing radical changes and increased collagenolytic activity and fibroblast undergoing phenotypic changes. Numerous studies have been carried out to understand the role of inflammation in progression of squamous cell carcinomas and cancers of other sites. Researchers have found that inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant transformation, invasion and metastasis. Inflammation also affects the immune surveillance and host response to therapy. Agrawal et al., 2011, in their study addressed role of host immune response in the form of

lymphocytic infiltration around the tumor cell nests. In their study, cases showing lymphocytic infiltration around tumor cell nests also revealed less number of elastic fibres on VVG staining and loss of collagen bundles in same area on ultrastructural examination. 25% of their cases showed minimal or no demonstrable elastic fibres possibly masked due to the dense lymphocytic infiltration around tumor cell nests.

We found similar results in present study where 13 cases out of 30 showed moderate to severe degree of lymphocytic infiltration and minimal distribution of elastic fibres around tumor cell nests. In the remaining 17 cases which showed mild response of lymphocytic infiltration, occasionally fragmented and very few elastic fibres were detected around tumor cell nests. It appears that the presence of lymphocytes in addition to lysis of collagen is an indicator of poor prognosis.

Present study indicates the importance of host immune response, the effects of this cell-mediated immunity on the ECM leading to increased elastic fibres proliferation. Extracellular matrix changes which indicate capacity of tumor cells to infiltrate can be easily studied using special stains.

5. Conclusion

Oral squamous cell carcinoma (OSCC) is most commonly seen in oral cavity in the Indian population. Not only tumour cells but also extracellular matrix (ECM) is prognostic indicator in OSCC. Routine histochemical stains such as Masson's trichrome and Verhoeff's van gieson which are easily accessible can be used to identify changes of ECM fibres in oral biopsies so as to advice surgeon regarding the propensity for invasion and metastasis. The present study demonstrated changes in the ECM as collagenolysis, increase in elastic fibre distribution preceded by lymphocytic infiltration.

6. Limitations and Future Studies

Similar cohort studies on large samples would help to identify these predictive factor and also to understand ECM changes which cause metastasis of tumour.

The present study showed that, Verhoeff's van gieson (VVG) stain for elastic fibres and Masson's trichrome stain for collagen fibres can be performed easily and are cost effective.

Extracellular matrix changes which indicate capacity of tumour cells to infiltrate can be easily studied using special stains. The present study showed that, Verhoeff's van gieson (VVG) stain for elastic fibres and Masson's trichrome stain for collagen fibres can be performed easily and are cost effective.

There exists a significant difference in histological grading systems of squamous cell carcinoma given by Broders and Anneroths's. The changes in extracellular matrix (ECM) of OSCC during different stages of tumorigenesis are studied by two different grading systems and both the grading systems showed similar ECM changes which are seen in present study as:

1. Squamous cell carcinoma of the buccal mucosa was most commonly seen.
2. Increased distribution of elastic fibres with the increasing histological grade of tumour.
3. Increased collagenolytic activity with increasing histological grade of tumour, indicating role of collagen in preventing migration of tumour cells.

4. Majority of cases showed mild degree of inflammatory response, especially in well differentiated OSCC, indicating the role of immune surveillance and host response in the initial stages of tumorigenesis.

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Table 1: Distribution of Elastic Fibres in different grades of OSCC (Broders Grading):

Distribution of Elastic Fibres	**Broders Grading**			**Total**
	Well Differentiated OSCC Grade I	**Moderately Differentiated OSCC Grade II**	**Poorly Differentiated OSCC Grade III**	**No. (%)**
	No. (%)	**No. (%)**	**No. (%)**	
Minimal	8(40%)	1(14.29%)	0	**9(30%)**
Moderate	6(30%)	5(71.42%)	1(33.33%)	**12(40%)**
Severe	6(30%)	1(14.29%)	2(66.67%)	**9(30%)**
Total	**20(66.67%)**	**7(23.33%)**	**3(10%)**	**30**

Value of X2=6.325, d.f. =4, Significant, p<0.05

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Table 2. Distribution of Elastic Fibres in different grades of OSCC (Anneroth's Histologic Grading):

Elastic Fibres	**Anneroths Histologic Grading of Malignancy of Tumor Host Relationship Grading**		**Total**
	Grade I	**Grade II**	
	No. (%)	**No. (%)**	
Minimal	5(45.45%)	4(21.05%)	**9(30%)**
Moderate	3(27.27%)	9(47.36%)	**12(40%)**
Severe	3(27.27%)	6(31.57%)	**9(30%)**
Total	**11(36.66%)**	**19(63.64%)**	**30**

