University of Dental Medicine, Yangon

IMMUNOHISTOCHEMICAL EXPRESSION OF CD44 AND ALDEHYDE DEHYDROGENASE 1 IN ORAL SUBMUCOUS FIBROSIS AND ORAL SQUAMOUS CELL CARCINOMA

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This thesis has been approved and passed by the Board of Examiner and witnessed by the members of the Jury for the degree of Doctor of Dental Science (Oral Medicine).

Examiner   Examiner   Examiner

Juror   Juror

DEDICATION

Dedicated to my teachers

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree of qualification of this and any other University or Institute of learning.

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ABSTRACT

In potentially malignant disorders and oral squamous cell carcinomas (OSCC), hyaluronan receptor cluster of differentiation 44 (CD44) and aldehyde dehydrogenase 1 (ALDH1) were often used as cancer stem cell (CSC) markers. Prevalence of OSCC was high in Asian countries, especially in South and Southeast Asia; cancers of the oral cavity were thought to progress from premalignant lesions, beginning as hyperplastic tissue and developing into invasive squamous cell carcinoma. Oral submucous fibrosis (OSMF) was a one of the well-known potentially malignant disorders that its prevalence rate was high in Asian continents and its malignant transformation rate was 7-30% with a long-term follow-up study recording an annual malignant transformation rate of 0.5%.

The aim of the present study was to identify and compare CD44 and ALDH1 protein markers in relation with pathological variance of OSMF and OSCC. Thirty specimens of OSMF and 34 specimens of OSCC patients with primary OSCC, from the Department of Oral Medicine, University of Dental Medicine, Yangon, were analyzed cross-sectional study for the expression of CD44 and ALDH1. CD44 was expressed in 80% of OSMF and 58.82% of OSCC samples while ALDH1 was expressed in 56.67% of OSMF and 55.88% of OSCC samples. CD44 and ALDH1 expression patterns were not completely overlapping within OSMF and OSCC cases. Co-expression of CD44 and ALDH1 in OSMF was 56.67% while 41.18% in OSCC. Kruskal-Wallis test for CD44 and ALDH1 in OSMF was 8.980 (p = .030) and 12.565 (p = .028) while Kruskal-Wallis test for CD44 and ALDH1 in OSCC was 8.304 (p = .081) and 5.900 (p = .207). CD44 and ALDH1 showed lesser expression in association with lymphoplasmacytic infiltration at the epithelial-connective tissue junction of OSMF. Correlation of

CD44+/ALDH1+ cells and lymphoplasmacytic infiltration in OSCC was statistically significant (p = .026).

In conclusion, CD44 and ALDH1 expression showed a more distinct distribution pattern in potentially malignant lesion, OSMF. However, these markers were not sufficient to precisely isolate the CSC subpopulation from the tumor bulk. Further protein marker was needed to precisely define the CSC subpopulations in OSMF and OSCC. The finding of ALDH1- and CD44-positive cells in epithelium of OSMF and in adjacent non-tumor epithelium as well as tumor portion of OSCC suggests that changes were already underway, as these enzymes tend to be present in cells with a high tumorigenic potential. Tumor-host immune reaction took part an important role in expression of these markers in oral squamous cell carcinoma.

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ABBREVIATION

AACR   American Association for Cancer Research

ALDH1   Aldehyde dehydrogenases 1

ANTE   Adjacent non-tumor epithelia

ASC   Adult stem cell

Bmi1   B cell-specific Moloney murine leukemia virus

                integration site 1

CA   California

CAR   Chimeric antigen receptor

CD   Cluster of differentiation

CD44v   CD44 various isoform

CIC   Cancer Initiating Cell

CKI   Casein kinase I

CRB   Crumbs

CSC   Cancer stem cell

DAPI   Diamidino-phenylindole

DNA   Deoxyribonucleic acid

ECM   Extracellular matrix

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EndMT	Endothelial-mesenchymal transition
ESC	Embryonic stem cell
ESCC	Esophageal squamous cell carcinoma
FACS	Fluorescent-activated cell sorting
FFPET	Formalin fixed paraffin embedded tissue
FGF	Fibroblast growth factor
GPCR	G-protein-coupled receptor
H&E	Hematoxylin & Eosin
HA	Hyaluronan/ hyaluronic acid
HAS	Hyaluronan synthase
Hh	Hedgehog
HLA	Human leukocyte antigen
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HSC	Hematopoietic stem cell
IARC	International Agency for Research on Cancer

