COMPARATIVE EVALUATION OF TENASCIN-C EXPRESSION IN ORAL SUBMUCOUS FIBROSIS AND ORAL SQUAMOUS CELL CARCINOMA: AN IMMUNOHISTOCHEMICAL STUDY.

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LIST OF ABBREVIATIONS

Abbreviation	Full form
OPMDs	Oral potentially malignant disorders
OSMF	Oral submucous fibrosis
ECM	Extracellular matrix
TIMP	Tissue inhibitor of metalloproteinase
OSCC	Oral squamous cell carcinoma
Tn	Tenascin
Tn-C	Tenascin-C
IHC	Immunohistochemical
GvHD	Graft-versus-host disease
TGM	Transglutaminase
HPV	Human papillomavirus
GMEM	Glioma mesenchymal extracellular matrix
Tn-R	Tenascin-R
Tn-X	Tenascin-X
Tn-W	Tenascin-W
et al	and others
LN	Laminin
IV C	Type IV collagen
HS-PG	Heparan sulfate proteoglycan
FN	Fibronectin
VN	Vitronectin
HSC	Highly invasive human oral squamous cell carcinoma cell
PTF	Peri-tumor fibroblasts cells
DIG	Digoxigenin
mRNA	Messenger Ribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
WHO	World health organization
GBM	Glioblastoma multiforme
DNA	Deoxyribonucleic acid
BM	Basement membrane
Tn-c_L	Large unspliced tenascin-C

Abbreviation	Full form
MSI1	Musashi homolog 1
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5
NANOG	Nanog homeobox
POU5F1	POU class 5 homeobox 1
Oct4	Octamer-binding transcription factor 4
SRY	Sex determining region Y
SOX2	SRY-box 2
STAT5	Signal transducer and activator of transcription 5
WNT	Wingless-related integration site
LP	Lichen planus
TNF	Tumor necrosis factor
ECJ	Epithelium and connective tissue junction
CT	Connective tissue
ESCC	Esophageal squamous cell carcinoma
CAFs	Cancer-associated fibroblasts
TAM	Tumor-associated macrophage
HIF1α	Hypoxia-inducible factor1α
OS	Overall survival
DFS	Disease-free survival
PDGFRα	Platelet-derived growth factor α
SMA	Smooth muscle actin
FSP1	Fibroblast-stimulating protein-1
HNSCC	Head and neck squamous cell carcinoma
Gal-1	Galectin-1
NM	Normal mucosa
MSR	Margin of surgical resecate
CD31	Cluster of differentiation 31
CD34	Cluster of differentiation 34
NSCLC	Non-small cell lung cancers
MVD	Microvessel density
Web	Website
NEPC	Neuroendocrine prostate cancer
TCGA	The cancer genome atlas
WNT	Wingless-related integration site
TLR	Toll-like receptor
SSc	Systemic sclerosis
CRC	Colorectal cancer

Abbreviation	Full form
CEA	Carcinoembryonic antigen
CA19-9	Carbohydrate antigen
BCD	Benign colonic disease
HD	Healthy donors
CCN3	Communication network factor 3
PHLF	Post hepatectomy liver failure
PHx	Rat model of partial hepatectomy
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
ELIS A	Enzyme-linked immunoassay
GB	Glioblastomas
KO	Knockout
TME	Tumor microenvironment
STAT3	Signal transducer and activator of transcription 3
Src	A non-receptor tyrosine kinase protein in humans is encoded by the SRC gene
AKI	Acute kidney injury
pH	Potential of Hydrogen
HRP	Horseradish peroxidase
DAB	3,3’ diaminobenzidine
PBS	Phosphate buffered saline
ml	Milliliter
°C	Degree Celsius
MS Office	Microsoft office
SPSS	Statistical package for social sciences
ANOVA	Analysis of variance
SD	Standard deviation
P-value	Probability value
NS	Statistically not significant
S	Statistically significant

Introduction

Worldwide, head and neck cancers constitute the sixth most common malignant tumors. It affects approximately 650,000 people and causing almost 350,000 cancer deaths annually. Oral cancer is the most frequent cancer of head and neck (1) and about 80% of oral cancers are preceded by oral potentially malignant disorders (OPMD) in India (2).

Oral Submucous Fibrosis (OSMF) is a potentially malignant disorder and predominantly seen in South Asian countries. OSMF is a chronic, insidious disease that affects the lamina propria of the oral mucosa. With the progression of the disease, deeper tissues in the submucosa of the oral cavity are involved resulting in loss of fibroelasticity (3). The principal causative factor for OSMF is areca nut alkaloid, arecoline that induces fibroblastic proliferation and increased collagen formation. It influences the deposition of extracellular matrix (ECM) by increasing the production of tissue inhibitor of metalloproteinase (TIMP1) and the effect is enhanced with the interaction of fibroblasts with keratinocytes (4).

Oral squamous cell carcinoma (OSCC) is the predominant form of oral cancer and accounts for 90% of all oral cancers (5). OSCC is a hyper-proliferative disorder. It is a heterogeneous cellular entity whose growth is dependent on mutual interactions between genetically altered tumor cells and the microenvironment i.e. ECM in which it dwells (6). These altered tumor cells have a propensity for invasion into the ECM (5).

ECM is an extremely active unit and also a pool of growth factors and bioactive molecules. It determines and controls the most essential behavior and characteristics of cells such as proliferation, adhesion, migration, polarity, differentiation, and apoptosis during organogenesis, embryogenesis, and wound healing as well as influence tumor behavior (7). Constituents of the ECM bond together to form a structurally stable complex that provides the mechanical properties of tissues. ECM components constitute collagenous proteins, non-collagenous proteins, elastin and glycoprotein (8). ECM glycoproteins chiefly constitute of fibronectin, tenascin, and undulin (9).

Tenascin (Tn) is a large oligomeric glycoprotein of the ECM. Tenascin is synthesized by fibroblasts, glial cells, muscle cells and epithelial cells (9). Tenascin is expressed in a spatially and temporarily restricted manner particularly in areas of epithelial-mesenchymal interaction during embryogenesis (10). There are four members of the Tenascin gene family: Tenascin-C, Tenascin-R, Tenascin-X, and Tenascin-W (11).

Tenascin- cytotactin (Tn-C) is the founding member of the gene family and the most hugely studied member of the family because of its anti-adhesive property and its expression in the stroma of certain tumors is associated with a poor prognosis (12). It plays a role in cell proliferation, invasion and metastasis by altering cell-to-cell and cell-extracellular matrix communication or interaction (10). It may also possess an autocrine growth factor-like property and may influence the proliferative changes in the subepithelial connective tissue (9). Tn-C participates in cell adhesion and motility, guidance along cell migration pathways, shedding of epithelial cells from surfaces, promotion of cell growth, demarcation of tissue boundaries and tissue modeling (13).

Similar processes are likely taking place in the abnormal growth and proliferation associated with potentially malignant disorders, and benign and malignant tumors. The tenascin expression in normal human mucosa is almost negative or minimal and its distinct upregulation in inflammation, tissue repair and in certain reactive situations such as in the stroma of OSMF and OSCC suggests its organizing role in pathological conditions. In OSMF which is an OPMD, Tn-C may influence the proliferation-associated changes in the subepithelial connective tissue as it possesses an autocrine growth factor- like property (9). Keratinocyte movement is required in OSCC for connective tissue invasion by oral cancer cells resulting in the up-regulation of Tn-C during the interaction (14). Hence, Tn-C expression can be correlated with proliferative organization of the overlying epithelium or to an epithelial-mesenchymal interaction and proliferation in OSMF and OSCC.

Since its discovery, Tn-C has been studied in benign and malignant tumors including gliomas, fibrosarcoma, osteosarcomas and a wide range of carcinomas occurring at different sites bearing to its potential medical implications for diagnosis, prognosis, and therapy (15). Tn-C has been studied in OSCCs and in other oral lesions like OSMF, oral fibrous hyperplasia, hyperkeratosis, epithelial dysplasia, lichen planus. To our best knowledge, Tn-C expression in different grades of OSCC and OSMF has not been studied before.

Thus, the present study attempts to comparatively determine the expression of the specific extracellular matrix protein Tenascin-C in different grades of OSMF and OSCC through an immunohistochemical (IHC) staining.

Aim and Objectives

The present study will be an attempt to analyze the comparative evaluation of Tenascin-C in Normal Oral Mucosa, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma with the following aim.

Aim

To analyze the comparative evaluation of Tenascin-C in Normal Oral Mucosa, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma.

The aim was fulfilled with the following objectives

Objectives

- To evaluate the expression of Tenascin-C in Normal oral mucosa.

- To evaluate the expression of Tenascin-C in different grades of Oral Submucous Fibrosis.

- To evaluate the expression of Tenascin-C in different grades of Oral Squamous Cell Carcinoma.

- To compare the expression of Tenascin-C in Normal oral mucosa, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma.

Review of Literature

Cancer is a global burden with a hasty increase in the incidence each and every year worldwide. Most of the cancers arise due to changes in lifestyle, including tobacco, smoking, and alcohol abuse (16). Cancer of the oral cavity remains a life‑ threatening disease for more than 50% of newly diagnosed patients. Despite readily available diagnostic tools, lack of awareness, and delayed prognosis have heightened the incidence of oral cancer (17). Most of the oral cancers are due to the accumulation of multiple genetic alterations in the squamous epithelial cells of the oral cavity. OSCCs are improbable to arise directly from the normal epithelium, it first in all prospect goes through a series of stages of initiation and promotion leading to oral potentially malignant disorder (OPMD) (18). In India, about 80% of OSCCs are preceded by OPMDs which include oral leukoplakia, erythroplakia, erythroleukoplakia, oral submucous fibrosis (OSMF), palatal lesions in reverse smokers, oral lichen planus, oral lichenoid reactions, graft-versus-host disease (GvHD), oral lupus erythematosus, and some hereditary conditions, such as dyskeratosis congenita and epidermolysis bullosa (3).

Oral Submucous Fibrosis

OSMF a potentially malignant disorder is a chronic, insidious disease that affects the lamina propria of the oral mucosa (3). The disease is unique in its presentation and geographical predisposition and almost always associated with the habit of areca nut chewing. The fibrosis in the submucosa is pathognomic of the condition, so the collagen and ECM component content of the tissue has been inspected constantly (19).

Arecoline, an areca nut alkaloid is now identified as the principal causative factor for OSMF. Arecoline appears to be involved in the pathogenesis of OSMF causing fibroblastic proliferation and increased collagen formation. Arecoline influences the deposition of ECM by increasing the production of tissue inhibitors of matrix metalloproteinases (TIMP)-1 and the effect is enhanced when fibroblasts are co-cultured with keratinocytes. Interaction of arecoline and keratinocytes may induce differentiation of myofibroblasts from fibroblasts. Transglutaminase-2 (TGM – 2) regulated by arecoline, stabilizes ECM protein by cross-linking and has been implicated in several fibrotic disorders. TGM – 2 expression is increased in OSMF suggesting arecoline influences ECM (4).

Oral Squamous Cell Carcinoma

Squamous cell carcinoma is the principal form of oral cancer and accounts for 90% of all oral cancers (5). Chief etiological and predisposing factors for OSCC include mostly tobacco chewing, smoking and drinking habits, and ultraviolet radiation (specifically for lip cancer), Other factors such as human papillomavirus (HPV) and Candida infections, nutritional deficiencies, and genetic predisposition have been also associated in etiology (20). These Etiologic factors are associated with carcinogens that are responsible for genetic transformation in the cell that allow excessive and unregulated proliferation resulting in neoplasm. OSCC like all other tumors has two basic components, transformed neoplastic epithelial squamous cells and reactive connective tissue stroma. Neoplastic epithelial cells largely determine the tumor's behavior and pathologic consequences but their growth and evolution are critically dependent on their stroma. There is interaction between neoplastic epithelial cells and stromal cells in the ECM that directly influences the growth of OSCC (21). OSCC contains a very specific form of ECM and the stroma surrounding it closely resembles that of healing wounds that promote the migration of cells (22).

Extracellular Matrix (ECM)

ECM is a highly dynamic and vital unit. It is also a pool of growth factors and bioactive molecules. It determines and controls the most essential behaviors and characteristics of cells such as proliferation, adhesion, migration, polarity, differentiation, and apoptosis during organogenesis, embryogenesis, and wound healing. It also provides an extracellular environment of active structural proteins and influence tumor behavior by significantly involving in invasion and metastasis (7). ECM is a complex network composed of an array of multidomain macromolecules of collagenous proteins, non-collagenous proteins, elastin and glycoprotein, each with discrete physical and biochemical properties (7,8). ECM glycoproteins chiefly constitute fibronectin, tenascin, and undulin (9).

Tenascin (Tn)

Tn is a large oligomeric glycoprotein of the extracellular matrix and produced by fibroblasts, glial cells, muscle cells, and epithelial cells. Synthesis of Tn is at specific times and locations during embryonic development. Tenascin was given different names as glioma mesenchymal extracellular matrix (GMEM) protein, myotendinous antigen, hexabrachion protein, cytotactin, and J1 by several laboratories that discovered it independently. The biochemical structure of a tenascin molecule, observed by rotary shadowing electron microscopy shows a hexabrachion consisting of six long, thin arms (10). (Fig 1) The modular design of the hexabrachion arm suggests the possibility of multiple independent functions.

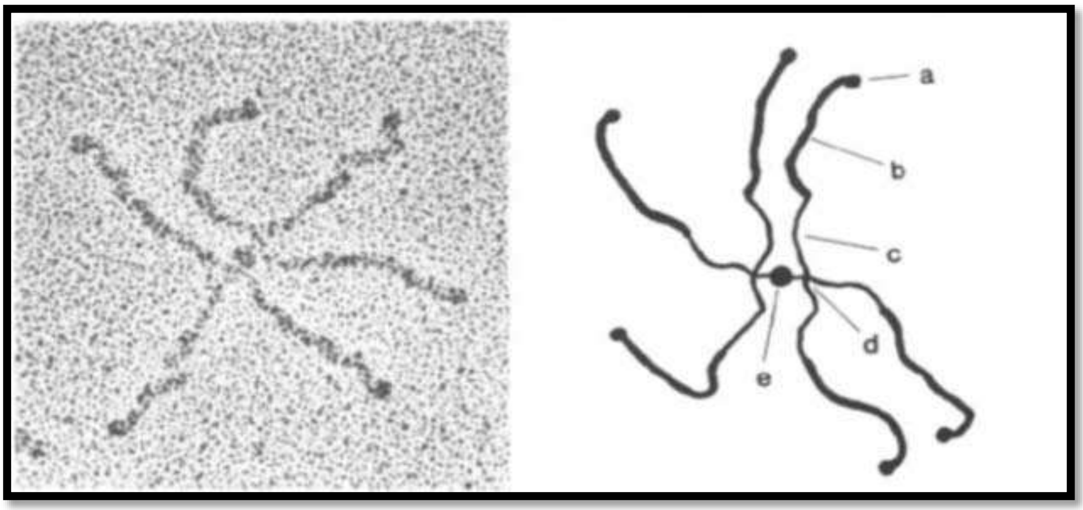


Fig 1: Rotary shadowed electron micrograph of a tenascin molecule hexabrachions, (250,000X). The lettered features diagrammatic representation (reprinted from Erickson & Iglesias 1 984) with features of the hexabrachion as (a) a terminal knob on each arm, (b) a thick distal segment, (c) a thin proximal segment, (d) a T-junction where three arms are joined to form a trimer, and (e) a central knob, where two trimers are joined to form the hexamer (10).

Currently, four tenascin members have been identified in mammals, which are Tenascin-C, Tenascin-R, Tenascin-X, and Tenascin-W. Tn-R is restricted to the nervous system and Tn-X is predominantly expressed in heart and skeletal muscle with distinct distribution and often reciprocal to that of Tn-C. A little is known about Tn-W compared to other members. (23) Tenascin-C (Tn-C) which is the founding member of the Tn family, was discovered in 1983 as a protein enriched in the stroma of gliomas and as a myotendinous antigen. The major sites of Tn-C expression are tendons, ligaments and the ECM of tumor stroma (24). It is a multifunctional protein, with only a specific subset of functions being employed in each tissue. As demonstrated by antibody inhibition in vivo, Tn engages in cell motility and migration which are closely related to and dependent on cell adhesion (10). Tn-C binds to fibronectin in a region around its major heparin-binding site preventing the interaction of cells with fibronectin through syndecan-4. This results in alteration in intracellular signaling pathways that are normally triggered by cell contact with adhesive ECM substrates.

Tn-C is again always strongly expressed in inflamed regions of the dermis locally just underneath the hyperproliferative epidermis suggesting that it precedes inflammation. Tn-C is strongly induced by various pro- and anti-inflammatory cytokines, and its de novo expression is a reliable molecular marker for acute inflammation. An early inflammatory response is almost always associated with enhanced Tn-C levels, both in the tissue and in plasma (24). The transformed or malignant epithelial cells in OSCCs appear to induce the fibroblasts of the underlying connective tissue to synthesize Tn-C (10,25).

Tn-C participates in cell adhesion and motility, guidance along cell migration pathways, shedding of epithelial cells from surfaces, promotion of cell growth, demarcation of tissue boundaries and remodeling of ECM (10). Similar changes are noted in OSCCs and OSMF. Tn-C is relatively a minor ECM component with a restricted distribution in most adult healthy tissues. Thus, Tn-C is a potentially useful marker for tissue remodeling in pathologies like OSCC and OSMF. The intensity of Tn-C staining can be correlated with the tumor grade and that the strongest staining mostly indicated poor prognosis. However, since some highly malignant tumors seem to lose Tn-C expression during progression, caution is required to use it for diagnosis (24).

Shrestha P, Sakamoto F, Takagi H, Yamada T, Mori M. (1994) (13) studied the expression of Tenascin immunoreactivity in leukoplakia and OSCC. In normal tissue specimens, tenascin immunoreaction appeared as a linear continuous lining at the immediate vicinity of the basement membrane. Hyperplastic epithelium in leukoplakia showed a distinct increase in tenascin immunoreactivity in the submucosa correlating with the degree of hyperplasia and/or dysplasia. The Tn reactivity in OSCC was most intense extending deeply into the underlying stroma with marked reaction especially around large tumor cell nests and the infiltrating tumor margin. However, in undifferentiated carcinoma, the connective tissue stroma showed traces of immunoreactivity. Positive immunoreactivity was seen around metastatic OSCC masses in regional lymph nodes. tenascin was unreactive in the stromal tissues infiltrated by inflammatory cells while those with desmoplastic changes were positive. The authors concluded that a boosted expression of tenascin may play a significant role during active phases of tumor cell proliferation and stromal changes in oral premalignant and malignant lesions.

Harada T, Shinohara M, Nakamura S, Oka M. (1994) (8) examined immunohistochemical expression of extracellular matrices (ECMs) laminin (LN), type IV collagen (IV C), heparan sulfate proteoglycan (HS-PG), fibronectin (FN), tenascin (Tn), decorin and vitronectin (VN) was in 112 primary tumors and 29 metastatic cervical lymph nodes in OSCC. In highly invasive primary tumors, they found the expression of LN, IV C and HS-PG along the tumor-stroma borderline at the basement membrane and decorin and VN expression at the invasive site in the tumor stroma were all significantly decreased. The expression of FN and Tn in the tumor stroma at the same site was markedly increased. In peri-tumor stroma in metastatic lymph nodes, LN, IV C, HS-PG, decorin, and VN were weakly expressed, while FN and Tn were strongly expressed. Thus, the staining pattern of the ECMs in the metastatic lymph nodes was similar to that in highly invasive primary tumors. Furthermore, in primary tumors of metastatic cases, the expression of LN, IV C, HS-PG, decorin, and VN obviously decreased, while the expression of FN and Tn increased when compared with those of the non-metastatic cases. Their investigation of ECMs in OSCC was valuable in predicting tumor behavior.