Value of X2=4.107, d.f.=4, significant, p<0.05

Table 3. Distribution of Collagen Fibres in different grades of OSCC(Broders Grading):

Collagen Fibre Changes	**Broders Grading**			**Total**
	Well Differentiated OSCC Grade I	**Moderately Differentiated OSCC Grade II**	**Poorly Differentiated OSCC Grade III**	**No. (%)**
	No. (%)	**No. (%)**	**No. (%)**	
Thick in Bundles	10(50%)	2(28.57%)	0	**12(40%)**
Thin and Separate	10(50%)	5(71.43%)	3(100%)	**18(60%)**
Total	**20(66.67%)**	**7(23.33%)**	**3(10%)**	**30**

Value of X2=3.214, d.f. =2, significant, p<0.05

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Table 4. Distribution of Collagen Fibres in different grades of OSCC (Anneroth's Histologic Grading)

Collagen Fibre Changes	**Anneroths Histologic Grading of Malignancy of Tumor Host Relationship Grading**		
	Grade I	**Grade II**	**Total**
	No. (%)	**No. (%)**	**No. (%)**
Thick in Bundles	4(36.36%)	8(42.10%)	**12(40%)**
Thin and Separate	7(63.63%)	11(57.89%)	**18(60%)**
Total	**11(36.66%)**	**19(63.33%)**	**30**

Value of X2=0.09, d.f.=1, Not Significant, p>0.05

Figure 1. Distribution Of Elastic Fibres (Mild) in WDSCC - VVG Stain (20x)

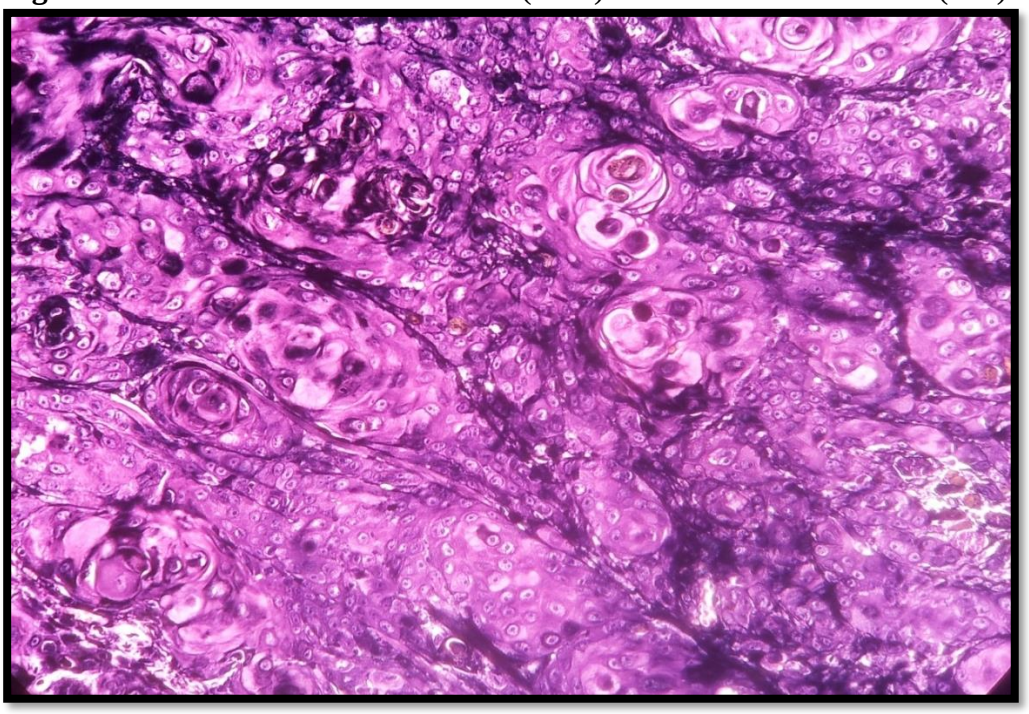

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Figure 2 : Distribution Of Elastic Fibres (Moderate) in MDSCC- VVG Stain (10x)