IgG	Immunoglobulin G
IHC	Immunohistochemistry
IPF	Idiopathic pulmonary fibrosis
iPSC	Induced pluripotent stem cell
ITF	Invasive tumor front
LKB1	Liver kinase-B1
MMP	Matrix metalloproteinases
MSC	Mesenchymal stem cell
NIH	National Institute of Health
NOD-SCID	Non-obese Diabetic-Severely Compromised Immunodeficient
NP	Nuclear Polymorphism
OC	Oral cancer
OSCC	Oral squamous cell carcinoma
OSMF	Oral submucous fibrosis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
PMOL	Potentially malignant oral lesions
RA	Retinoic acid

RTK	Receptor tyrosine kinase
SC	Stem cell
SCRIB	Scribble
SPSS	Statistical Package for the Social Science
TGF	Transforming growth factor
TIC	Tumor initiating cell
TIMP	Tissue inhibitor of matrix metalloproteinases
TNM	Primary Tumor, Regional lymph node metastasis and distant metastasis
UDM	University of Dental Medicine
USA	United States of America
WHO	World Health Organization
Wnt	Wingless –related integration site

Chapter 1

INTRODUCTION

Cancer is the leading cause of death worldwide and the second leading cause of death in the United States (Kung *et al*. 2008). Over the past 30 years, the global burden of cancer has more than doubled. There were 7 million deaths from cancer in 2008. Affected by the still growing and aging world population, this figure is expected to increase to 17 million annually by 2030 (World Cancer Report, 2008).

Worldwide, **oral cancer** is the sixth commonest type of malignancy and its incidence is rising. 90% of oral cancers are mostly squamous cell carcinoma (Shah *et al.*, 1995). Despite advances in treatment, which have improved quality of life, survival rates have not improved significantly in more than 30 years. Mortality from this disease remains high because of development of metastases and the emergence of therapy-resistance local and regional recurrences. After receiving standard therapy a subset of patients fail to respond the treatment, or their cancer recurs (Prince and Ailles, 2008).

The occurrence of oral cancer is not clearly known in Myanmar, where betel quid chewing habits are widely spread. Oral cancer stood at the 6th position in males and 10th in females, contributing to 3.5% of whole body cancers. There was a male predominance with a ratio of 2.1:1. Their most frequent site was the tongue, followed by the palate, which was different from that in other countries with betel quid chewing habits. About 90% of male and 44% of female patients had habitual backgrounds of chewing and smoking for more than 15 years (Oo *et al*., 2011).

Local–regional relapse after definitive therapy is a major cause of morbidity and mortality in patients with head and neck squamous cell carcinoma (HNSCC). Many clinical and pathological prognostic factors have been described in HNSCC, such as tumor stage, lymph node involvement, postsurgical margin and histological grade; however these factors lack sensitivity and accuracy in the clinical setting and, with the exception of disease stage, are infrequently used to guide treatment decisions (Ginos *et al.*, 2004).

Most oral squamous cell carcinomas are preceded by clinical premalignant lesions and conditions like oral leukoplakia, erythroplakia, and oral submucous fibrosis (OSMF) (Van der Waal, 2009). OSMF, now globally accepted as an Asian disease, has one of the highest rates of malignant transformation amongst potentially malignant oral lesions and conditions. The hallmark of the disease is submucosal fibrosis that affects most parts of the oral cavity, pharynx and upper third of the esophagus leading to dysphagia and progressive trismus due to rigid lips and cheeks (Angadi and Krishnapillai, 2012).

Most of the earlier studies have focused on the prevalence of epithelial dysplasia in OSMF. It has so far been the most reliable indicator for predicting potential malignant transformation of an oral precancerous lesion though new markers are emerging (Warnakulasuriya, 2001).