Tiitta O, Happonen RP, Virtanen I, Luomanen M. (1994) (26) studied the distribution of tenascin in oral premalignant lesions and squamous cell carcinoma by using Immunofluorescence. Tn was distributed as a continuous thin, delicate line at the basement membrane in normal buccal and palatal mucosa, the ventral tongue, the floor of the mouth and the gingiva. A distinct zone of enhanced Tn immunoreactivity was observed immediately beneath the epithelium of hyperkeratosis without dysplasia. An enhancement of the stromal Tn expression was observed in dysplasias of various degrees and most obvious in carcinoma in situ lesions. In most invasive carcinoma cases the Tn expression was intense, extending deeply into the underlying stroma often covering total stroma. They predominantly noted the strongest immunoreaction at the advancing edges of the tumor. They considered the triggering factor of epithelial origin for stromal Tn enhancement and suggested the enhanced expression of Tn was symbolic of its role in organizing and remodeling the stroma to support active epithelial proliferation and migration. However, they also thought that inflammation is linked with Tn expressions.

Reichart P, van Wyk CW, Becker J, Schuppan D (1994) (27) studied the distribution of procollagen type III, collagen type VI and tenascin in biopsy specimens of OSMF from the buccal mucosa of 19 Indian women using the immunogold-silver staining technique. Tenascin was noted very faintly at the subepithelial basement membrane. The study showed that in OSMF procollagen type III and collagen type VI were expressed in a specific pattern which allowed a clear differentiation between fibrotic areas and adjacent apparently normal connective tissue stroma. The authors explained the stiffness of the oral mucosa in patients with OSMF to loss of procollagen type III, and therefore a probable predominance of collagen type I in collagen fiber bundles, and an almost complete loss of collagen type VI. The immunohistochemical findings provided evidence that the process of fibrosis starts in the deeper subepithelial connective tissue stroma and not close to the subepithelial basement membrane.

Ramos D, et al (1997) (6) identified Tn-c and one of its integrin receptors, αvβ6, in normal oral mucosa and oral OSCC specimens. Both Tn-C and αvb6 were not expressed in normal oral mucosa. They studied 2 human oral squamous cell carcinoma cell lines: the highly invasive (HSC)-3 cells, and the poorly invasive OSCC-25 cells. They determined that the adhesion of these cells to Tn-C involves both α2 and αv integrins. Fibroblast conditioned medium was required by oral OSCC cells to migrate on Tn-C. This migration was blocked by anti-α2 and anti-αv antibodies and was partially inhibited by antibodies to hepatocyte growth factor, epidermal growth factor and transforming growth factor-β1. The poorly invasive OSCC-25 cells formed αvβ6-positive focal contacts when seeded on Tn-C however the HSC-3 cells did not. Tn-C was secreted by HSC-3, OSCC-25 and peri-tumor fibroblasts cells (PTF) into the culture medium. However, only murine Tn-C could be identified in the reactive stroma adjacent to the resulting tumor nests when HSC-3 cells were inoculated into the floor of the mouth of nude mice, that demonstrated that HSC-3 cells do not secrete Tn-C in vivo. They finally concluded that αvβ6 and Tn-C are newly expressed in oral squamous cell carcinoma and that the tumor-stromal environment is influential in OSCC behavior.

Ramos D, Chen B, Regezi J, Zardi L, Pytela R (1998) (22) examined mild to moderate dysplasia or carcinoma in situ oral biopsy specimens to study Tn-C expression and found that carcinoma in situ is the stage at which Tn-C becomes widely expressed, suggesting it may be involved in the initial stages of tumor progression. To study Tn-C matrix production in vitro, they used an invasive OSCC cell line (HSC-3) and peri-tumor fibroblasts (PTF). Neither cell type organized a Tn-C matrix when cultured alone; however, when co-cultured with HSC-3 cells, PTF was able to assemble a Tn-C matrix. PTF retained the ability to organize a Tn-C matrix when separated from the HSC-3 cells by a semi-permeable membrane, indicating that cell-cell contact is not necessary for the Tn-C matrix organization and suggesting that soluble factors may be involved. Moreover, PTF were induced to assemble Tn-C matrices when grown in medium conditioned by both the PTF and HSC-3 cells. Antibodies to FN and to the first FN type III repeat blocked both FN and Tn-C matrix assembly, indicating that the Tn-C matrix organization is dependent on an FN template. Antibodies to α5, αv and β1 integrins also blocked Tn-C matrix formation. When seeded onto FN matrices, the co-cultures were unaffected by the anti-integrin and anti-FN antibodies and were able to organize a Tn-C matrix. Their results suggested that the progression of malignant oral SCC is accompanied by an alteration of the normal ECM to one rich in Tn-C and that the organization of a Tn-C matrix is dependent on both the OSCC cells and the PTF for soluble signals.

Eleanor M, Halfter W, and Liverani D. (1998) (28) investigated the distribution of tenascin in normal skin and healing skin wounds in rats by immunohistochemistry. In normal skin, they found tenascin was sparsely distributed, predominantly in association with basement membranes. In wounds, there was an obvious increase in the expression of tenascin at the wound edge in all levels of the skin. There was also principally strong tenascin staining at the dermal-epidermal junction beneath migrating, proliferating epidermis. Tenascin was observed throughout the matrix of the granulation tissue, which was present in full-thickness of wounds but was not detectable as wound contraction was complete in the scar. The distribution of tenascin was spatially and temporally different from that of fibronectin, and tenascin appeared before laminin beneath migrating epidermis. Tn was not entirely co-distributed with myofibroblasts, the contractile wound fibroblasts. In electron microscopic studies of wounds, Tn was restricted in the basal lamina at the dermal-epidermal junction. Tn was distributed homogeneously or bound to the surface of collagen fibers in the extracellular matrix of the adjacent dermal stroma. Tn appeared in the dermis underlying the migrating epithelium but fibronectin was not detected in cultured skin explants where epidermis migrated over the cut edge of the dermis. This demonstrated that migrating, proliferating epidermis induces the production of Tn. The concluded that Tn was important in wound healing and is subject to quite different regulatory mechanisms than is fibronectin.

Häkkinen L, et al (1999) (29) studied integrin α9 subunit and tenascin expression in oral leukoplakia, lichen planus, and squamous cell carcinoma. They found that in areas of the subepithelial connective tissue Tn-C expression was increased in mild inflammatory reaction as compared with the corresponding normal tissue, whereas in lesions that showed heavy inflammation in the connective tissue, the expression of Tn-C was decreased. They attributed the reduced expression of Tn-C in the heavily inflamed areas in their study to the degradation of Tn-C by proteolytic enzymes present abundantly in the inflammatory infiltrate. Tn-C can antagonize certain functions of fibronectin, including T-cell activation by complexity and reciprocity in such interactions.

Carnemolla B, et al (1999) (30) reported a human antibody fragment, Tn11, derived from a phage library with a high affinity for the spliced repeat C by preparing a digoxigenin (DIG)-labeled cRNA probe specific for the cTn-C and performed in situ hybridization on glioblastoma cryostat sections. The results demonstrate that the cTn-C isoform was produced by tumoral cells, even though not all tumoral cells produce the cTn-C isoform. This repeat was extremely abundant in high-grade astrocytoma (grade III and glioblastoma), especially around vascular structures and proliferating cells. They stated that the antibody appeared to have the potential for the development of a therapeutic agent for patients with high-grade astrocytoma.

Swindle S, et al (2001) (31) described a new class of ligand for the epidermal growth factor (EGF) receptor (EGFR) found within the EGF-like repeats of tenascin-C. Select EGF-like repeats of tenascin-C provoked mitogenesis and EGFR autophosphorylation in an EGFR-dependent manner. Micromolar concentrations of EGF-like repeats induced EGFR autophosphorylation and activated extracellular signal-regulated, mitogen-activated protein kinase to levels analogous to those brought by subsaturating levels of known EGFR ligands. When the ligands were tethered to inert beads, EGFR-dependent adhesion was noted and simulating the physiologically relevant presentation of tenascin-C as hexabrachion, which suggested an increase in avidity similar to that seen for integrin ligands upon surface binding. They further established specific binding to EGFR by immunofluorescence detection of EGF-like repeats bound to cells and cross-linking of EGFR with the repeats. Both of these interactions were eliminated upon opposition by EGF and enhanced by dimerization of the EGF-like repeat. They concluded that they were successful to identify a new class of "insoluble" growth factor ligands and a novel mode of activation for growth factor receptors.

Regezi J, et al (2002) (14) evaluated the expression of tenascin and β6 integrin in in-situ and invasive squamous cell carcinomas from the floor of the mouth by assessing staining intensity, pattern, and co-localization. Tenascin was highly expressed at the epithelial-mesenchymal interface of both in-situ and invasive carcinomas. β6 was expressed in basal keratinocytes of all in situ and invasive lesions but was not evident in any of the control epithelia. There was no significant difference in staining of in situ and invasive carcinomas, but there was a significant difference in staining between these lesions and controls. Staining was colocalized in serial sections, supporting a receptor-ligand relationship. Both tenascin and β6 were weakly expressed in dysplastic areas adjacent to carcinomas suggesting that changes in the expression of these proteins occur prior to the invasive phenotype. They conclude that tenascin and β6 are overexpressed in in-situ and invasive floor of the mouth carcinomas, but that lapse of the basement membrane by neoplastic epithelial cells requires additional changes to the keratinocyte molecular profile.

Herold-Mende C, et al (2002) (32) studied changes in Tn-C expression in human gliomas to correlating these changes with tumor progression and to explain the functional role of the glioma cell-specific Tn-C isoform pool. Tn-C expression was analyzed immunohistochemically in eighty-six glioma tissues of different World Health Organization (WHO) grades. The examination of the influence of the specific Tn-C isoforms produced by glioblastoma cells on proliferation and migration was done in vitro using blocking antibodies recognizing all isoforms. Overall tenascin-C expression was increased with tumor malignancy. All glioblastoma tissues expressed perivascular staining of Tn-C around tumor-supplying blood vessels whereas, in WHO II and III gliomas, perivascular Tn-C staining appeared less frequently. The appearance of perivascular Tn-C correlated significantly with a shorter disease-free time suggesting prognostic value Tn-C for an earlier tumor recurrence. Analysis of proliferation and migration in the presence of blocking antibodies suggested an inhibition of proliferation by around 30% in all 3 glioblastoma cell cultures, as well as a decrease in migration of 30.6–46.7%. So, they concluded that the endogenous pool of Tn-C isoforms in gliomas supports tumor cell proliferation and tumor cell migration.

Leins A et al (2003) (33) conducted a systematic immunohistochemical investigation of Tn-C distribution patterns in normal human brain tissue and in a large variety of brain tumors. They assessed immunoreactivity for Tn-C localization within tumor cells, blood vessels, and ECM using three different monoclonal antibodies (clones BC2, BC4, and Tn2). In control human brains, they noted a significant difference in the expression of Tn-C when comparing gray with white matter. Tn-C was found in the white matter of the frontal, temporal, parietal, and occipital lobes and especially strong in the hippocampus. In astrocytomas of different grades (WHO Grade 2–4), Tn-C immunopositivity was found to varying degrees in the cellular and stromal components of the tumor and in tumor-associated vessels. Glioblastomas expressed strong immunopositivity in the vessels and moderate immunopositivity of the ECM. In anaplastic astrocytomas (WHO Grade 3) a statistically significant reduction of Tn-C immunopositivity in tumor-associated vessels or ECM was observed compared with Glioblastoma multiforme (GBM) (WHO Grade 4). Patients who had GBM lesions and lacked Tn-C immunopositivity in the ECM had a significantly longer survival (median 28 months; standard error, 7.8 months) compared with patients who had GBM lesions with Tn-C immunopositivity (median 12 months; standard error, 1.6 months). In meningiomas, the neoplastic cells, the ECM of the tumor, and the vessels were Tn-C negative. In schwannomas the tumor cells were Tn-C negative; whereas, in 50% of tumors, the vessels and the ECM of regressively altered tumor areas were positive. In metastatic carcinomas, the tumor cells were negative; occasionally vessels were stained positive for Tn-C. Focal areas of the ECM, often accompanied by fibrotic changes, were immunopositive for Tn-C. The most persistent Tn-C immunopositivity was observed in the ECM of the fibrotic stroma in highly malignant brain tumors and along the tumor border, especially in high-grade astrocytomas. Their results suggested that Tn-C expression may be correlated with the grade of malignancy in astrocytic tumors and that the presence or absence of Tn-C expression in the stroma of astrocytic tumors may play a mysterious role in shortening or prolonging, respectively, the survival of patients.

Daniels D et al (2003) (34) probed antigens presented by a monolayer of tumor cells for their ability to interact with a pool of aptamers. They used a glioblastoma-derived cell line, U251 as the target for systematic evolution of ligands by exponential enrichment by using a single-stranded DNA library. They isolated specifically interacting oligonucleotides, and biochemical strategies were used to identify the protein target for one of the aptamers. Then they characterize the interaction of the DNA aptamer, GBI-10, with tenascin-C which is believed to be involved in both embryogenesis and oncogenesis pathways. The systematic evolution of ligands by exponential enrichment appeared to be a successful strategy for former identification of targets of biological interest within complex systems.

Utsunomiya M et al (2005) (35) studied the expression of eight ECM molecules namely Tenascin, Perlecan, Fibronectin, Collagen types I, III, and IV, Laminin and Elastin in oral submucous fibrosis by histochemistry, immunohistochemistry, and in situ hybridization. They found Tn was immunolocalized in a band-like fashion beneath the epithelial basement membrane (BM) in the early stage. However, the expression of Tn band disappeared completely in the intermediate and advanced stages. They detected mRNA signals for tenascin faintly in the basal cells of the oral epithelium and fibroblasts in the juxtaepithelial zone in the early stage but not in the intermediate and advanced stages. The linear deposit of tenascin in the BM zone in the early stage of OSF and its production by basal cells in the oral epithelium were different from those of other ECM molecules. Their results indicated that Tn is a variable marker for identification of the early stage of OSMF, even when histological features are not yet very characteristic in terms of fibrosis.

Franz M et al (2006) (36) demonstrated complex formation of the laminin-5(Ln-5) ϒ2 chain and large unspliced tenascin-C($Tn\text{-}c_L$) in oral squamous cell carcinoma in vitro and in situ to implicate sequential modulation of extracellular matrix in the invasive tumor front. They examined OSCC tissue specimens of different malignancy grades by means of confocal laser scanning microscopy and revealed different patterns of Ln-5 and $Tn\text{-}c_L$ co-localization implicating complex formation in vivo also. A ribbon-like colocalization was detected in subepithelial basement membranes around well-differentiated OSCC parts and tumor clusters. Furthermore, a fibrillar Ln-5 ϒ2 chain/ Tn-c$_L$ co-localization occurred in the carcinoma stroma beneath tumor clusters. Additionally, at the site of ruptured basement membranes, there were dot or strand-like co-deposits of both molecules, but co-localization was only rarely detectable. They suggested that the different patterns of co-deposition and co-localization of Ln-5/ ϒ2 and Tn-c$_L$ represented a successive modulation and reorganization of the ECM in the tumor/ stroma interface and thus reflected different stages of OSCC invasion.

Oskarsson T et al (2011) (37) studied Tn producing breast cancer cells that metastasized the lungs. They found that the expression of Tn-c is associated with the aggressiveness of pulmonary metastasis. Cancer cell-derived Tn-c promoted the survival and outgrowth of pulmonary micrometastases. Tn-C enhanced the expression of stem cell signaling components, Musashi homolog 1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5). MSI1 is a positive regulator of NOTCH signaling, and LGR5 is a target gene of the WNT pathway. Tn-C modulation of stem cell signaling occurred without affecting the expression of transcriptional enforcers of the stem cell phenotype and pluripotency, namely Nanog homeobox (NANOG), POU class 5 homeobox 1 (POU5F1), also known as OCT4, and SRY-box 2 (SOX2). Tn-C protected MSI1-dependent NOTCH signaling from inhibition by signal transducer and activator of transcription 5 (STAT5) and selectively enhanced the expression of LGR5 as a WNT target gene. Cancer cell-derived Tn-C remained essential for metastasis outgrowth until the tumor stroma took over as a source of Tn-C. Their findings link Tn-C to pathways that supported the fitness of metastasis-initiating breast cancer cells and highlighted the relevance of Tn-C as an extracellular matrix component of the metastatic niche.

Maraee A, Farag A, El Tahmody M, Metawea H. (2014) (38) evaluated the expression of Tn-C in lichen planus (LP) by immunohistochemical study to highlight its hypothesized role in the etiopathogenesis of LP. 20% of the controls showed low-intensity dermal staining, 50% showed low-intensity basal epidermal staining, whereas 30% of the biopsies were negative for Tn-C. There was significant dermal upregulation of Tn-C in the skin of LP cases compared with that of controls; however, they found no statistically significant difference regarding epidermal expression. Tn-C expression was increased in hypertrophic as compared to classic and guttate types due to the effect of epidermal hyperplasia and proliferating epithelium in hypertrophic LP. There was a significant association between Tn-C expression and LP presented with itching and thus they speculated that Tn-C might play a role in the escalation of the itch sensation in LP through the TNFa–IL-31 pathway. They concluded that Tn-C may play a role in the etiopathogenesis of LP through its immune-modulatory effect.

Tak J, Rao NN, Chandra A, Gupta N (2015) (9) analyzed tenascin C expression in early, moderate and advanced grades of oral submucous fibrosis (OSMF). Most of the OSMF cases showed tenascin expression at the junction of epithelium and connective tissue (ECJ) and in deeper connective tissue (CT). Its expression was diverse in different grades as well as around inflammatory cells, fibroblast and endothelial cells in the same tissue section. Tn was expressed as bright and continuous deposition at ECJ in early and moderate stages of OSMF suggesting either proliferative organization within the overlying epithelium or at an epithelial- mesenchymal interaction. However, a weak immunoreactivity of tenascin at ECJ was observed in the advanced stage of OSMF which they related to an altered fibroblast or a genetic modulation in fibroblast cells. They concluded that Tn is expressed differentially with the progression of disease.

Yang Z, et al (2016) (39) explored the clinicopathological significance of Tn-C as a prognostic determinant of esophageal squamous cell carcinoma (ESCC). They examined the presence of isoforms using western Blotting in ESCC patient tissues and cell lines and investigated Tn-C immunohistochemical expression in 136 ESCC tissue samples. They also determined the clinical relevance of Tn-C expression and the correlation between Tn-C expression and expression of other factors related to cancer-associated fibroblasts (CAFs). Both 250 and 350 kDa sized isoforms of Tn-C were identified in all four CAFs derived from esophageal cancer tissues. Tn-C expression was higher in ESCC than in adjacent non-tumor esophageal epithelium ($p < 0.001$). Tn- C expression in ESCC stromal fibroblasts was associated with a patient’s age, tumor (pT) stage, lymph node metastasis, clinical stage, and cancer recurrence. They correlated Tn-C expression in cancer cells with an increase in tumor-associated macrophage (TAM) population, cancer recurrence, and hypoxia-inducible factor1α (HIF1α) expression. In the Cox proportional hazard regression model, Tn-C overexpression in cancer cells and stromal fibroblasts was a significant independent hazard factor for overall survival (OS) and disease-free survival (DFS) in ESCC patients in both univariate and multivariate analyses. Furthermore, they positively correlated Tn-C expression in stromal fibroblasts of the ESCC patients with platelet-derived growth factor α (PDGFRα), PDGFRβ, and smooth muscle actin (SMA) expression. The 5-year OS and DFS rates were curiously lower in patients with positive expressions of both Tn-C and PDGFRα ($p < 0.001$), Tn-C and PDGFRβ ($p < 0.001$), Tn-C and SMA ($p < 0.001$), Tn-C and fibroblast activation protein (FAP) ($p < 0.001$), and Tn-C and fibroblast-stimulating protein-1 (FSP1) ($p < 0.001$) in ESCC stromal fibroblasts than in patients with negative expressions of both Tn-C and one of the above mentioned CAF markers. They concluded that Tn-C is a reliable and significant prognostic factor in ESCC and may thus be a potent ESCC therapeutic target.