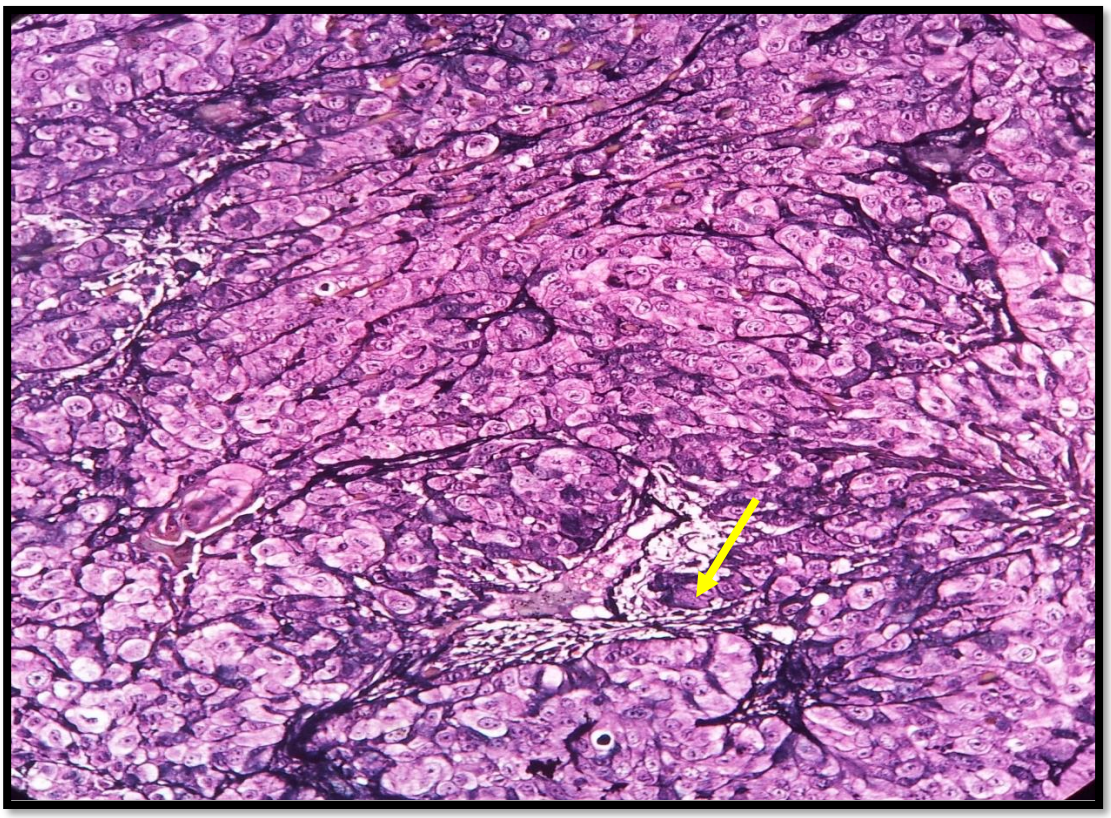

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Figure 3 : Excessive Deposition of bundled Elastic Fibres in PDSCC - VVG Stain (20x)