Sundquist E, et al (2017) (40) studied Tn-C and fibronectin (FN) immunohistochemically as prognosticators for early-stage tongue cancer to determine treatment. They found that the expression of Tn-C and FN in the stroma, but not in the tumor cells, proved to be excellent prognosticators both in all stages and in early-stage cases. Among the early stages, when stromal Tn-C was negative, the 5-year survival rate was 88%. Similarly, when FN was negative, cancer deaths were not observed and five-year survival rates for rich expression of Tn-C and FN were 43% and 25%, respectively. They then concluded that stromal Tn-C and, especially, FN expressions differentiate patients into low- and high-risk groups. They suggested surgery alone might be adequate when stromal FN is negative in early-stage primary tumors bur advised aggressive treatments when both Tn-C and FN are abundant.

Leppänen J, et al (2017) (41) evaluated the expression of Tn-C and fibronectin in esophageal adenocarcinoma and its precursor stages. They found stromal Tn-C and fibronectin expression in all evaluated lesion types. The expression of both molecules increased from gastric metaplasia towards adenocarcinoma ($p<0.05$). In carcinomas, Tn-C expression in the bulk was associated with T-stage ($p=0.006$), presence of lymph node (p=0.004) and distant organ metastases (p=0.007). Abundant tenascin-C expression was associated with poor survival (p=0.034) in univariate analysis. Fibronectin expression was associated with T-stage (p=0.030). Expression of Tn-C or fibronectin in the tumor invasive front was not associated with clinicopathological variables or survival. No significant correlation with tumor/stroma percentage, cancer-associated fibroblasts or mean vascular density was observed with either Tn-C or fibronectin. They concluded that Tn-C and fibronectin are upregulated in esophageal adenocarcinoma when compared to Barrett's esophagus and dysplasia. Increased Tn-C expression is associated with metastasis and poor prognosis in esophageal adenocarcinoma.

Zivicova V et al (2018) (42) detected distinct changes in gene-expression profiles in specimens of tumors and transition zones of Tn-C positive/-negative head and neck squamous cell carcinoma (HNSCC). They analyzed tissue specimens of each anatomical site by immunofluorescent detection of Tn-C, fibronectin (Fn) and galectin-1 (Gal-1) as well as by microarrays. They histopathologically demonstrated that TnC+Fn+Gal-1+ co-expression occurs more frequently in samples of HNSCC (55%) than in normal mucosa (NM) (9%; p<0.01). Contrary, the Tn-C–Fn+Gal-1– (45%) and Tn-C–Fn–Gal-1– (39%) status occurred with significantly (p<0.01) higher frequency than in HNSCC (3% and 4%, respectively). In margin of surgical resecate (MSR), different immunophenotypes were distributed rather equally (TnC+Fn+Gal-1+=24%; Tn-C–Fn+Gal-1=36%; Tn-C–Fn–Gal-1–=33%), differing to the results in tumors (p<0.05). They used the absence/presence of Tn-C for stratification of patients into cohorts without a difference in prognosis, to comparatively examine gene-activity signatures. Microarray analysis revealed the expression of several tumor progression associated genes in Tn-C+ HNSCC tumors and a strong upregulation of gene expression assigned to lipid metabolism in MSRs of Tn-C– tumors, while NM profiles remained similar. They pointed out the plasticity of gene-expression profiles without necessarily bearing prognostic relevance and were relevant in principle for considerations of relating differences detected on this level to clinical parameters.

Rzechonek A et al (2018) (43) analyzed the correlation of expression of Tn-C with the markers of vascular endothelial cells, CD31 and CD34, and clinicopathological data in Non-small cell lung cancers (NSCLC). They carried out immunohistochemical reactions on paraffin sections using mouse monoclonal antibodies anti-Tn-C, anti-CD31, and anti-CD34, with the use of Autosteiner Link-48. Their findings showed a positive correlation between Tn-C expression and CD31(+) and CD34(+) microvessel density (MVD) ($r=0.456$, $p<0.0001$; $r=0.296$, $p<0.01$, respectively). They concluded that Tn-C may be involved in angiogenesis in NSCLC.

Anindhita M, Dharmaningputri NGA, Susantono DP, Antarianto RA (2018) (44) evaluated preputial skin regenerative capacity by using three specific immunohistochemical markers- Ki-67 for epidermal cells proliferative capacity, Oct4(octamer-binding transcription factor 4) for presence of pluripotent stem cells in preputial skin and Tn-C for tissue repair prognosis in surgical incision margin. They observed that the mean of Ki-67 positive basal keratinocyte in the inner mucosal epithelium was relatively higher than the outer cutaneous epithelium indicating higher proliferative capacity in inner mucosa epidermal cells. 85.7% of tenascin-c positive samples exhibited normal inflammation resolution suggesting normal tissue repair outcomes. 80% preputial skin samples had positive Oct4 cells, specifically at sites previously reported as skin stem cell's niche i.e. the sebaceous gland, hair root follicle, blood vessel lumen, and hypodermis layer of the skin. So, they concluded that regenerative capacity of post circumcision preputial skin is indicated by higher proliferation activity in inner mucosa epidermal layer, normal tissue repair outcome as indicated by the majority of Tn-C expression at wound incision area and presence of Oct-4+ cells in majority of post-circumcision preputial skin.

Ming X, et al (2018) (45) conducted a meta-analysis to quantitatively assess the prognostic roles of tenascin-C for patients with cancer. They systematically searched all published studies about the role of tenascin-C in cancers on PubMed, Web of Science, Cochrane Library, and Embase. The association between tenascin-C expression level and overall survival of patients with cancer was analyzed by pooled hazard ratio with 95% confidence intervals. They used a pooled odds ratio with 95% confidence intervals to investigate the association between tenascin-C expression level and clinicopathologic features of patients with cancer. They performed a trial sequential analysis to obtain the required information size. They found the pooled hazard ratio of 18 trials was 1.73 (95% confidence interval: 1.29-2.32, P < .001) for overall survival, suggesting that elevated tenascin-C expression strongly predicted poor prognosis among patients with various cancers. Simultaneously, elevated tenascin-C expression was also significantly associated with lymph node metastasis (odds ratio ¼ 2.42, 95% confidence interval: 1.79-3.26, P < .001). However, they observed no significant correlation between the tenascin-C expression and distant metastasis (odds ratio ¼ 1.72, 95% confidence interval: 0.86-3.44, P ¼ .127). They considered Tenascin-C as a promising unfavorable prognostic factor in human cancers and likewise, it could be used as a monitoring indicator for poor prognosis in a wide range of cancers.

Mishra P et al (2019) (46) meta-analyzed alterations of the Tn-C gene in prostate cancer using publicly available databases (cBioportal Version 2.2.0, http://www.cBioportal.org/index. do). The analysis identified Tn-c alterations or gene amplification significantly in the neuroendocrine prostate cancer (NEPC) dataset (Trento/Broad/ Cornell), which was further validated in other prostate cancer datasets, including The Cancer Genome Atlas (TCGA) prostate cancer (2015). In the TCGA prostate cancer dataset, high Tn-C alteration revealed a strong association with high diagnostic Gleason score. Genomic alterations of Tn-c were also significantly associated ($P < 0.05$) with the expression level of genes from NOTCH, SOX and WNT family, implicating a link between Tn-C and poorly differentiated aggressive phenotype in NEPC. TCGA prostate adenocarcinoma cases with Tn-C alteration also demonstrated a prominent decrease in disease-free survival ($P = 0.0637$). Their findings indicated a possible association of Tn-C to the aggressive subtype of prostate cancer.

Xu C et al (2019) (47) studied Tn-C as an endogenous toll-like receptor (TLR) 4 ligand responsible for maintaining persistent organ fibrosis in systemic sclerosis (SSc). They found elevated levels of Tn-C in SSc skin biopsy samples, and serum and SSc fibroblasts, and in fibrotic skin tissues from mice. Exogenous Tn-C stimulated collagen gene expression and myofibroblast transformation via TLR4 signaling. Mice lacking Tn-C showed attenuation of skin and lung fibrosis and accelerated fibrosis resolution. Their results identified Tn-C as an endogenous danger signal that was upregulated in SSc and driven TLR4-dependent fibroblast activation, and by its persistence impeded fibrosis resolution. They further suggested that disrupting this fibrosis amplification loop might be a viable strategy for the treatment of SSc.

Zhou M et al (2019) (48) evaluated the diagnostic value of S100A9 and Tn-C levels as colorectal cancer (CRC) biomarkers in several ways, including through screening tests, differentiation tests, combination with existing biomarkers (CEA and CA19-9), and serum level measurements before and after surgery. They found that the serum levels of S100A9 were 22.32 (14.88-29.55) ng/ml, 10.02 (5.83-14.15) ng/ml and 10.05 (7.68-15.34) ng/ml in the CRC, benign colonic disease (BCD) and healthy donors (HD) groups, respectively. The serum levels of Tn-C were 4.30 (2.12-6.04) ng/ml, 1.60 (1.06-2.30) ng/ml and 2.00 (1.37-3.00) ng/ml in the CRC, BCD and HD groups, respectively. Significantly higher levels of both biomarkers (S100A9 and Tn-C) were found in CRC patients (both $p<0.001$). They observed both S100A9 and Tn-C levels were superior to CEA and CA19-9 levels as CRC diagnostic biomarkers; the combination of S100A9, Tn-C and CEA levels was an excellent biomarker with 79.8% sensitivity and 89.6% specificity. The serum levels of S100A9 and Tn-C in CRC patients were significantly lower after surgery than before surgery ($p<0.01$). They determined that S100A9 and Tn-C levels could serve as diagnostic biomarkers of colorectal cancer.

Ma D, et al (2019) (49) established CD47 homozygous deletion (CD47_/_) in human and mouse Glioblastomas (GB) cells and investigated the impact of eliminating the "don't eat me" signal on tumor growth and tumor– tumor microenvironment (TME) interactions. CD47 knockout (KO) did not significantly change tumor cell proliferation in vitro but significantly increased phagocytosis of tumor cells by macrophages in cocultures. They observed that CD47 KO was increased Tn-C in xenografts, which they further examined in vitro. CD47 loss of function upregulated Tn-C expression in tumor cells via a Notch pathway–mediated mechanism. The depletion of Tn-C in tumor cells boosted the growth of CD47_/_ xenografts in vivo and decreased the number of TAM. Tn-C knockdown also inhibited phagocytosis of CD47_/_ tumor cells in cocultures. Furthermore, Tn-C stimulated the release of pro-inflammatory factors including Tn-Fa via a Toll-like receptor 4 and STAT3-dependent mechanism in human macrophage cells. Their results reveal a vital role for Tn-C in immunomodulation in brain tumor biology and demonstrate the prominence of the TME extracellular matrix in affecting the antitumor function of brain innate immune cells. Their findings link Tn-C to CD47-driven phagocytosis and demonstrate that Tn-C affects the antitumor function of brain TAM, facilitating the development of novel innate immune system-based therapies for brain tumors.

Hawkins A et al (2019) (50) detected microenvironmental factors drive Tn-C and src cooperation to promote invadopodia formation in Ewing Sarcoma. They found that microenvironmental stress upregulated Tn-C expression and this was reduced with the application of the Src inhibitor dasatinib, suggesting that Tn-C expression and Src activation cooperate to promote the invasive phenotype. Their work reported the impact of stress-induced Tn-C expression on enhancing cell invadopodia formation, and provided evidence for a feed-forward loop between Tn-C and Src to promote cell metastatic behavior, and highlighted a pathway by which microenvironment-driven Tn-C expression could be therapeutically targeted in Ewing sarcoma.

Ozkan H et al (2019) (51) evaluated serum levels and urinary excretion of Tn-C and tissue inhibitors of metalloproteinases-1 (TIMP-1) in Acute Kidney Injury (AKI). They obtained two samples of serum and urine, first at the beginning of the study and second samples were obtained at discharge when renal function improved. They found that serum TIMP-1 concentrations (admission and discharge) were higher in patients than healthy controls ($p = 0.0001$ for both comparisons). Tn-C excretion in spot urine was significantly higher in healthy controls than at the admission levels of the patient group ($p = 0.036$). However, TIMP-1 excretion in spot urine was lower in healthy controls than in admission and discharge levels of the patient group ($p = 0.0001$ for both comparisons). They conclude that Tn-C and TIMP-1 might have a role in the pathophysiology of AKI and suggested that further investigations are needed in the same field.

Material and Methods

The present study titled “Comparative Evaluation of Tenascin-C Expression In Oral Submucous Fibrosis And Oral Squamous Cell Carcinoma: An Immunohistochemical Study.” was carried out in the department of oral pathology and microbiology after approval by the institutional ethics committee.

Type of Study: Comparative, retrospective, analytical, observational study.

Study Duration: The study was carried out from May 2018 to October 2019 over a period of 18 months.

Materials:

The materials used for the study were as under:

1. **Paraffin-embedded tissue sections** clinico-histopathologically diagnosed as Oral Submucous Mucous Fibrosis (OSMF) and Oral Squamous Cell Carcinoma (OSCC) from archives.

2. **Olympus Trinocular Research Microscope** (Model – BX-51)

3. **Armamentarium and reagents for immunohistochemical detection of Tenascin**

    - Glassware – Glass bottles, measuring jars, beakers, stirring rods
    - Optiplus Microscope Slides- BioGenex positively charged Glass slides (size 25 × 75 × 1.0 mm)
    - Hot plate
    - Water bath with thermometer
    - Glass coverslips
    - Analytical balance
    - pH meter
    - Incubator (56º- 60ºC)
    - Refrigerator (4ºC)
    - Staining Jars
    - Humidity chamber
    - Microtips and Micropipettes
    - E Z Antigen Retrieval system
    - Plastic vials for storage and making dilutions
    - Primary monoclonal mouse antihuman antibody for Tenascin-C (Novus Biologicals - a Bio-Techne brand)
    - Polymer-HRP Detection Kit/DAB (BioGenex)

The criteria for inclusion and exclusion for the paraffin-embedded tissue sections were as followed.

Inclusion Criteria

All the archived paraffin blocks of specimens that were clinico-histopathologically diagnosed as OSMF and OSCC from the department of oral pathology and microbiology.

1. **Grading of OSMF**

   The criteria grading OSMF was according to **Utsunomiya H et al** (52)

   i. Grade I: Early stage
   ii. Grade II: Intermediate stage
   iii. Grade III: Advanced stage

2. **Grading of OSCC**

   The criteria grading OSCC was according to **Bryne's Classification** (53)

   i. Well Differentiated Squamous cell carcinoma
   ii. Moderately Differentiated Squamous cell carcinoma
   iii. Poorly Differentiated Squamous cell carcinoma

Exclusion Criteria

- OSMF subjects with other potentially malignant disorders (based on clinical record).

SAMPLE SIZE

Formula for sample size

$$n = \delta_{\alpha,\beta}\left[\sum_{i=1}^{r}\sum_{j=1}^{c}\frac{(p_{ij} - p_{i.}p_{.j})^2}{p_{i.}p_{.j}}\right]^{-1}$$

where δ is the non-centrality parameter, pij, pi. and p.j are cell probability and marginal probabilities. For a 95% confidence level and 80% power, the value of δ is 10.83. Using the data from the study, the Pearson's chi-square statistic was 0.58. The study has three groups. In order to have Pearson's statistic near 0.1, the estimated **sample size is 108**. Thus, **36** subjects were included in each group.

Study Groups

Samples were divided into 3 groups: (n=108)

Group number	Study sample grouping	Size
Control Group I	Normal oral mucosa	36 samples
Group II	Oral Submucous fibrosis	36 samples
	OSMF Grade I	12
	OSMF Grade II	12
	OSMF Grade III	12
Group III	Oral Squamous Cell Carcinoma	36 samples
	Well Differentiated OSCC	12
	Moderately Differentiated OSCC	12
	Poorly Differentiated OSCC	12

OSMF: Oral submucous fibrosis, OSCC: Oral Squamous Cell Carcinoma

Method For Immunohistochemical Staining Of Tenascin

The immunohistochemical staining was carried out in the department of Oral Pathology And Microbiology.

Reagent Preparation

Prior to staining the following reagents were prepared.

a. **Phosphate Buffered Saline (PBS) pH 7.4, 0.05 M.**

For 1 Liter

- Sodium dihydrogen phosphate - 3.4gm.
- Disodium hydrogen phosphate- 12.0 gm.
- Sodium chloride - 8.5 gm.
- Distilled water - 1000 ml.

b. **Preparation of Antigen Retrieval Solution (pH -6)**

For 500 ml.

Stock Solution 1

Citric Acid Buffer solution - 2.1 gm in 100 ml distilled water. (at 2 - 8^{o}C)

Stock Solution 2

Sodium citrate Buffer solution - 2.94 gm in 100 ml distilled water. (at 2 - 8^{o}C)

Working Antigen retrieval solution was made by mixing both the stock solutions

Stock solution 1 (9 ml) with stock solution 2 (41 ml). Add distilled water to make up 500 ml, the pH of the solution obtained was around 6.

Procedure For Immunohistochemical Staining Of Tenascin-C

All the selected archived paraffin blocks of specimens of normal oral mucosa (from tissue removed during routine surgical procedures) and sections clinic-histopathologically diagnosed as OSMF and OSCC, and positive and negative controls from the department of oral pathology and microbiology were processed, stained and evaluated for the expression of Tn-C by immunohistochemical method.

Following protocol was followed:

1. The slides were dehydrated by increasing grades of alcohol and brought to distilled water.
2. Treated with hydrogen peroxide to eliminate endogenous peroxidase activity.
3. Antigen retrieval with tri-sodium citrate for Tenascin-C was done with primary antibody (monoclonal).
4. Sections were rinsed with PBS for five minutes.
5. Blocking antibody was applied, incubated for 20 minutes at room temperature, residual fluid thrown off.
6. Primary antibody was applied for 60 minutes at room temperature.
7. After rinsing, the slides were incubated with a biotin-conjugated secondary antibody at 20-37°C for 20 minutes.
8. After rinsing and incubation, proceeded with chromogen of final developmental DAB or use DAB Kit (Control the degree of staining with regular microscopy).
9. The slides were stained and differentiated in hematoxylin.

Methods Of Data Collection

Using light microscopy, brown color expression was considered for Tn-C positive expression by three independent observers. Evaluation for intensity of tenascin-C was done according to the study by **Regezi J et al** (14):

0: almost negative;

1+: weak positive staining;

2+: distinct positive staining;

3+: extensive positive staining.

Statistical Methods Employed

The data obtained was compiled on an MS Office Excel Sheet (v 2010) and was subjected to statistical analysis using SPSS v 21.0, IBM (Statistical package for social sciences) software.

For all the statistical tests, $P<0.05$ was considered to be statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%.

Ethical Issues Involved - None

COLOR PLATE 1

Figure 2: Positively charged microscopic slides

Figure 3: Glassware and pipettes

COLOR PLATE 2

Figure 4: Antigen retrieval system

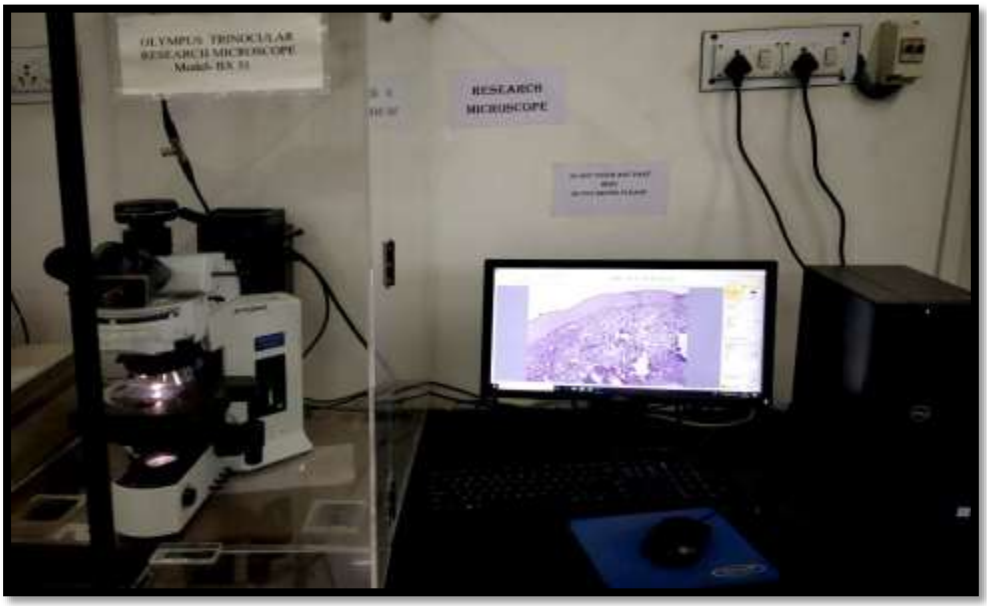


Figure 5: Research microscope with attachment for photomicrography.

COLOR PLATE 3

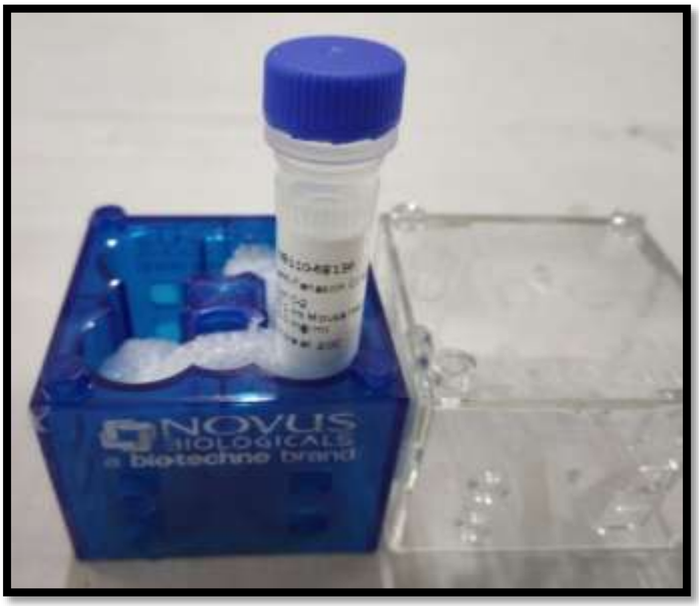


Figure 6: Primary monoclonal mouse antihuman antibody for Tenascin-C (Novus Biologicals - a Bio-Techne brand)

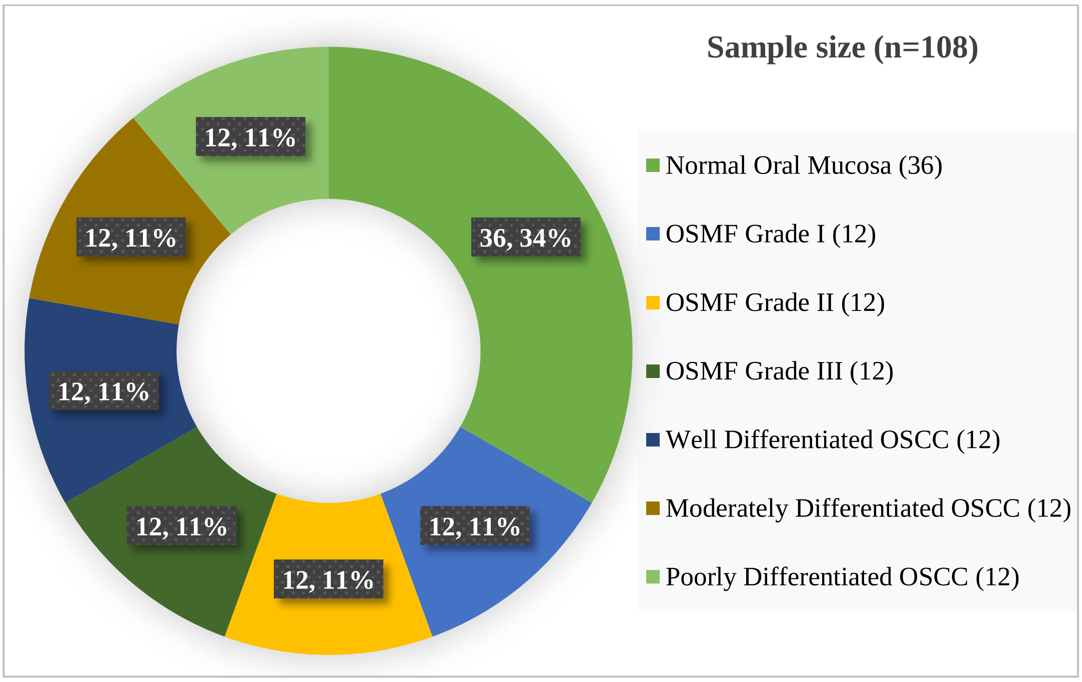
Figure 7: Super sensitive polymer-HRP/ DAB detection system (BioGenex)

Observations and Results

The present comparative, retrospective, analytical, observational study consisted of a total of 108 samples. Out of these samples, 36 were of each clinico-histopathologically diagnosed as oral submucous fibrosis OSCC. Out of 36 samples of OSMF, 12 samples each of OSMF grade-I, OSMF grade -II and OSMF grade -III. Among the 36 samples of OSCC, 12 samples were each of well-differentiated OSCC, moderately-differentiated OSCC, and poorly-differentiated OSCC. 36 samples of normal oral mucosa were considered as a control group for comparative purposes.

Study Group

Graph 1: Pie chart showing the distribution of sample size



Each study group was evaluated for the expression of Tenascin-C in respective of the intensity of staining in the connective tissue stroma of immunohistochemically stained sections. Intensity for the Tn-C was observed in all three groups by 3 observers under low power view (10x) with constant high/ full intensity of light under light microscope.

Kendall's tau-b test was applied to minimize the inter-observer variability for intensity. The data were collected, tabulated and analyzed by SPSS v21.0, IBM (Statistical package for social sciences) software and the statistical significance was tested at a 5% level.

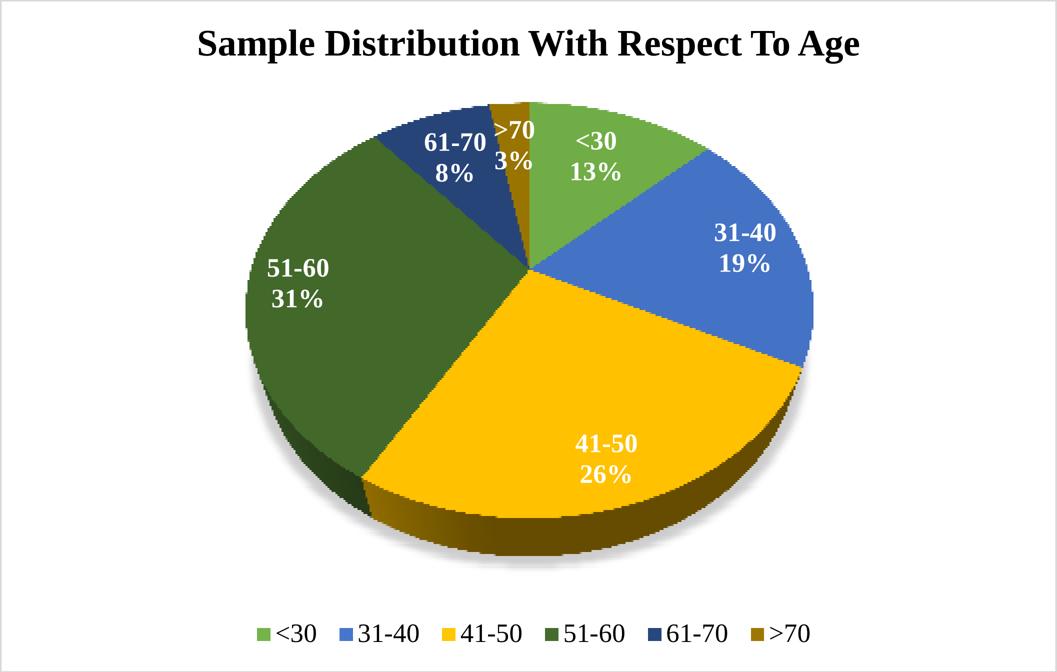
The observations of the present study are explained in detail in the following sections.

Age distribution in the study

Table 2: Distribution of study sample with respect to the age range

Age Range (years)	No. of Samples	Percentage
<30	14	12.96 %
31-40	21	19.44%
41-50	28	25.92%
51-60	33	30.55%
61-70	9	08.33%
>70	3	02.77%
Total	108	100.0%

Graph 2: Pie chart of distribution of study sample with respect to the age range



Comments (Table 2 and Graph 2)

- Among total sample of 108 subjects, 14 (12.96%) subjects were below 30 years of age, 21 (19.44%) subjects were in age range 31-40 years of age, 28 (25.92%) subjects were in age range 41-50 years of age, 33 (30.55%) subjects were in age range 51-60 years of age, 9 (8.33%) subjects were in age range 61-70 years of age, and 3 (2.77%) subjects were above 70 years of age.

Table 3: Distribution of mean age in the study

Group	Mean age (Years)	Standard deviation (Years)
Group I- Normal oral mucosa (control group)	42.5428	11.5333
Group II - OSMF	40.6944	12.6915
Group III - OSCC	54.9444	11.2069
Total	46.0605	11.8106

Comments (Table 3)

The mean age of the study group was 46.06 years with a standard deviation of 11.81 years.

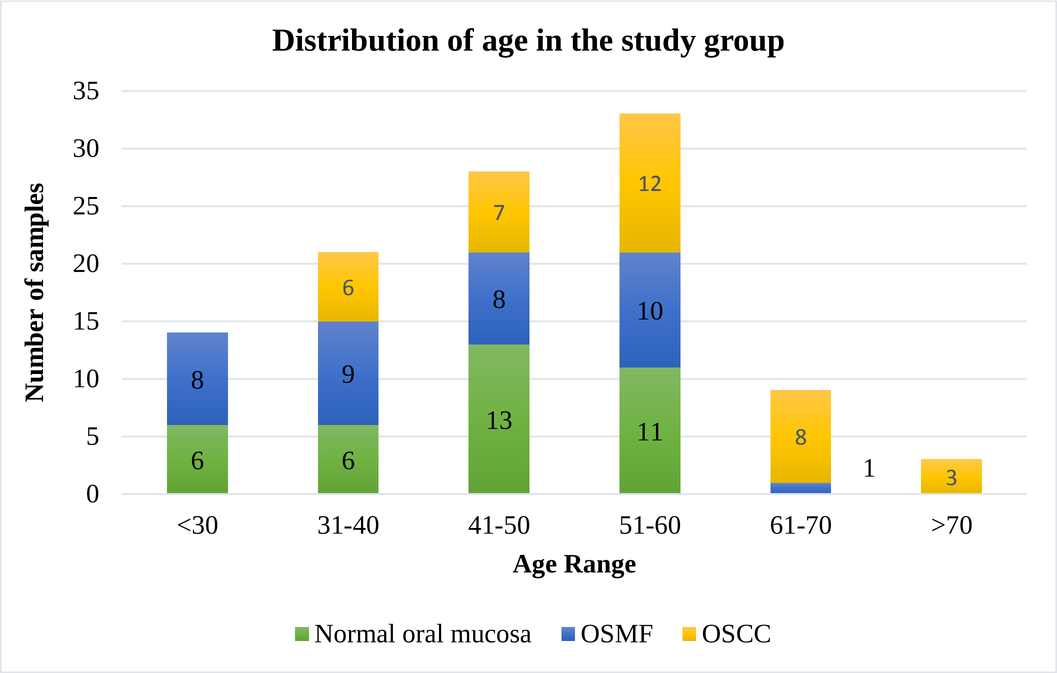
- The mean age in the control group was 42.54 years with a standard deviation of 11.53 years.
- The mean age of subjects with OSMF included in the study was 40.69 years with a standard deviation of 12.69 years.
- The mean age of subjects with OSCC included in the study was 54.94 years with a standard deviation of 11.20 years.

Table 4: Correlation of age distribution in the study group

Age Range	No. of samples (percent)			P-value
	Group I Normal oral mucosa	Group II OSMF	Group III OSCC	
<30	6 (16.7%)	8 (22.22%)	0 (0%)	0.0032 (S)
31-40	6 (16.7%)	9 (25%)	6 (16.7%)	
41-50	13 (36.11%)	8 (22.22 %)	7 (19.44%)	
51-60	11 (30.5%)	10 (27.77%)	12 (33.33%)	
61-70	0 (2.8%)	1 (2.8%)	8 (22.22%)	
>70	0 (0%)	0 (0%)	3 (08.33%)	
Total	36 (100%)	36 (100%)	36 (100%)	

Chi square Test. X^2 =29.3485. S: Significant ($P<0.05$). OSMF: Oral Submucous Fibrosis, OSCC: Oral Squamous Cell Carcinoma

Graph 3: Bar diagram of distribution of age in the study group



Comments (Table 4 and Graph 3)

- In control group, 6 (16.7%) subjects were below 30 years of age, 6 (16.7%) subjects were in the age range 31-40 years, 13 (36.11%) subjects were in age range 41-50 years, and 11 (30.5%) subjects were in age range 51-60.
- In group II, 8 (22.22%) subjects were below 30 years of age, 9 (25%) subjects were in age range 31-40 years, 8 (22.22%) subjects were in age range 41-50 years, and 10 (27.77%) subjects were in age range 51-60, and 1 (2.8%) subject was in age range 61-70 years.
- In group III, 6 (16.7%) subjects were in age range 31-40 years, 7 (19.44%) subjects were in age range 41-50 years, 12 (33.33%) subjects were in age range 51-60 years 8 (22.22%) subjects were in age range 61-70 years, and 3 (8.33%) were above 70 years of age.
- Correlation of age distribution was analyzed in the study group by Chi-square test and was found to be statistically significant P= 0.032 (P<0.05)

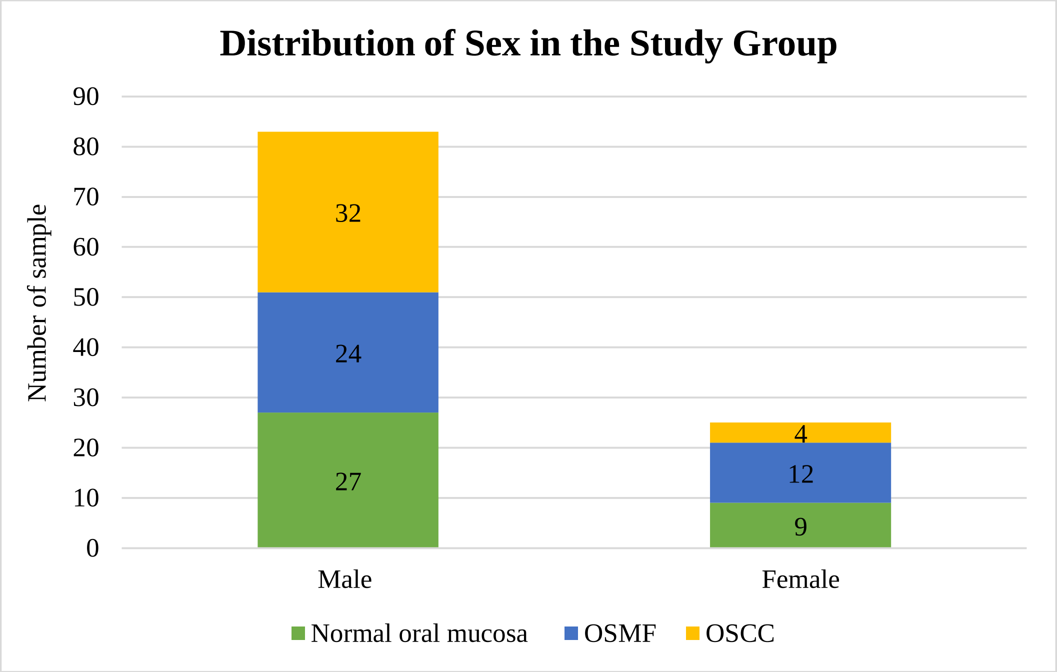
Sex distribution in the study

Table 5: Correlation of sex distribution in the study group

Group	**No. of samples (percent)**	**No. of samples (percent)**	**Total**	**P-value**
	Male	**Female**		
Group I Normal oral mucosa (control group)	27 (75%)	9 (25%)	36	0.073 (NS)
Group II OSMF	24 (66.66%)	12 (33.33%)	36	
Group III OSCC	32 (88.9%)	4 (11.1%)	36	
TOTAL	**83** (76.85%)	**25** (23.31%)	108	

Chi-square Test. X^2 =5.1007. NS: Nonsignificant (P>0.05). OSMF: Oral Submucous Fibrosis, OSCC: Oral Squamous Cell Carcinoma

Graph 4: Bar diagram of distribution of sex in the study group



Comments (Table 5 and Graph 4)

- Among the total sample of 108 subjects, 83 (76.85%) subjects were males and 25 (23.31%) were females.
- In the control group, 27 (75%) subjects were males while 9 (25%) were of females.
- Among Groups II and Group III (disease group), 24 (66.66%) subjects were males and 12 (33.33%) subjects were females in the OSMF while in OSCC, 32 (88.9%) subjects were males and 4 (11.1%) subjects were females.
- Correlation of sex distribution was analyzed in the study groups by Chi-square Test and was found to be statistically insignificant P= 0.073 ($p>0.05$)

Tn-C Expression in Group I -Normal Oral Mucosa (control group)

The Tn-C expression in normal oral mucosa was observed at epithelial connective tissue junction (ECJ) as a very faint linear band of brown staining of almost negative intensity. Thus, the overall staining score for normal oral mucosa was calculated to be 0 intensity.