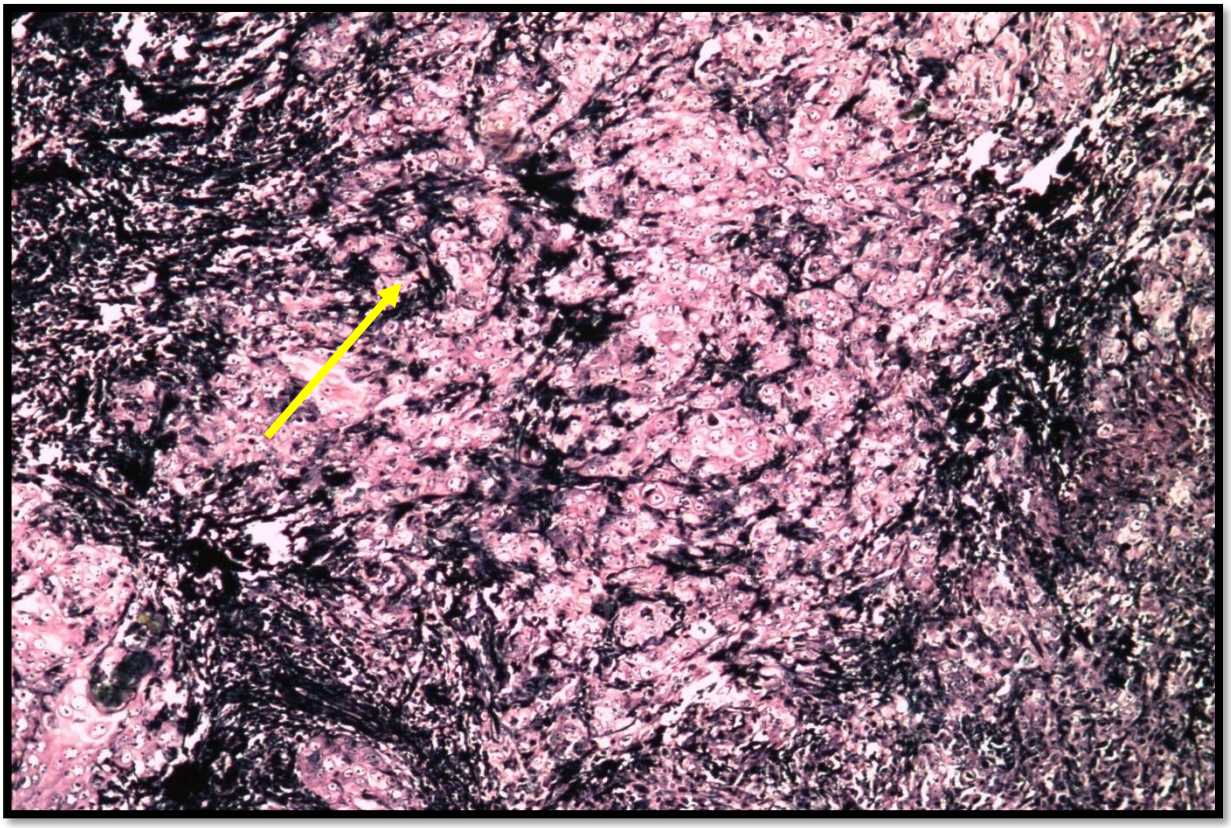

Figure 4 : Excessive and fragmented Elastic Fibres in PDSCC- VVG Stain (10x)

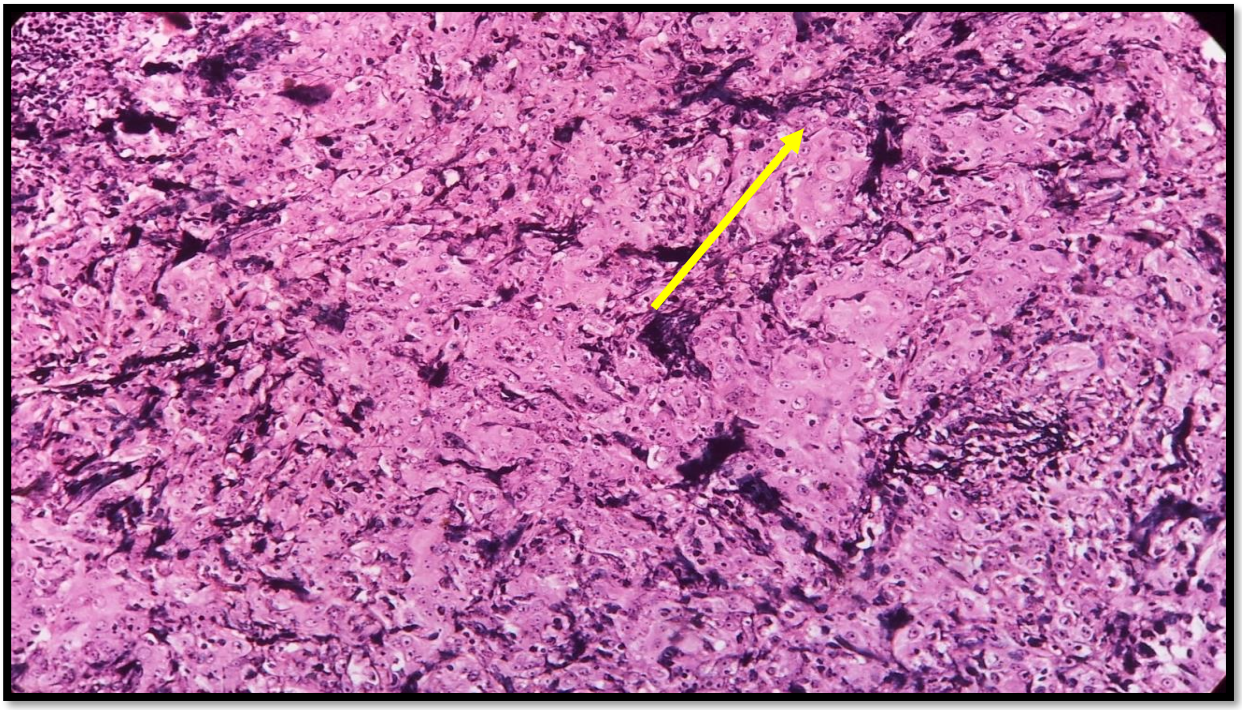

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Figure 5 : Collagen fibres thick & bundled in WDSCC-Masson's Trichrome Stain (10x)