The Tn-C expression in different grades of OSMF (Group II)

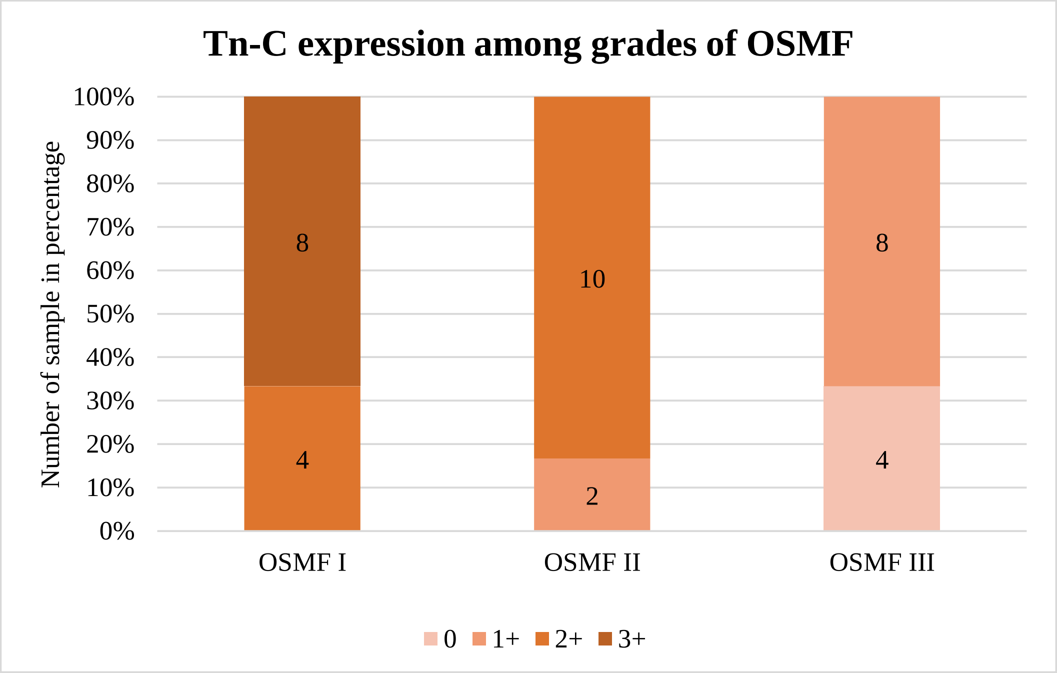
The Tn-C expression in three grades of OSMF was observed as a band at the ECJ. The expression varied from grade to grade. It was evaluated and analyzed significantly by the Chi-Square test.

Table 6: Tn-C expression among grades of OSMF (Group II)

OSMF GRADES	Intensity of Tn-C Expression				Total	P-Value
	0	**1+**	**2+**	**3+**		
OSMF I	0	0	4 (33.33 %)	8 (66.66%)	12	0.001 (S)
OSMF II	0	2 (16.66%)	10 (83.33%)	0	12	
OSMF III	4 (33.33%)	8 (66.66%)	0	0	12	
Total	4 (11.11%)	10 (27.77%)	14 (38.88%)	8 (22.22%)	**36**	

Chi-Square, X^2 =45.257 $P < 0.05$, Significant (S). OSMF: Oral Submucous Fibrosis, Tn-C: Tenascin-C

Graph 5: Bar diagram of Tn-C expression among grades of OSMF (Group II)



Comments (Table 6 and Graph 5)

- In OSMF grade I, 4 (33.33 %) samples showed 2+ (distinct positive) staining and 8(66.66%) samples showed 3+ (extensive positive) staining.

- In OSMF grade II, 2 (16.66%) samples showed 1+ (weak positive) staining and 10 (83.33%) samples showed 2+ (distinct positive) staining.
- In OSMF grade III, 4 (33.33 %) samples showed 0 (almost negative) staining and 8(66.66%) samples showed 1+ (weak positive) staining.
- Among total 36 samples of OSMF, 4 (11.11%) samples showed 0 (almost negative) staining, 10 (27.77%) samples showed 1+ (weak positive) staining, 14 (38.88%) samples showed 2+ (distinct positive) staining, and 8 (22.22%) samples showed 3+ (extensive positive) staining irrespective of grades.
- The analysis of the intensity of Tn-C expression between grades of OSMF was statistically significant with P=0.001 (< 0.05)

The Tn-C expression in different grades of OSCC (Group III)

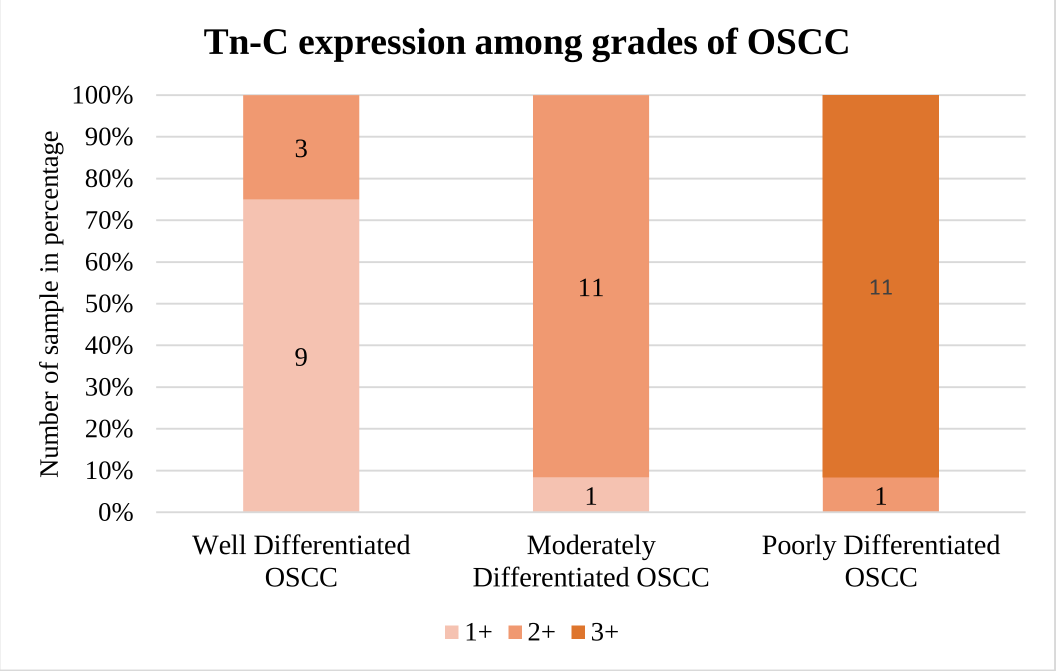
The Expression of Tn-C in well-differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC were observed widespread in the tumor stroma. Intensity was varied with varying degrees of differentiation. The intensity was evaluated and analyzed statistically by the Chi-Square test.

Table 7: Tn-C expression among grades of OSCC (Group III)

OSCC GRADES	Intensity of Tn-C Expression			Total	P-Value
	1+	2+	3+		
Well Differentiated OSCC	9 (75%)	3 (25%)	0	12	**0.001 (S)**
Moderately Differentiated OSCC	1 (8.33%)	11 (91.66%)	0	12	
Poorly Differentiated OSCC	0	1 (8.33%)	11 (91.66%)	12	
Total	10 (27.77%)	15 (41.66%)	11 (30.55%)	36	

Chi-Square, $X^2 = 47.800$, $P < 0.05$, Significant (S). OSCC: Oral Squamous Cell Carcinoma, Tn-C: Tenascin-C

Graph 6: Bar diagram of Tn-C expression among grades of OSCC



Comments (Table 7 and Graph 6)

- In well-differentiated OSCC, 9 (75%) samples showed 1+ (weak positive) staining and 3 (25%) samples showed 2+ (distinct positive) staining of Tn-C expression.
- In moderately differentiated OSCC, 1 (8.33%) sample showed 1+ (weak positive) staining and 11 (91.66%) samples showed 2+ (distinct positive) staining of Tn-C expression.
- In poorly differentiated OSCC, 1 (8.33%) sample showed 2+ (distinct positive) staining and 11 (91.66%) samples showed 3+ (extensive positive) staining of Tn-C expression.
- Among total 36 samples of OSCC, 10 (27.77%) samples showed 1+ (weak positive) staining, 15 (41.66%) samples showed 2+ (distinct positive) staining, and 11 (30.55%) samples showed 3+ (extensive positive) staining of Tn-C expression irrespective of grades of differentiation.
- The analysis of the intensity of Tn-C expression between grades of OSCC was statistically significant with P=0.001 (< 0.05)

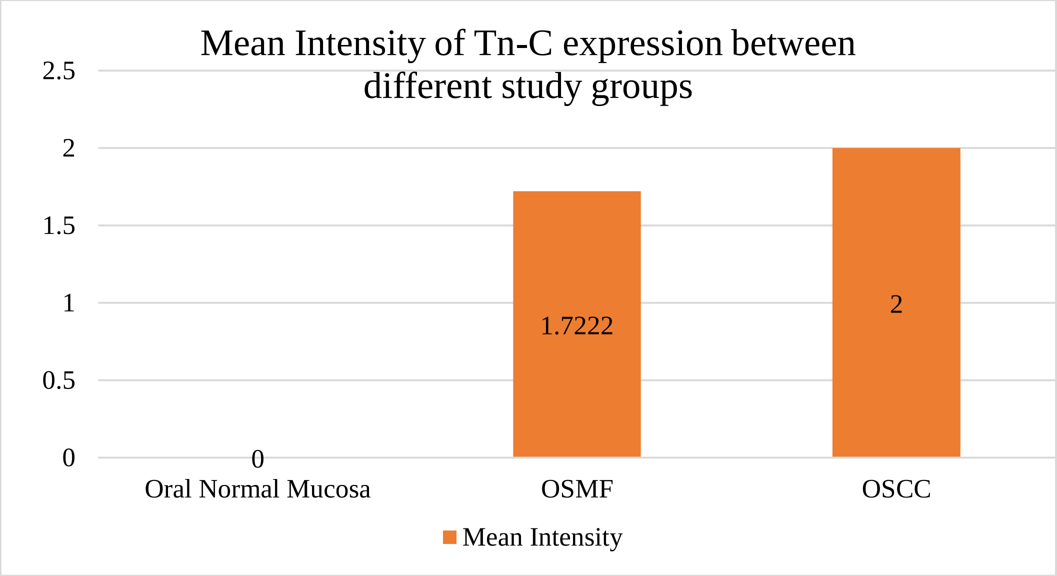
Comparison of Tn-C expression in normal oral mucosa, OSMF and OSCC.

Table 8: Tn-C expression among Normal Oral Mucosa, OSMF, and OSCC

Study group	N	Mean Intensity of Tn-C expression	Std. Deviation	P-Value
Normal Oral Mucosa (Control group)	36	0	0.0	0.0064 (S)
OSMF (Group II)	36	1.7222+	0.45799	
OSCC (Group III)	36	2+	0.37673	

Two-Tailed Test. Degree of Freedom=70. S: Significant ($P<0.05$). OSMF: Oral Submucous Fibrosis, OSCC: Oral Squamous Cell Carcinoma, Tn-C: Tenascin-C

Graph 7: Bar diagram of Tn-C expression among Normal Oral Mucosa, OSMF, and OSCC



Comments (Table 8 and Graph 7)

- Mean intensity of Tn-C expression staining normal oral mucosa was 0, OSMF was 1.7222+ which is between 1+ (weak positive staining) and 2+ (distinct positive staining) and OSCC was 2+ (distinct positive staining).
- Comparative analysis of Tn-C expression between normal oral mucosa, OSMF and OSCC irrespective of histopathological grading was statistically significant ($P<0.05$).

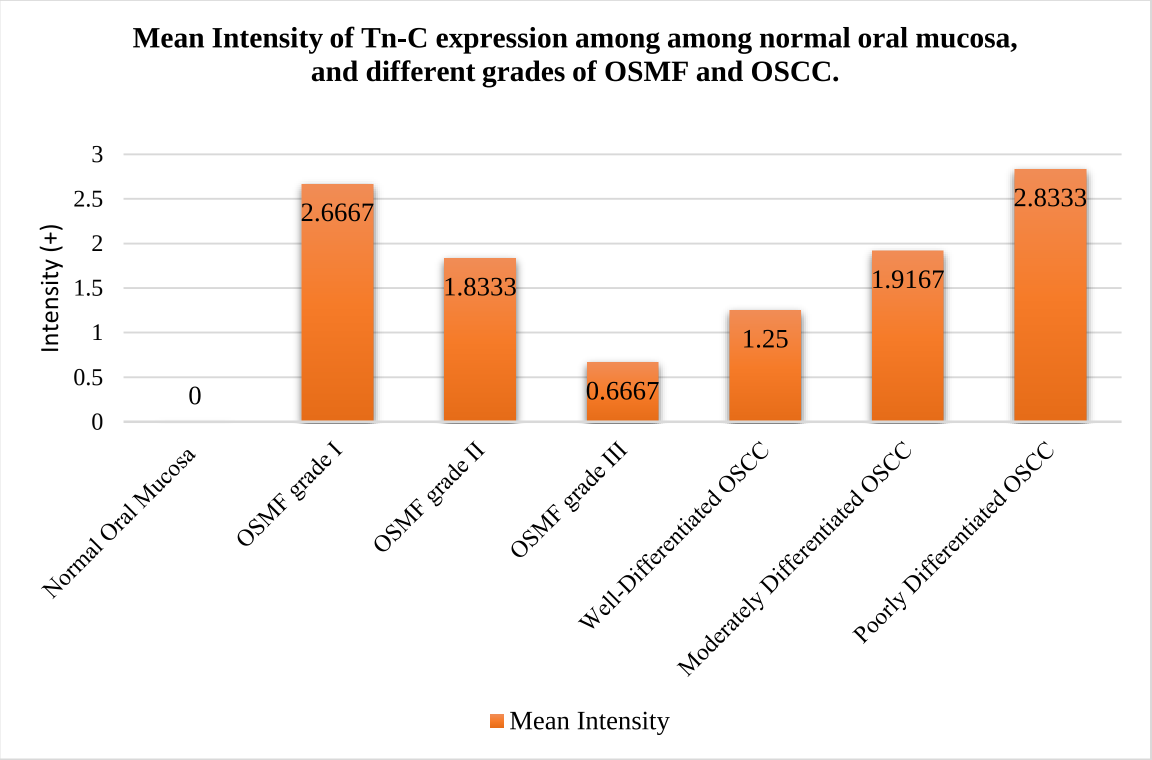
Comparative evaluation of Tn-C expression among all the study groups was then studied. The mean of observed intensity with standard deviation in each group was evaluated and analyzed statistically by ONE-WAY ANOVA.

Table 9: Comparative evaluation intensity of Tn-C expression among normal oral mucosa, and different grades of OSMF and OSCC.

Study group	**N**	**Mean Intensity of Tn-C expression**	**Std. Deviation**	**Std. Error**	**P VALUE**
Normal Oral Mucosa (Control group)	36	0	0.0	0	0.001 **(S)**
OSMF grade I	12	2.6667+	0.49237	0.14213	
OSMF grade II	12	1.8333+	0.38925	0.11237	
OSMF grade III	12	0.6667+	0.49237	0.14213	
Well-Differentiated OSCC	12	1.2500+	0.45227	0.13056	
Moderately Differentiated OSCC	12	1.9167+	0.28868	0.08333	
Poorly Differentiated OSCC	12	2.8333+	0.38925	0.11237	
Total	108	1.5952+	1.03107	0.11250	

Degree of Freedom=6.04, ANOVA (F) = 82.862. Significant ($P<0.05$). OSMF: Oral Submucous Fibrosis, OSCC: Oral Squamous Cell Carcinoma, Tn-C: Tenascin-C

Graph 8: Bar diagram of mean intensity of Tn-C expression among normal oral mucosa, and different grades of OSMF and OSCC.



Comments (Table 9 and Graph 8)

- The mean intensity of Tn-C expression in normal oral mucosa was 0 as the expression was almost negative.
- The mean intensity of Tn-C expression in OSMF grade I was 2.66+ which is between 2+ (distinct positive staining) and 3+ (extensive positive staining) with a standard deviation of 0.49237.
- The mean intensity of Tn-C expression in OSMF grade II was 1.83+ which is between 1+ (weak positive staining) and 2+ (distinct positive staining) with a standard deviation of 0.38925.
- The mean intensity of Tn-C expression in OSMF grade III was 0.66+ which is between 0 (almost negative) and 1+ (weak positive staining) with a standard deviation of 0.49237.

- The mean intensity of Tn-C expression in well-differentiated OSCC was 1.2500+ which is between 1+ (weak positive staining) and 2+ (distinct positive staining) with a standard deviation of 0.45227.
- The mean intensity of Tn-C expression in moderately differentiated OSCC was 1.9167+ which is between 1+ (weak positive staining) and 2+ (distinct positive staining) with a standard deviation of 0.28868.
- The mean intensity of Tn-C expression in poorly differentiated OSCC was 2.8333+ which is between 2+ (distinct positive staining) and 3+ (extensive positive staining) with a standard deviation of 0.38925.
- The mean intensity of Tn-C expression was highest in poorly differentiated OSCC and lowest in OSMF grade III among disease groups.
- The mean intensity of Tn-C expression decreases with an increase in grade of OSMF but increases from well-differentiated OSCC to poorly differentiated OSCC. Comparative analysis of mean intensity of Tn-C expression among normal oral mucosa, and different grades of OSMF and OSCC was statistically significant with $p=0.001$ (<0.05)

Hypothesis

Tn-C immunoreactivity was expressed distinctly in the study group as there was enhanced Tn-C expression in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma as compared to Normal Oral Mucosa.

Thus, from the above results, the null hypothesis was disproved.

COLOR PLATE 4

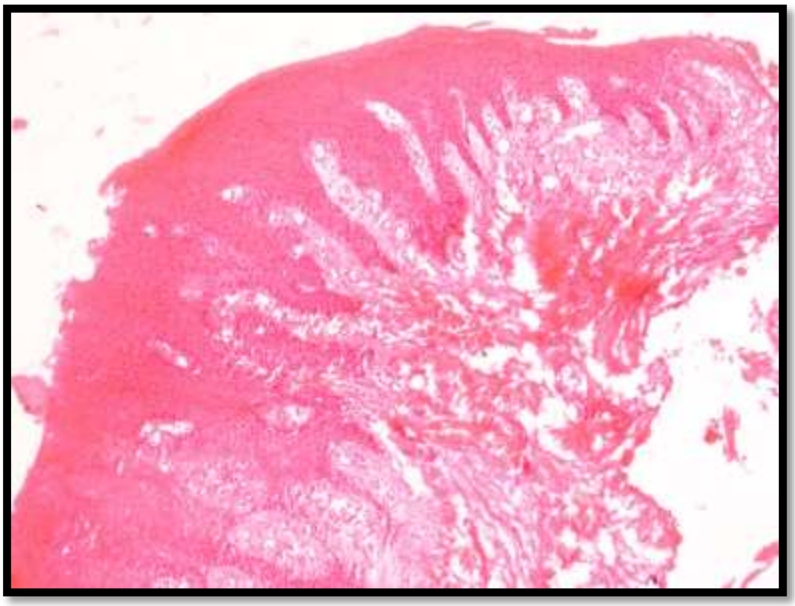


Figure 8: Normal Oral Mucosa H&E stain (10x)

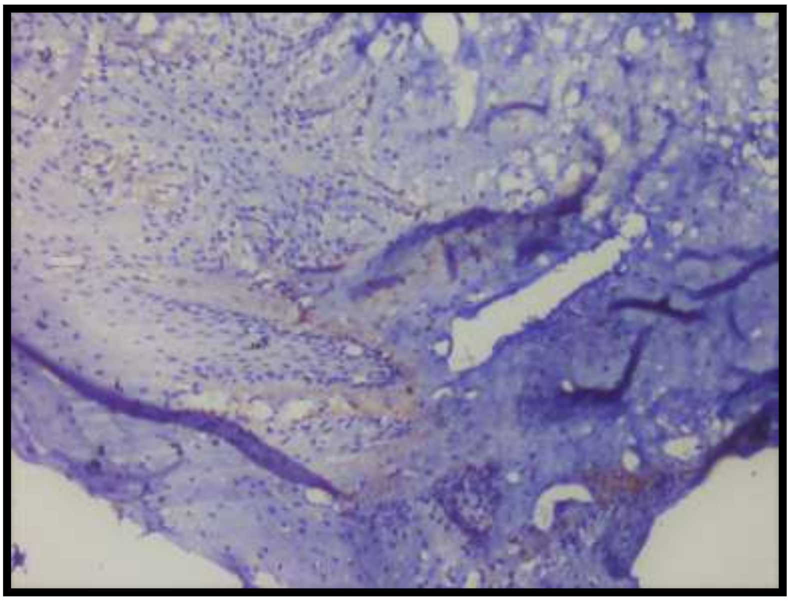


Figure 9: IHC expression of Tn-C at ECJ in Normal Oral Mucosa as a faint band of immunoreactivity which is considered as almost negative. (20X)

COLOR PLATE 5

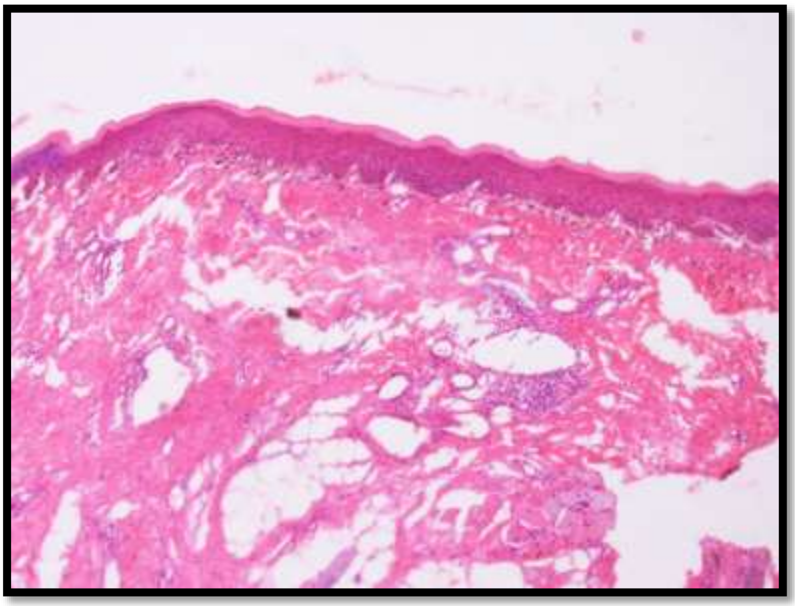


Figure 10: OSMF Grade-I H&E stain (10X)

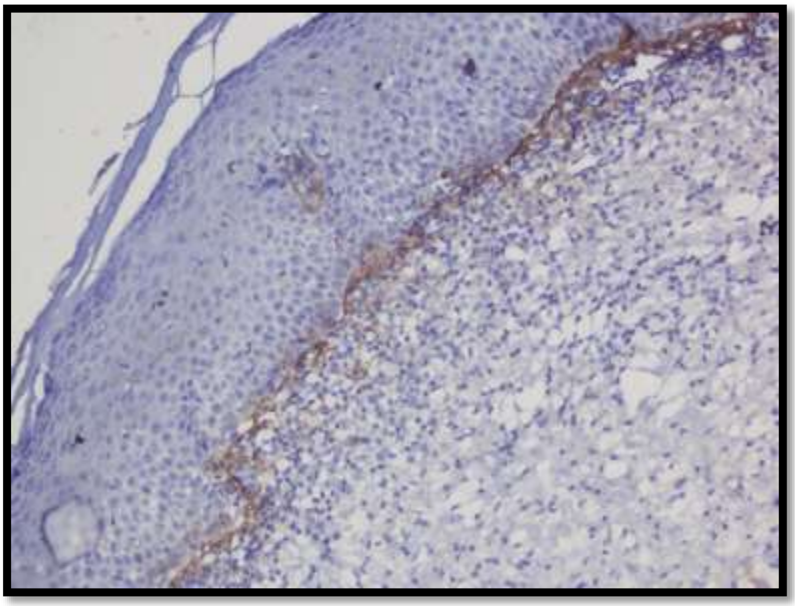


Figure 11: IHC expression of Tn-C in OSMF Grade-I as subepithelial band of extensive immunoreactivity. (20X)

COLOR PLATE 6

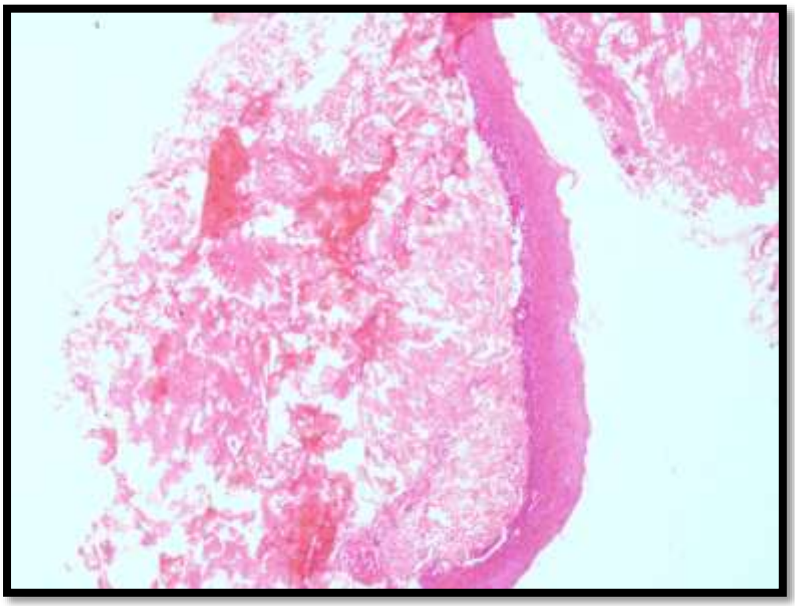


Figure 12: OSMF Grade-II H&E stain (10X)

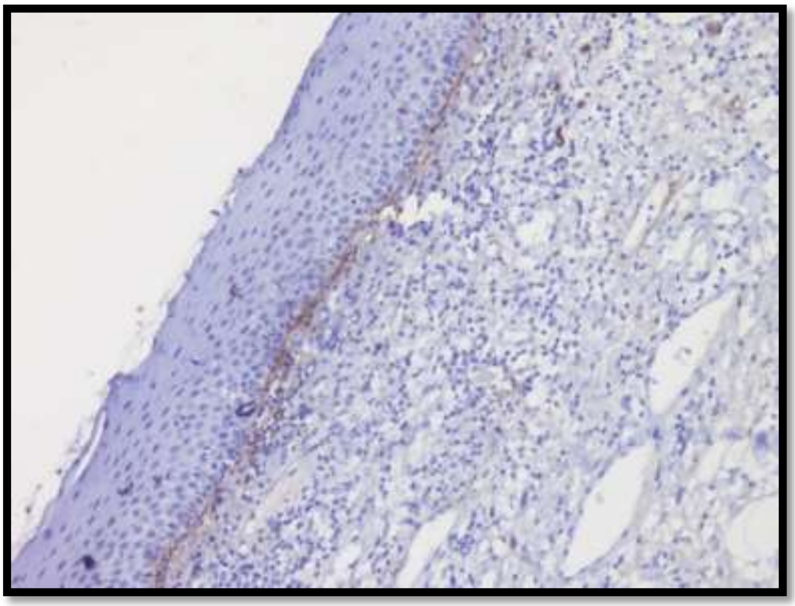


Figure 13: IHC Tn-C expression in OSMF Grade-II as subepithelial band of immunoreactivity with distinct intensity. (20X)

COLOR PLATE 7

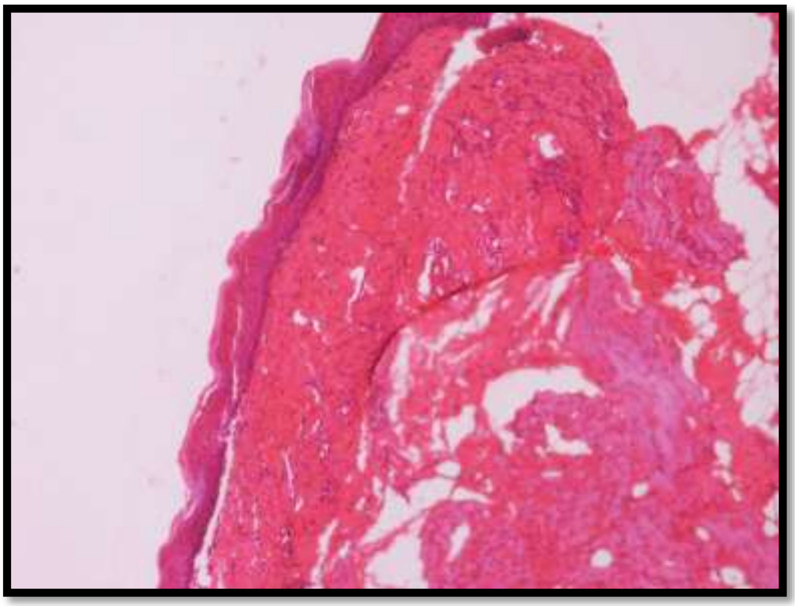


Figure 14: OSMF Grade-III H&E stain (10X)

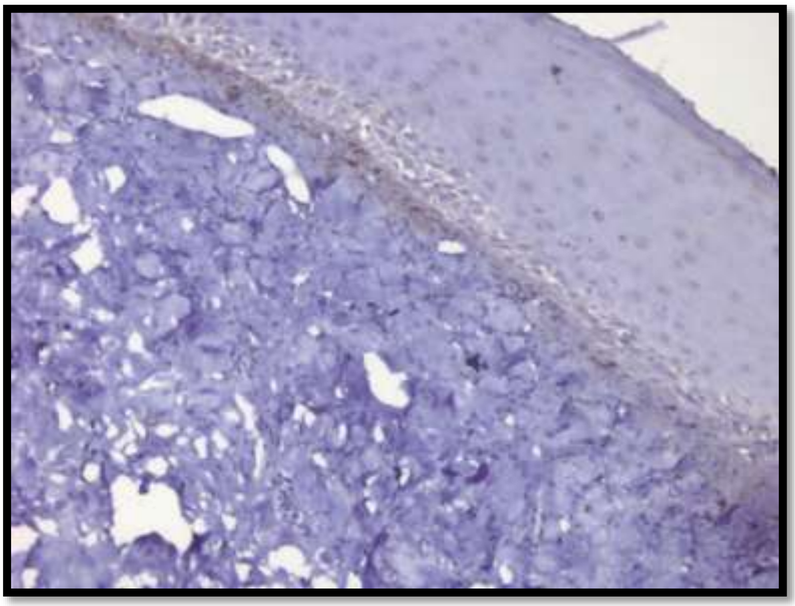


Figure 15: IHC expression of Tn-C in OSMF Grade-III as subepithelial band of weak immunoreactivity. (20X)

COLOR PLATE 8

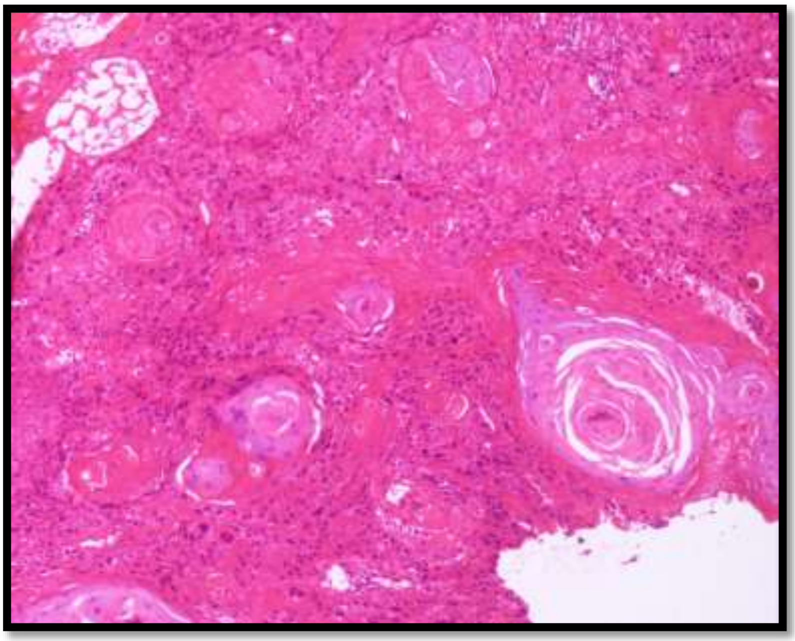


Figure16: Well Differentiated Squamous Cell Carcinoma, H&E stain (10X)

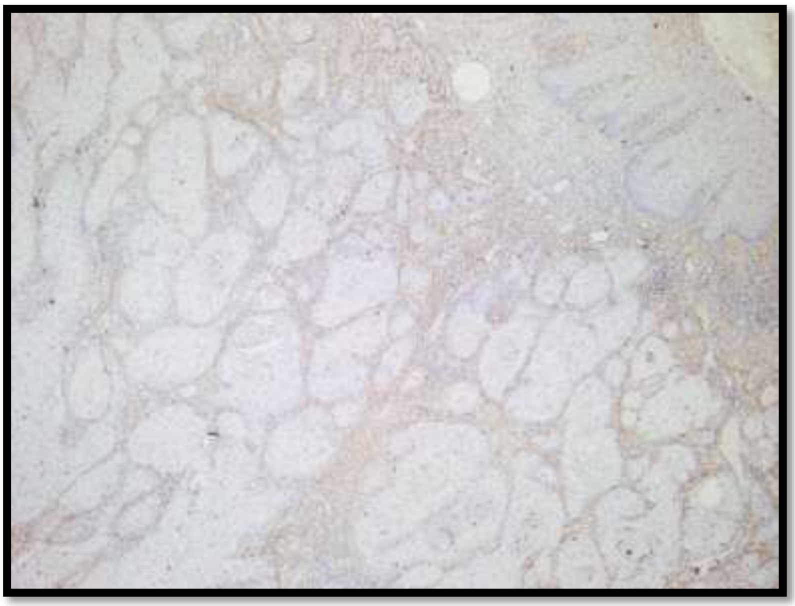


Figure 17: IHC expression of Tn-C in Well Differentiated Squamous Cell Carcinoma with weak intensity. (4X)

COLOR PLATE 9

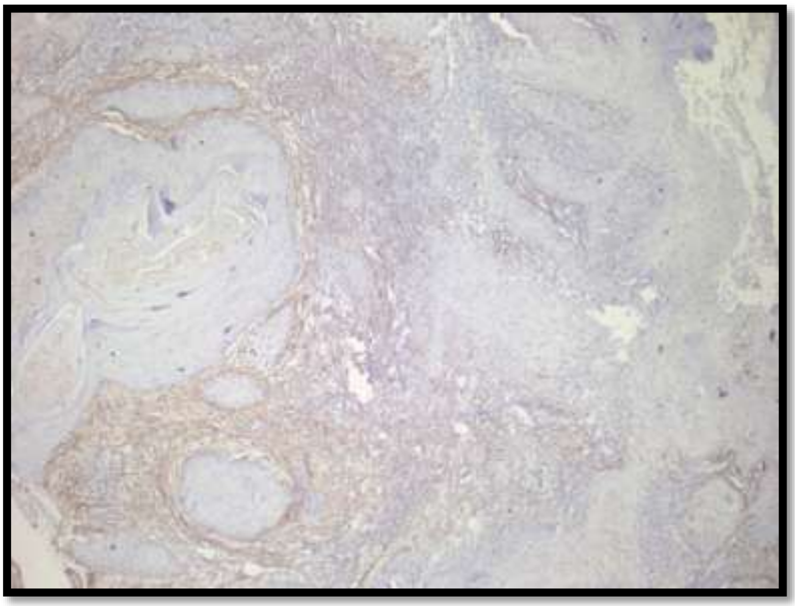


Figure 18: IHC expression of Tn-C in Well Differentiated Squamous Cell Carcinoma. (10X)

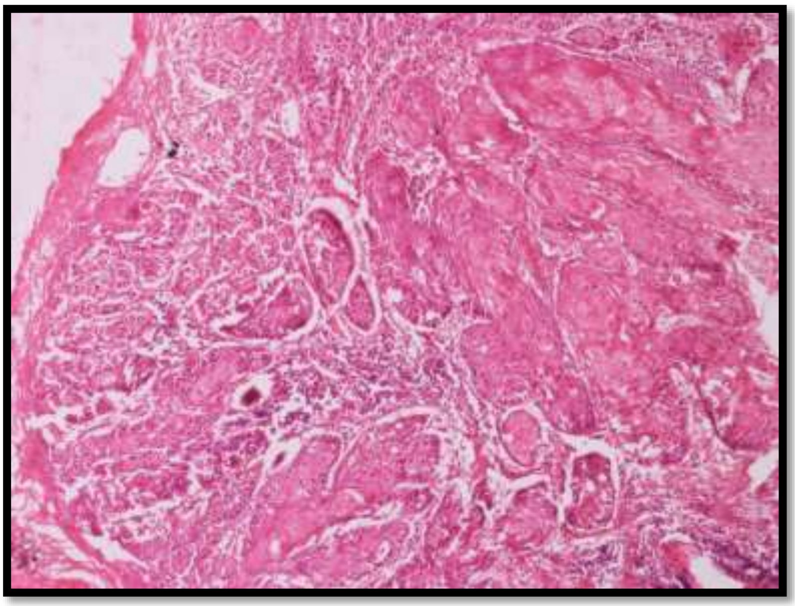


Figure 19: Moderately Differentiated Squamous Cell Carcinoma, H&E stain (10X)

COLOR PLATE 10

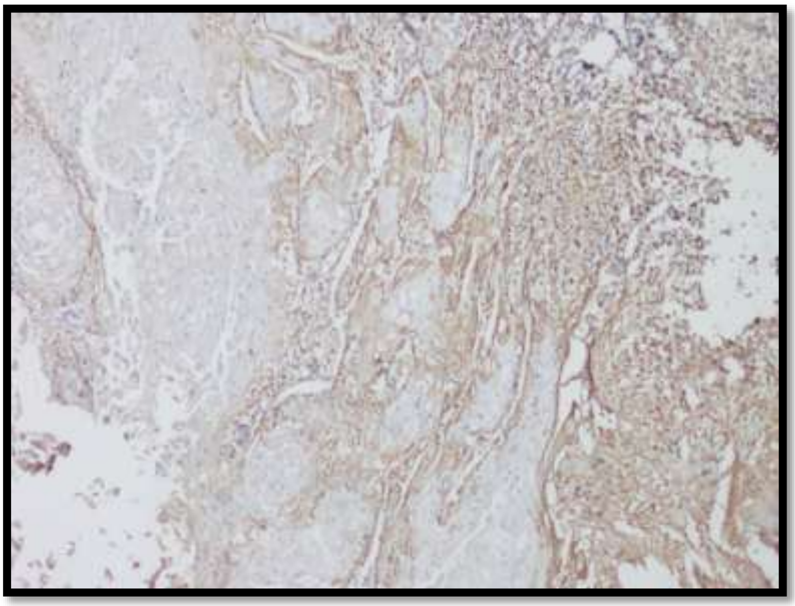


Figure 20: IHC expression of Tn-C in Moderately Differentiated Squamous Cell Carcinoma with distinct intensity. (10X)