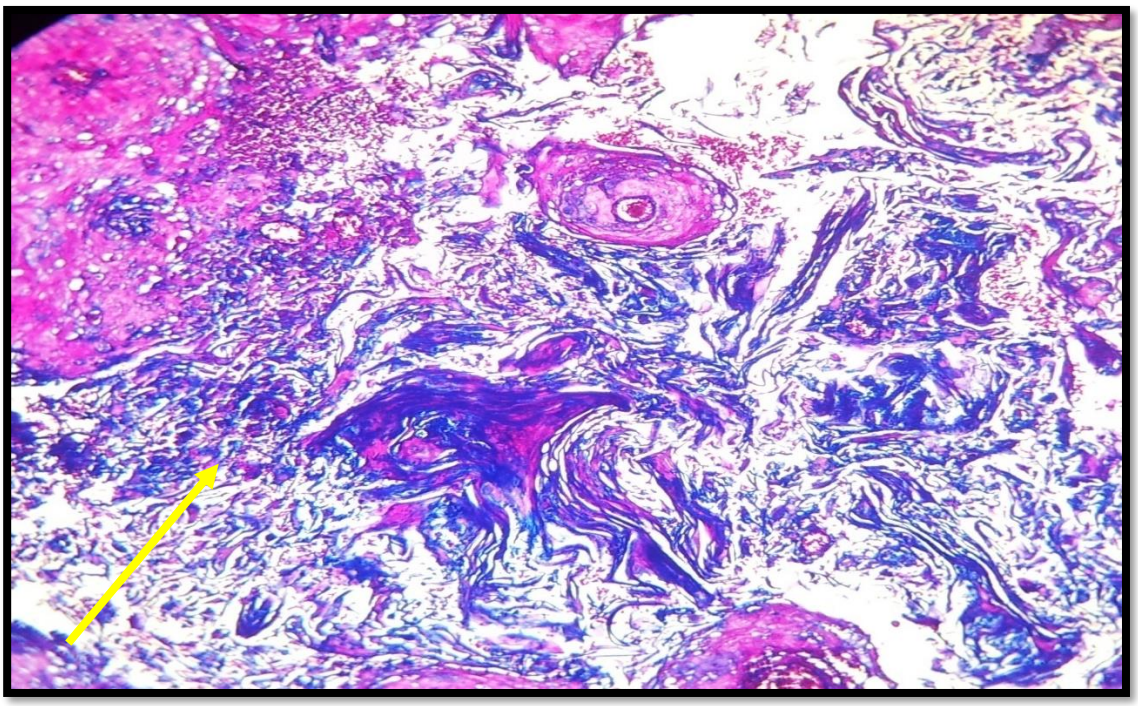

Figure 6 : Collagen fibres thick & bundled in WDSCC- Masson's Trichrome Stain (20x)