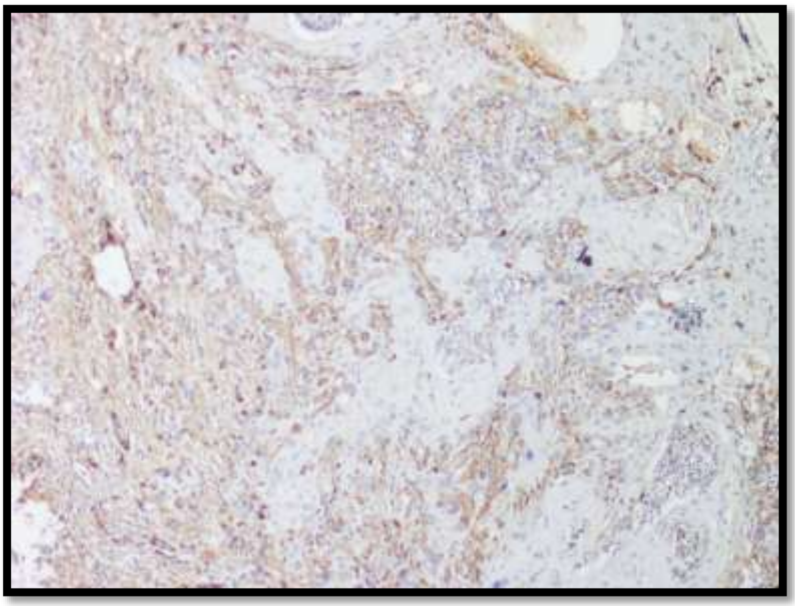


Figure 21: IHC expression of Tn-C in Moderately Differentiated Squamous Cell Carcinoma with distinct intensity. (20X)

COLOR PLATE 11

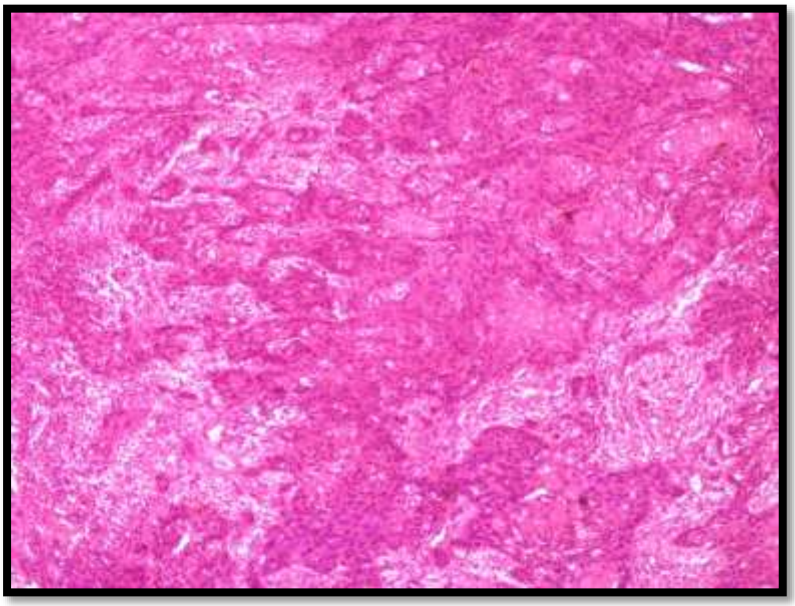


Figure 22: Poorly Differentiated Squamous Cell Carcinoma, H&E stain (10X)

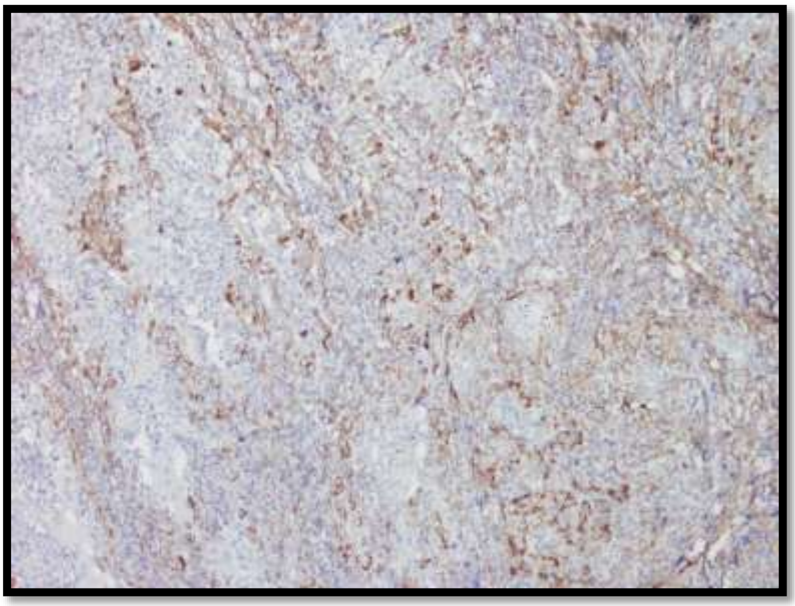


Figure 23: IHC expression of Tn-C in Poorly Differentiated Squamous Cell Carcinoma with extensive intensity. (10X)

COLOR PLATE 12

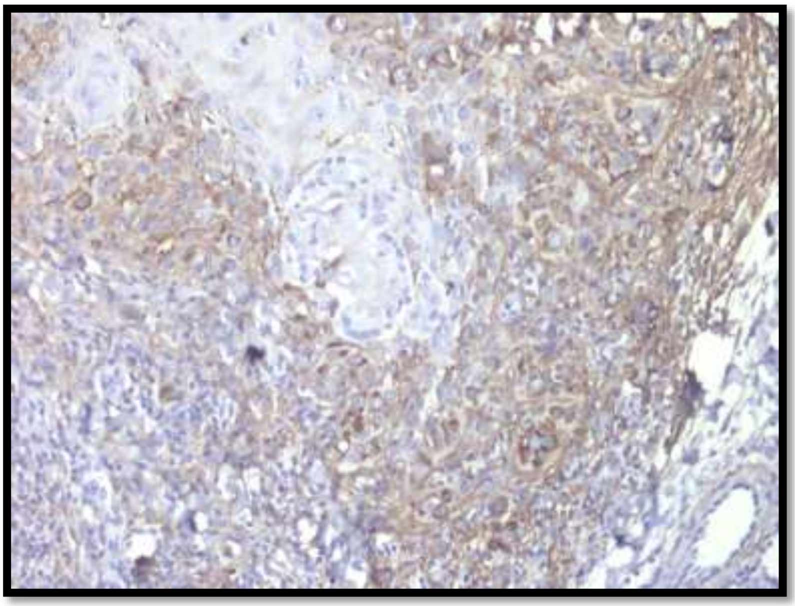


Figure 24: IHC expression of Tn-C in Poorly Differentiated Squamous Cell Carcinoma with extensive dark brown expression. (20X)

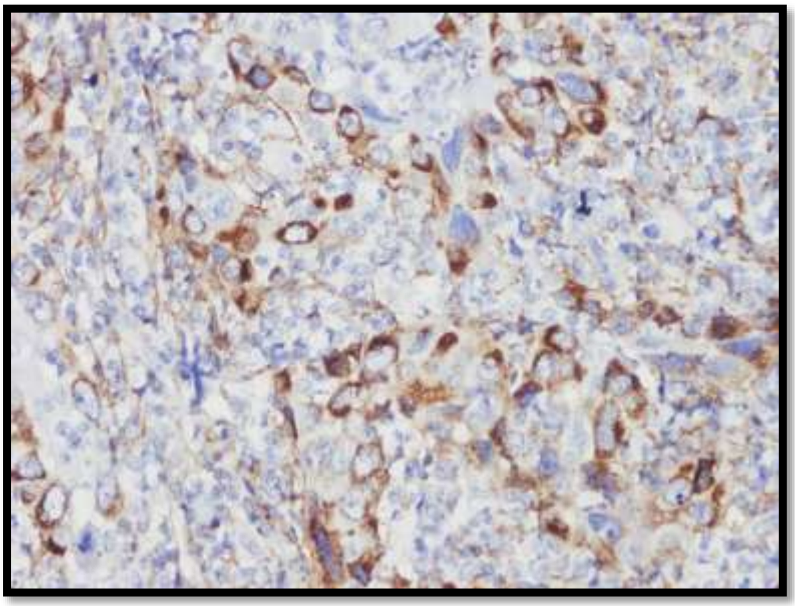


Figure 25: IHC expression of Tn-C in Poorly Differentiated Squamous Cell Carcinoma with extensive intensity and dark brown expression. (40X)

Discussion

Oral cancer is the sixth most common cancer reported globally having an annual incidence of over three lakh cases, 62% of which arise in developing countries. In India, oral cancer accounts for 30% of all cancers in the country. Cancer pertaining to the oral cavity is the 8th most frequent cancer in the World among males and 14th most frequent among females. The variation in incidence and pattern of the disease can be attributed to the combined effect of aging as well as regional differences in the prevalence of disease-specific risk factors (54). Oral cancer is the most frequent cancer of head and neck (1) and about 80% of oral cancers are preceded by oral potentially malignant disorders (OPMD) in India (2).

Multi-step theory of carcinogenesis describes the development of oral cancer from potentially malignant mucosal lesions to invasive malignant changes which causes serial histological and clinical alterations. The term ‘potentially malignant disorders’ (OPMD) used by WHO is more widely accepted today as it conveys that not all lesions described under this term may transform into cancer, rather, there is a family of morphological alterations among which some may have an increased risk for malignant transformation. Oral leukoplakia, erythroplakia, erythroleukoplakia, oral submucous fibrosis (OSMF), palatal lesions in reverse smokers, oral lichen planus, oral lichenoid reactions, graft-versus-host disease (GvHD), oral lupus erythematosus, and some hereditary conditions, such as dyskeratosis congenita and epidermolysis bullosa are included in OPMDs (3).

OSMF is an OPMD with a malignant transformation rate of 7%–12% (55). It is a chronic, insidious disease that affects the lamina propria of the oral mucosa with unique in its presentation and geographical predisposition (19). Clinically, blanching of mucosa, loss of normal pigmentation, a burning sensation in the mucosa on consumption of spicy food, initiation in limited mouth opening are early features of OSMF. As the disease progress, fibrous bands in the oral mucosa are palpable, tongue becomes rigid with limited mobility, palate may appear pale with horizontal bands across the soft palate, uvula may be shrunken or deformed, loss of papillae, cheeks are sunken, ultimately leading to termination of mouth opening. The severity of the disease is generally measured objectively by assessing mouth opening reduction. This disease has a significant impact on the quality of life of affected subjects (3).

An areca nut alkaloid, arecoline is the chief causative factor for fibroblastic proliferation and increased collagen formation leading to fibrosis in the submucosa which is pathognomic for OSMF. Arecoline may take part in ECM remodeling as it influences the deposition of ECM by increasing the production of tissue inhibitors of matrix metalloproteinases (TIMP)-1. Interaction of arecoline and keratinocytes induce differentiation of myofibroblasts from fibroblasts. Arecoline regulates transglutaminase-2 (TGM – 2) that is involved in several fibrotic changes (4).

Over 95% of oral cancers are OSCCs, which is a multistep and multifactorial process involving field carcinogenesis and intraepithelial clonal spread. The frequency of OSCCs in India remains one of the highest in the World, being 30-50% of the total global cancer incidence. In large part, it is attributed to exposure to carcinogens such as tobacco (56). Notably, the great morbidity and mortality rates of this disease have not improved in decades. Hence, early recognition is imperative to improve oral cancer survival rates, preserve function and enhance aesthetic and psychological outcomes. OSCC is of significant public health importance in India. Firstly, it is diagnosed at a later stage resulting in low treatment outcomes and considerable cost to the patients who are typically of low socioeconomic status. Secondly, rural areas have inadequate access to trained health care services. Thus, earlier detection of OSCC offers the best chance for long term survival and has the potential to improve the healthcare outcomes. Efforts to increase the body of literature on the knowledge, etiology and regional distribution of the risk factors of this disease have begun to gain momentum. Oral cancer will remain a major health problem and efforts towards its early detection as well as prevention will reduce this burden (54).

Chief etiological and predisposing factors for OSCC include mostly tobacco, smoking and drinking habits, and ultraviolet radiation (specifically for lip cancer). Other factors such as human papillomavirus (HPV) and candida infections, nutritional deficiencies, and genetic predisposition have been also associated with etiology (20). Predominantly males are affected between the sixth and seventh decades of life. The most affected sites are the tongue, oropharynx, lip, floor of mouth, gingiva, hard palate, and buccal mucosa (57). Small lesions are often asymptomatic or may present with vague symptoms like growth or ulcer, but locally advanced lesions usually present with pain, halitosis, difficulty in speech, deglutition and mastication (58). The exact reason for the delay in early detection of OSCC is unknown, but ignorant social and health-related behavior and tumor characteristics are some of the difficulties (20).

Progression in OSCC is linked with a reorganization or remodeling of ECM. Epithelial tumor cells invade underlying connective tissue by the degradation of the basement membrane. Several molecular events such as gain and loss of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation, and initiation of angiogenesis occur that result in tumor spread (59). ECM offers a protein substrate for tumor cell detachment and motility that results in proliferation and invasion of tumor cells. Growth and differentiation of OSCC result from a very essential course of epithelial-mesenchymal interactions (13). Metastatic progression of OSCC is driven by genetic and epigenetic alterations in cancer cells, but supportive signals from the surrounding microenvironment is essential (60).

ECMs are significantly involved in invasion, progression, and metastasis of OSCC (8) as well as in the pathogenesis of OSMF (61). ECM being an extremely active unit and source of growth factors and bioactive molecules determines and controls cell proliferation, adhesion, migration, polarity, differentiation, and apoptosis (7). An extracellular matrix glycoprotein, Tenascin C (Tn-C) belongs to a family of tenascin. Tn-C is the most widely studied member accounting for a very restricted pattern of expression but an enormously diverse range of functions (62). Tn-C being a complex multifunctional protein can, directly and indirectly, influence cell behavior. Tn-C contributes to cell adhesion and motility, guidance along cell migration pathways, shedding of epithelial cells from surfaces, promotion of cell growth, demarcation of tissue boundaries and remodeling of ECM. (10)

Tenascin-C can interact with matrix constituents, soluble factors, cell surface proteins as well as pathogenic components that give it the ability to bind to more than 25 different molecules by far identified. Functionally, Tenascin-C interacts with fibronectin and could be known as an anti-adhesive or adhesion modulating protein (63). Tn-C interaction with fibronectin in a region around its major heparin-binding site prevents adhesion of cells with fibronectin through syndecan-4. The bond formed by cells with Tn-C is weaker than that formed with fibronectin (15) facilitating loss of adhesion and enhancement of migration and metastasis of tumor cells (45).

Reciprocal interactions of epithelial cells and fibroblasts in the microenvironment consisting of paracrine factors such as ECM proteins and cytokines control expression of Tn-C. Tn-C also acts as a growth stimulant in endothelial cell proliferation that aids in angiogenesis (25). Tn-C has an effect on the proliferation of cancer-associated fibroblasts.

Tenascin-C also affects the immune system by inhibiting T-cell proliferation and interfere with leukotriene B4 induced chemotaxis of PMNs and monocytes. These effects of Tn-C could be beneficial in the case of injuries during wound healing (64). Tn-C is expressed in a spatially and temporally manner in wound healing. It is present throughout the matrix of the granulation tissue, which filled the full-thickness of wounds but was not traced in the scar after complete wound contraction (28). Its expression in cancer tissues whereas is often markedly high and continued for a long period of time (25).

Tn-C is determined in a wide range of processes from oligomerization to induction of mitogenic responses, cell migration, cell attachment, cell spreading, focal adhesion, cell survival, matrix assembly, neurite outgrowth and potentiation, and protease and pro-inflammatory cytokine synthesis. The distribution of Tn-C is characteristically limited in adult tissues; however, it is re-expressed in pathological lesions undergoing tissue remodeling, such as inflammation, tissue repair, and cancers (62).

Hsia H et al (11) in a mini-review mentioned Tenascin as multifunctional and mysterious protein, and the inability to identify all specific functions of tenascins labeled it as “talented proteins in search of functions”. Since its discovery, Tn-C has been studied in benign and malignant tumors including gliomas, fibrosarcoma, osteosarcomas and a wide range of carcinomas occurring at different sites bearing potential medical implications for diagnosis and therapy (10). Tn-C has been studied in OSCCs and in other oral lesions like OSMF, oral fibrous hyperplasia, hyperkeratosis, epithelial dysplasia, lichen planus and so on. To our best knowledge, comparative evaluation of Tn-C expression has not been studied in different grades of OSCC and OSMF before.

The present study was an attempt to carry out a comparative evaluation of Tn-C expression in different grades of OSMF and OSCC by immunohistochemistry. For this study, a total of 108 paraffin blocks of oral lesions were studied. These were divided into three groups. Group I consisted of 36 paraffin blocks of normal oral mucosa which is the control group. Group II consisted of 36 paraffin blocks of clinico-histopathologically proven OSMF divided as 12 each in 3 grades of OSMF. Group III consisted of 36 paraffin blocks of clinico-histopathologically proven OSCC. Group III was further divided into 12 paraffin blocks of well differentiated squamous cell carcinoma, 12 paraffin blocks of moderately differentiated squamous cell carcinoma and 12 paraffin blocks of poorly differentiated squamous cell carcinoma. The blocks were sequentially processed, stained and evaluated for the expression of Tenascin-C by immunohistochemical method.

The mean age of the overall population was 46.06 years with a standard deviation of 11.81 years. The mean age in the control group was 42.54 years with a standard deviation of 11.53 years. OSMF subjects included in the study had a mean age of 40.69 years with a standard deviation of 12.69 years while OSCC subjects had a mean age of 54.94 years with a standard deviation of 11.20 years.

According to the studies by **R. Shenoi** (65), the occurrence range of OSCC is in the 50 to 60 years age group. According to **Hande A et al** (55)**,** the highest occurrence range of OSMF is in 18 to 56 years age group.

Out of 36 patients with normal oral mucosa, 27 (75%) were males and 9 (25%) were females; out of 36 patients with OSMF, 24 (66.66%) were males and 12 (33.33%) were females, and out of 36 patients with OSCC, 32 (88.9%) were males and 4 (11.1%) were female. Considering the disease group of OSMF and OSCC groups, 56 (77.77%) were males and 16 (22.22%) were females. This signifies that males were affected more as compared to females.

In studies by **Hande A et al** (55), R. **Shenoi et al** (65)**, and Pires F et al**20), it has been mentioned that these diseases occur more commonly in males than in females as is evident in the present study.

In the present study, Tn-C immunoreactivity in normal oral mucosa (control group) was expressed as a very faint linear band of immunoreactivity at the interface of epithelium and connective tissue, and so the expression was considered as almost negative. Similar results were observed in previous studies by **Tak J et al** (9)**, Shrestha P et al** (13)**, Tiitta O et al** (26)**, Ha¨kkinen L et al** (29)**.**

On evaluation of Tn-C in grades of OSMF, the immunoreactivity was observed at the epithelial connective tissue interface as a band with varying intensity of expression with respect to the OSMF grading. In OSMF grade I, the subepithelial bright band of Tn-C immunoreactivity was observed with a mean intensity of 2.66+ which is between 2+ (distinct positive staining) and 3+ (extensive positive staining). In OSMF grade II, the subepithelial band of Tn-C expression immunoreactivity of light brown color was observed with a mean staining intensity of 1.83+ which is between 1+ (weak positive staining) and 2+ (distinct positive staining). In OSMF grade III, a weak subepithelial band of Tn-c immunoreactivity was observed with a mean intensity of 0.66+ which is between 0 (almost negative) and 1+ (weak positive staining). This signifies that Tn-C expression decreases with the increase in the grade of OSMF.