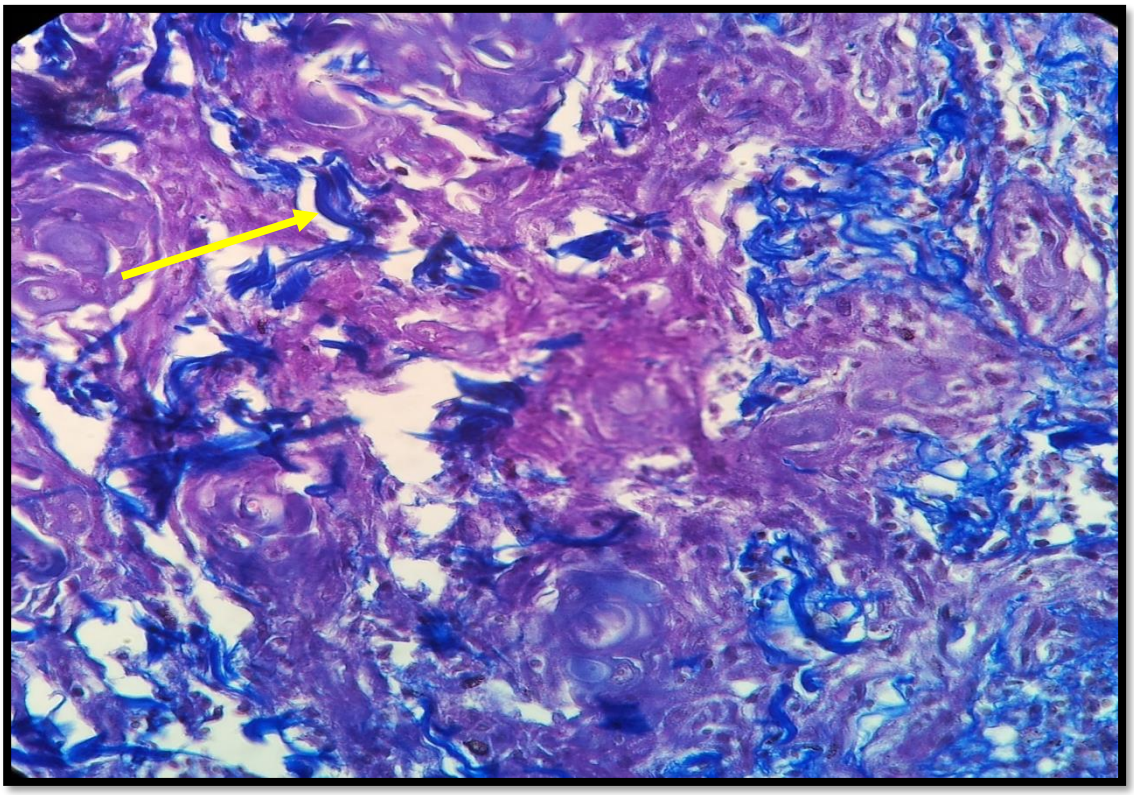

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Figure 7 : Thin & separated Collagen fibres in stroma of MDSCC-Masson's Trichrome Stain (20x)

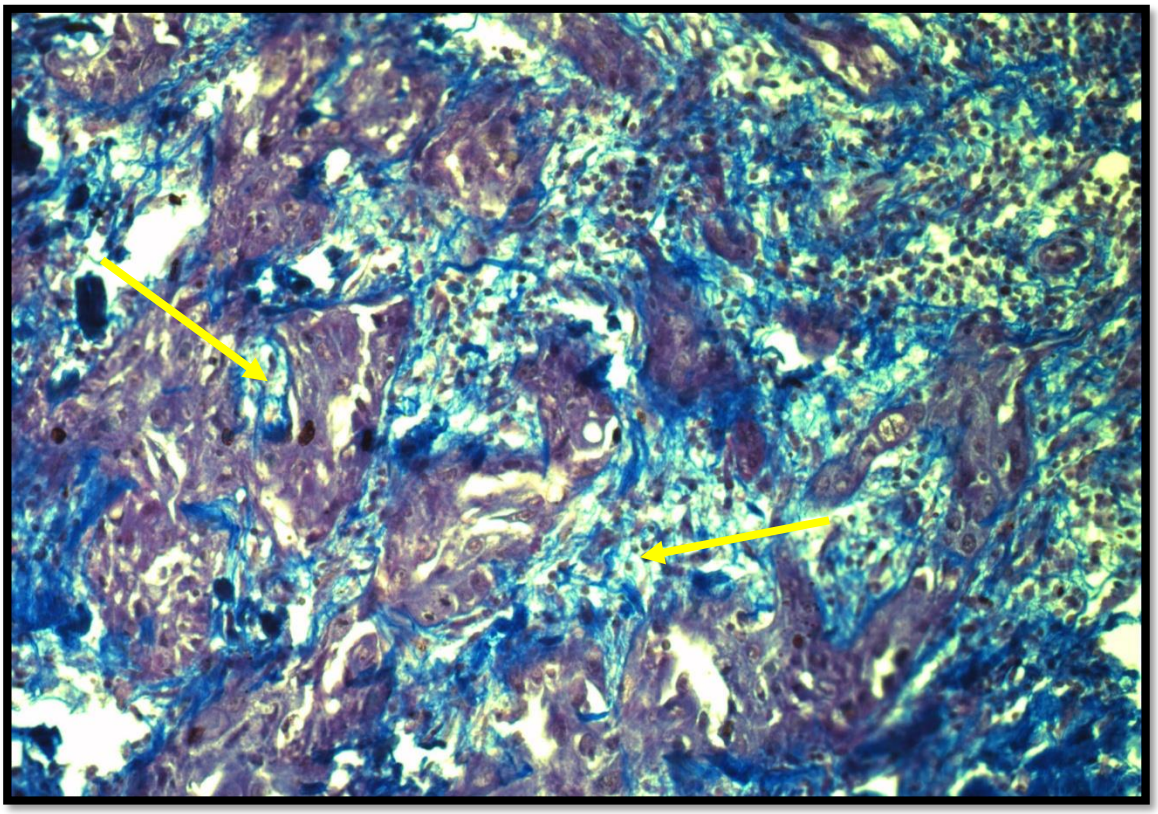

Figure 8 : Thin & separated Collagen fibres in stroma of PDSCC - Masson's trichrome Stain (20x)

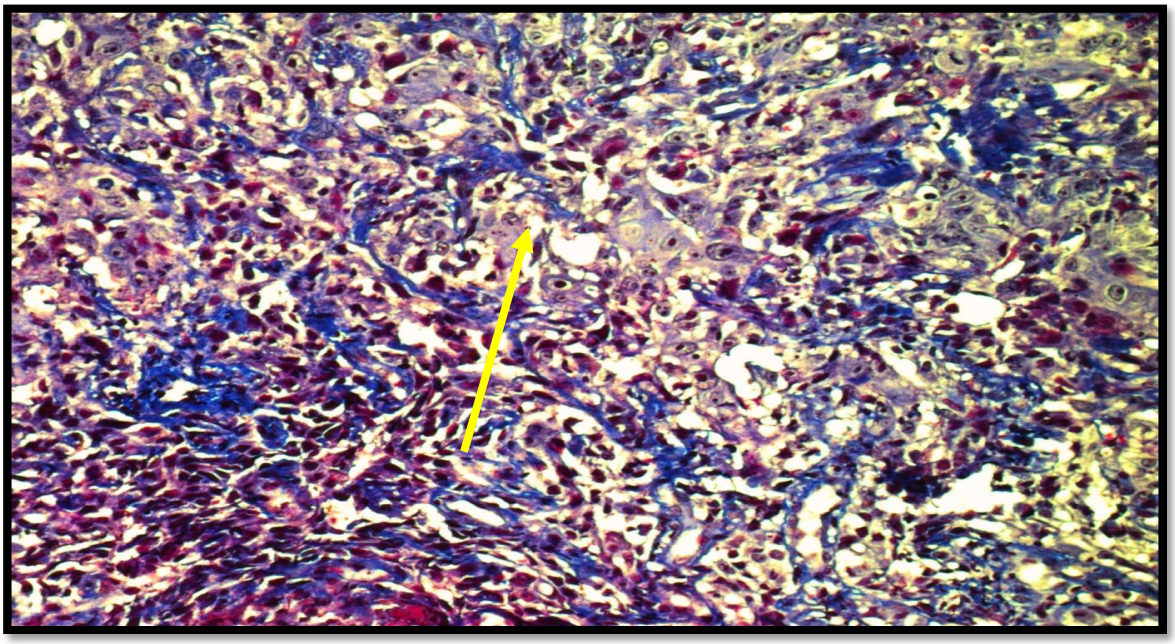

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Figure 9: Lymphocytic infiltration (Mild) around tumour cell nests in WDSCC (20x)