Similar results were observed by **Tak et al** (9) in an IHC study where 30 samples of OSMF with 10 each of histopathologically proven early, moderate and advanced [Classification by **Utsunomiya et al** (35)] cases of OSMF were studied for Tn-C expression. They observed that early and moderate OSMF cases exhibited a bright and continuous band of Tn-C immunoreactivity at ECJ signifying either proliferative organization within the overlying epithelium or an epithelial‑ mesenchymal interaction. While in the advanced case of OSMF, they observed a weak immunoreactivity of tenascin at ECJ and enhanced expression in the extracellular matrix of deeper connective tissue. They suggested that tenascin protein in the deeper region of the connective tissue was most likely related to an altered fibroblast or a genetic modulation in fibroblast cells.

In a study by **Utsunomiya et al** (35), 30 OSMF cases 10 in each grade of three, were examined for expression and or deposition modes of Tenascin along with seven other ECM molecules by histochemistry, immunohistochemistry, and in situ hybridization methods. The authors observed that tenascin, perlecan, fibronectin, and collagen Type III were characteristically enhanced in the lamina propria and the submucosal layer of the early stage. However, in the intermediate stage, the Tn-C expression was reduced significantly in the submucosal layer but other molecules were irregularly deposited around the muscle fibers. In the advanced stage, the ECM depositions completely decreased and replaced entirely with collagen type I only. Their gene expression levels varied with the progression of fibrosis and the mRNA signals were confirmed in fibroblasts in the submucosal fibrotic areas. Their results suggested that the steps of ECM remodeling in OSMF are similar to each phase of usual granulation tissue formation.

The results of the present study were in accordance with these previous studies as the expression of Tn-C decreases with the increase in grading. As mentioned above in a study by Utsunomiya et al, mRNA signals for tenascin were faintly detected in the basal cells of the oral epithelium and fibroblasts in the juxta-epithelial zone in the early stage. However, in the intermediate and advanced stages, no expression of mRNA for tenascin either in epithelial cells or in subepithelial connective tissues was detected (35). This suggests that there is epithelial and mesenchymal interaction at the ECJ in the early stage but the interaction is reduced as the disease progresses. This can be explained as the expression of Tn-C is controlled by reciprocal interactions of epithelial cells and fibroblasts (25) and there is a decrease in fibroblasts number with the progression of the disease (52). Furtherly, Utsunomiya et al 35) concluded that OSMF is an organizing process of granulation tissue formed by oral mucosa due to chewing stimulation. Similarly, **Angadi et al** (66) also suggested that OSMF actually represents a failed wound-healing process of the oral mucosa after constant chronic injury that results in scarring and fibrosis (66). Tn-C is extensively expressed in the granulation tissue in the wounds but not traced after wound contraction in the scarring (28). Thus, variation of Tn-C expression in different grades of OSMF can be correlated with fibrosis and scarring, and the number of fibroblasts.

In OSCC, the expression of Tn-C was observed in the stroma. Tn-C expression was distinct with strong immunoreactivity at places while some stromal areas of the same tissue remained unreactive or mildly reactive in a few specimens. The mean intensity of Tn-C expression in well-differentiated OSCC was 1.25+ which is between 1+ (weak positive staining) and 2+ (distinct positive staining), the mean intensity of Tn-C expression in moderately differentiated OSCC was 1.9167+ which is between 1+ (weak positive staining) and 2+ (distinct positive staining), and the mean intensity of Tn-C expression in poorly differentiated OSCC was 2.8333+ which is between 2+ (distinct positive staining) and 3+ (extensive positive staining). This signifies that the Tn-C expression increased with the increase in the grades of OSCC.

Shrestha P et al (13) in an IHC study observed Tn expression in 5 specimens of normal oral mucosa, 22 specimens of leukoplakia and 36 specimens of OSCC. They observed increased in Tn immunoreaction in leukoplakia compared with normal oral mucosa and correlated it with the increasing degree of hyperplasia, hyperkeratosis and in particular, dysplasia. They observed a heterogenous Tn reaction between OSCCs. Liner Tn immunoreactivity was frequently observed at the tumor-stromal interface in well and moderately differentiated OSCCs. In poorly differentiated OSCCs, the Tn expression was in traces but widespread with irregular stromal reaction. They observed negative or low Tn reactivity in tumor-mesenchymal interface densely infiltrated by inflammatory cells suggesting that the inflammatory cells may degrade or disorganize the Tn by producing specific enzymes or some unknown Tn inhibition factor. They concluded that the Tn expression was low in poorly as compared with well and moderately differentiated OSCCs and suggested that the state of differentiation of tumor cell determine the level of stromal reaction.

In a study by **Tiitta O et al** (26), extensive Tn reaction was noted in tumor stroma of most of the OSCC and intense immunoreactive rim was noted surrounding the advancing tumor cells islets suggesting selective distribution of Tn at the sites of active cellular migration. In 6 out of 49 OSCCs, Tn was expressed in the neoplastic epithelial cells in addition to the stromal reaction to which they concluded that transformed epithelial cells take part in the synthesis of Tn. As marked reaction was observed in both poorly and well differentiated OSCCs, they concluded that degree of differentiation may not influence the intensity of Tn reaction.

The observations by **Shrestha P et al** (13) and **Tiitta O et al** (26) in OSCCs were in contrast to the present study as significant variability was observed with the degree of differentiation of OSCC. The results of **Shrestha P et al** (13) were based on observation of the Tn immunoreactivity pattern. However, in the present study Tenascin-C, the anti-adhesive molecule member of the Tenascin family has been specifically evaluated.

Ming X et al (45) conducted a meta-analysis to quantitatively assess the prognostic role of Tn-C for patients diagnosed with cancer of different types and sites such as esophageal squamous cell carcinoma, tongue cancer, lung cancer, prostate cancer, colorectal carcinomas, breast carcinoma, posterior fossa ependymoma, clear cell renal cell carcinoma, supratentorial ependymomas, bladder cancer, pancreatic cancer. They suggested that Tn-C played a key role in carcinogenesis and its high expression was associated with poor prognosis in a variety of malignant tumors.

Regezi J et al (14) evaluated for the expression of Tn and β6 integrin in in situ and invasive squamous cell carcinomas from the floor of the mouth. Tenascin was highly expressed at the keratinocyte and connective tissue interface in situ and invasive carcinomas suggest Tn is synthesized by keratinocytes for cell movement. Tenascin and β6 integrin were also colocalized in in situ carcinoma and invasive OSCC suggesting biological interaction.

Yang Z et al (39) studied Tn-C expression in esophageal squamous cell carcinoma (ESCC) tissues with varying degrees of differentiation. They observed stromal reactivity to Tn-C in 58.3% (14/24) cases of well differentiated ESCC, 68.6% (59/86) in moderately differentiated ESCC and 80.8% (21/26) cases of poorly differentiated ESCC. Tn-C reactivity in cancer cells was observed in 50% cases of well differentiated ESCC, 57% (49/86) in moderately differentiated ESCC and 53.8% (14/26) cases of poorly differentiated ESCC. They correlated the overexpression of Tn-C in stromal fibroblasts and cancer cells and indicated that Tn-C is an independent predictor of poor prognosis in ESCC patients.

There are few researches that have correlated Tn-C expression in OSCC with degree of differentiation. In the present study, the upregulation of Tn-C in poorly differentiated OSCC is correlated with the degree of dysplasia as it is more in poorly differentiated OSCC. The expression of Tn-C is increased along the degree of dysplasia (40) Tn-C shows extensive reaction at the invasive tumor front of cancer which is correlated to the presence of highly dysplastic cells with lower degree of dissociation (59). In poorly differentiated OSCCs, the cancer cells are mostly discreetly widespread in the tumor stroma due to loss in intercellular attachment. **Ohtsuka et al** (67) mentioned that anti-adhesion and inhabitation of cell attachment are the most prominent effects of Tn-C that favor motility and invasion of cancer cells. Thus, the upregulated expression of Tn-C in poorly differentiated OSCC in the present study can be correlated to the degree of dysplasia and anti-adhesive property of Tn-C.

Ha¨kkinen L et al (29) observed an obvious increased in accumulation of Tn at the subepithelial connective tissue in OSCC, in lichen planus, in mucosal hyperkeratosis without dysplasia, and in dysplastic oral mucosa as compared to normal oral mucosa. They observed that Tn immunoreactivity was strikingly reduced in areas of dense inflammatory infiltrate. Tn is potently upregulated by mesenchymal cells, keratinocytes and OSCC cell lines by inflammatory cytokines. However, they suggested that the reduced expression of TN in the heavily inflamed areas in the study may result from degradation of Tn by proteolytic enzymes existing abundantly in the inflammatory infiltrate.

Harada T et al (8) studied seven different ECM including Tn in primary OSCCs on the basis of nodal metastasis. They observed Tn expression at the invasive front of primary tumors in highly invasive and metastatic OSCC. An extensive Tn expression was observed in the metastatic lymph nodes, especially those with extra-nodal spread. They, therefore, suggested that the Tn expression is associated with invasive and metastatic potential of OSCC as well as with dysplastic changes. In addition, they observed weak Tn reactivity in cases with marked infiltrating lymphocytes regardless of invasive grade suggesting that Tn may be regulated by infiltrating lymphocytes, probably lymphocyte-derived cytokines, along with invasive potential of tumor cells.

In the present study, the association of reduced Tn-C reactivity with lymphocytic infiltrate provides a correlation with reduced expression of Tn-C in well differentiated OSCCs where marked host response is produced with dense inflammatory cell infiltrate. Whereas, in poorly differentiated OSCC there is mild inflammatory infiltrate with extensive expression of Tn-C.

On comparing the expression of Tn-C in normal oral mucosa, OSMF, and OSCC, there was marked upregulation in expression in OSMF and OSCC compared to normal oral mucosa. The mean intensity of Tn-C expression was 1.7222+ in OSMF and 2+ in OSCC irrespective of gradings. The results in the present were in accordance with **Tak J et al** (9)**, Shrestha P et al** (13)**, Titta O et al** (26)**, Ha¨kkinen L et al** (29)**.** Thus**, increased expression of Tn-C in OSCC and OSMF** than in normal oral mucosa signifies that Tn-C plays a role its pathogenesis.

Limitations

- A relatively larger sample size would be helpful for better confirmation of the results.
- A follow-up of the patients would have evaluated the correlation between Tn-C expression and prognosis.

Future scope

- Future research on Tn-C reactivity in other potentially malignant disorders will define the specific role of Tn-C in its induction and pathogenesis.
- More research on Tn-C in varying degrees of OSCC along with TNM staging could provide a clinicopathological correlation of Tn-C reactivity.
- The targeted therapy against Tn-C could be helpful to achieve a better prognosis for OSCC.

Conclusion

Oral cancer has become one of the leading causes of death in not only India but globally. Pertaining to its multistep complex carcinogenesis, it has become impossible for a human to find a cure. Efforts are being made for early detection, control cancer progression and decrease mortality. Oral squamous cell carcinoma is the most common oral malignancy and is mostly preceded by oral potentially malignant disorders like oral submucous fibrosis. Extracellular matrix plays a significant role in the pathogenesis of both OSCC and OSMF by providing a medium for important regulatory signals for cellular responses during disease progression and even metastasis in OSCCs.

Tenascin-C, a large oligomeric glycoprotein of ECM is known to be involved in proliferation, adhesion, cell to cell and cell to stromal interaction, migration, polarity, differentiation, and apoptosis during embryogenesis, organogenesis, wound healing as well as in carcinogenesis. The spatial and temporal expression of Tn-C in biological and pathological conditions suggests that its functions are designed at the right time and place.

Thus, in the present study, an immunoreactivity of Tn-C was evaluated in OSMF and OSCC as well as in normal oral mucosa. The Tn-C expression is comparatively analyzed in histopathological grades of OSMF and OSCC. In normal oral mucosa, Tn-C was observed as a faint linear immunoreactivity at the epithelial connective tissue junction.

In OSMF, decrease in Tn-C expression from early to advanced grade can be correlated with the decrease in Tn-C producing fibroblasts which are essential for epithelial-mesenchymal interaction. Also, if the stroma of OSMF resembles granulation tissue of wound healing where Tn-C expression is abridged after scarring then Tn-C expression may be similarly reduced after fibrosis or scarring in OSMF. In OSCC, Tn-C expression was enhanced but upregulated from well differentiated to poorly differentiated OSCC. Abundant Tn-C expression in poorly differentiated OSCC is correlated with the amplified degree of dysplasia, detached tumor cells, and anti-adhesive property of Tn-C. Whereas reduced Tn-C immunoreactivity in well differentiated OSCC can be correlated with dense inflammatory cell infiltrate. In general, the strong upregulation of Tn-C in OSCC suggests that Tn-C takes part in ECM remodeling, epithelial-mesenchymal interaction, proliferation and dissociation of dysplastic cells. Thus, the present study concludes that the extracellular matrix protein Tn-C may play a role in the pathogenesis of OSCC.

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MASTER CHART

Sr. No	Age	Sex	Clinico-histopathological Diagnosis	Intensity		
				Observer 1	Observer 2	Observer 3
1	23	M	Normal	0	0	0
2	45	M	Normal	0	0	0
3	42	M	Normal	0	0	0
4	36	M	Normal	0	0	0
5	34	M	Normal	1+	1+	1+
6	39	M	Normal	0	0	0
7	48	M	Normal	0	0	0
8	21	M	Normal	0	0	0
9	31	F	Normal	0	0	0
10	46	F	Normal	0	0	1+
11	50	M	Normal	0	0	0
12	49	M	Normal	0	1+	0
13	27	F	Normal	0	0	0
14	51	M	Normal	0	0	0
15	46	M	Normal	1+	1+	0
16	43	M	Normal	0	0	0
17	31	M	Normal	0	0	1+
18	25	M	Normal	0	0	0
19	20	F	Normal	0	1+	1+
20	38	M	Normal	0	0	0
21	44	M	Normal	0	0	0
22	41	M	Normal	1+	0	0
23	56	F	Normal	0	0	0
24	21	M	Normal	1+	1+	0
25	45	M	Normal	0	0	0
26	54	M	Normal	0	0	0
27	56	M	Normal	0	0	0
28	45	F	Normal	0	0	0
29	52	F	Normal	0	0	0
30	54	F	Normal	0	0	0
31	60	M	Normal	0	0	0
32	51	M	Normal	0	0	0
33	54	M	Normal	1+	1+	0
34	51	F	Normal	0	0	0
35	60	M	Normal	0	0	0

Sr. No	Age	Sex	Clinico-histopathological Diagnosis	Intensity		
				Observer 1	**Observer 2**	**Observer 3**
36	48	M	Normal	0	0	0
37	52	F	OSMF I	3+	3+	3+
38	26	M	OSMF I	3+	3+	3+
39	21	M	OSMF I	3+	2+	2+
40	41	M	OSMF I	3+	3+	3+
41	32	M	OSMF I	2+	2+	2+
42	51	M	OSMF I	3+	3+	2+
43	32	M	OSMF I	3+	2+	2+
44	23	M	OSMF I	3+	3+	2+
45	23	M	OSMF I	3+	3+	3+
46	30	M	OSMF I	3+	3+	3+
47	26	M	OSMF I	3+	2+	2+
48	56	F	OSMF I	3+	3+	3+
49	45	M	OSMF II	2+	2+	1+
50	23	M	OSMF II	3+	2+	2+
51	46	M	OSMF II	1+	1+	1+
52	23	M	OSMF II	2+	2+	1+
53	51	M	OSMF II	2+	2+	2+
54	65	M	OSMF II	3+	2+	2+
55	33	F	OSMF II	2+	1+	1+
56	36	M	OSMF II	3+	2+	2+
57	31	M	OSMF II	2+	2+	1+
58	42	F	OSMF II	2+	2+	2+
59	51	F	OSMF II	2+	2+	1+
60	36	M	OSMF II	2+	1+	2+
61	35	M	OSMF III	1+	1+	0
62	50	M	OSMF III	0	0	1+
63	32	F	OSMF III	1+	0	1+
64	51	M	OSMF III	1+	1+	1+
65	60	M	OSMF III	0	0	0
66	31	M	OSMF III	1+	1+	1+
67	47	F	OSMF III	1+	1+	0
68	56	M	OSMF III	0	0	0
69	58	M	OSMF III	1+	1+	1+
70	60	M	OSMF III	0	0	0
71	49	F	OSMF III	0	1+	1+
72	41	F	OSMF III	1+	1+	1+

Sr. No	Age	Sex	Clinico-histopathological Diagnosis	Intensity		
				Observer 1	Observer 2	Observer 3
73	39	M	Well Differentiated OSCC	2+	2+	2+
74	46	M	Well Differentiated OSCC	1+	2+	1+
75	48	M	Well Differentiated OSCC	1+	1+	1+
76	59	M	Well Differentiated OSCC	1+	1+	1+
77	39	M	Well Differentiated OSCC	2+	2+	2+
78	36	M	Well Differentiated OSCC	1+	1+	1+
79	46	F	Well Differentiated OSCC	1+	1+	1+
80	45	M	Well Differentiated OSCC	1+	1+	1+
81	39	M	Well Differentiated OSCC	1+	1+	1+
82	47	M	Well Differentiated OSCC	1+	1+	1+
83	36	M	Well Differentiated OSCC	1+	1+	1+
84	55	M	Well Differentiated OSCC	2+	1+	2+
85	56	M	Moderately Differentiated OSCC	2+	2+	2+
86	60	M	Moderately Differentiated OSCC	2+	1+	1+
87	68	M	Moderately Differentiated OSCC	2+	2+	2+
88	70	M	Moderately Differentiated OSCC	2+	2+	2+
89	66	M	Moderately Differentiated OSCC	2+	2+	2+
90	64	M	Moderately Differentiated OSCC	2+	2+	2+
91	59	M	Moderately Differentiated OSCC	2+	2+	2+
92	55	M	Moderately Differentiated OSCC	2+	1+	2+
93	53	F	Moderately Differentiated OSCC	2+	2+	3+
94	61	M	Moderately Differentiated OSCC	2+	2+	2+
95	68	M	Moderately Differentiated OSCC	3+	2+	2+
96	52	M	Moderately Differentiated OSCC	2+	2+	2+
97	38	F	Poorly Differentiated OSCC	2+	2+	2+
98	61	M	Poorly Differentiated OSCC	3+	3+	3+
99	66	M	Poorly Differentiated OSCC	3+	2+	3+
100	75	M	Poorly Differentiated OSCC	3+	3+	3+
101	71	M	Poorly Differentiated OSCC	3+	3+	3+
102	56	M	Poorly Differentiated OSCC	3+	3+	3+
103	49	M	Poorly Differentiated OSCC	3+	2+	3+
104	76	F	Poorly Differentiated OSCC	3+	3+	3+
105	57	M	Poorly Differentiated OSCC	3+	3+	3+
106	48	M	Poorly Differentiated OSCC	3+	3+	3+
107	60	M	Poorly Differentiated OSCC	3+	3+	2+
108	54	M	Poorly Differentiated OSCC	3+	3+	3+