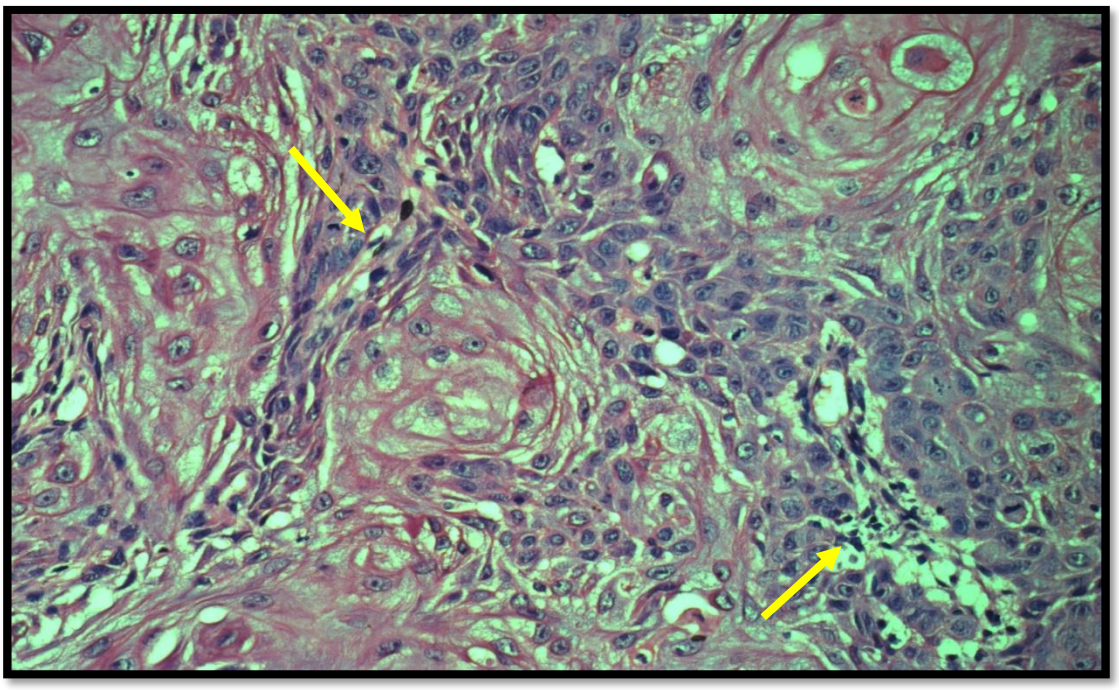
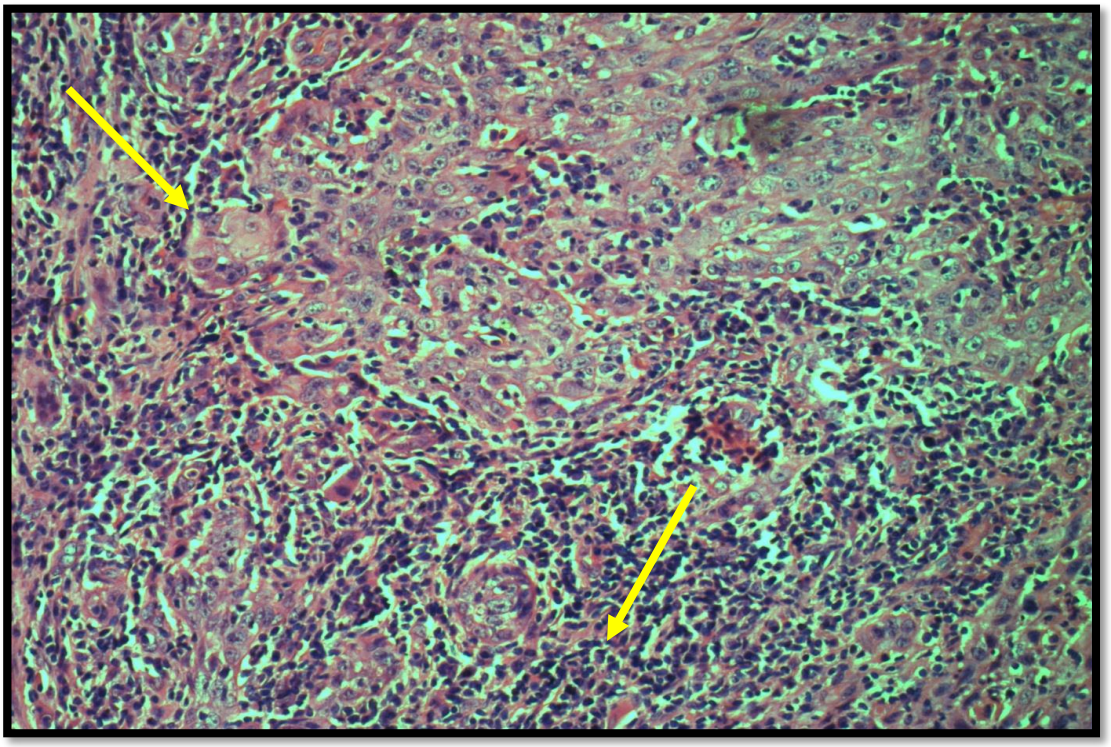


Figure 10 : Lymphocytic infiltration(Severe), absence of collagen around tumour cells in MDSCC (10x)



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