The reported malignant transformation rate of OSMF to oral squamous cell carcinoma (OSCC) is 7–13% with a long-term follow-up study recording an annual malignant transformation rate of 0.5% (Murti *et al.*, 1985).

Cancer stem cells were first identified in 1997 when a research group from the University of Toronto transferred a few blood stem cells from human leukemia patients into mice and watched leukemia develop in the mice. Stem cell-

like cells have also recently been found in breast and brain tumors. Like normal stem cells, tumor stem cells exist in very low numbers, but they can replicate and give rise to a multitude of cells. Unlike normal stem cells, however, cancer stem cells lack the controls that tell them when to stop dividing. Traditional chemotherapy kills off the majority of the tumor cells, but if any of the cancer stem cells survive the treatment, the cancer may return. Research into the differences in gene expression between normal and tumor stem cells may lead to treatments where the root of the problem—the cancer stem cell—is targeted (The National Academies, 2006).

According to the consensus of an American Association for Cancer Research (AACR) workshop in 2006, CSC is defined as: a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor (Clarke *et al*., 2006). It is important to note that this defines CSC functionally as the cancer cell with stem cell-like properties including self-renewal and pluripotency. These two essential properties provide cancer with long-lasting CSC to give rise to heterogeneous progenies to maintain the tumor or recapitulate the tumor elsewhere (metastasis) or after treatment (relapse).

To facilitate the identification and purification of normal stem cells and CSCs expression, some specific cell surface markers has been investigated, and several **stem cell markers** could be shared by CSCs in multiple human tumor types. The standard procedures for the isolation of CSCs have been similar in many investigations. Among the most used *in vivo* models is the fractionation of tumor cells using cell-surface markers with stem cell characteristics followed by their implantation into Non-obese Diabetic-Severely Compromised Immunodeficient (NOD-SCID) mice to let xenograft growth. The main surface

marker phenotypes associated with stem cell characteristics include CD44 and ALDH1 (Krishnamurthy *et al*., 2010).

CD44 is a cell surface glycoprotein receptor for hyaluronic acid (HA), and it seems to be involved in cell adhesion, migration, and metastasis of cancer cells (Shipitsin *et al*., 2007). It is one of the main components of extra-cellular matrix and most notably of the fundamental substance of conjunctive tissues, which displays important biological properties and a significant role in crucial physiological processes especially when cellular plasticity is involved such as inflammation, immune reactions, angiogenesis and would healing. It is also strongly involved in neoplastic cells migration and therefore in metastatic spreading of malignant tumors. The CD44 cell-surface marker has been used to identify putative CSCs in many tumor types, such as breast tumors (Shipitsin *et al*., 2007), prostate (Collins *et al*., 2005), pancreatic (Li *et al*., 2007) and head and neck carcinomas (Prince *et al*., 2007).

The **aldehyde dehydrogenase (ALDH)** family of enzymes is comprised of cytosolic isoenzymes that oxidize intracellular aldehydes and contribute to the oxidation of retinol to retinoic acid in early stem cell differentiation (Yoshida, 1992). High ALDH1 activity has been used to isolate normal hematopoietic and central nervous system stem cells (Hess *et al*., 2004).

ALDH1 activity has also been found in stem cells derived from hematopoietic malignancies including multiple myeloma and acute myeloid leukemia (Matsui *et al*., 2004) successfully isolated breast cancer stem cells (CSCs) / cancer initiating cells (CICs) by using ALDH1 activity for the first time. Chen *et al*., (2009) reported isolation of cancer stem cells/cancer initiating cells (CSCs/CICs) from HNSCC by using ALDH1 activity. Krishnamurthy *et al*., (2010) observed that ALDH positive cells are consistently localized within close

proximity of blood vessels; studies in hematopoietic stem cells suggest that the vascular niche can promote cell survival signals and could make them resistant to chemotherapies; moreover, antiangiogenic agents such as bevacizumab, have been shown to mediate depletion in the CSC, and these data could suggest therapeutic strategies including antiangiogenic agents. These reports indicate that ALDH expression may be an important new marker for the isolation of CSCs/CICs.

ALDH1 expression was significantly associated with malignant transformation in a large series of patients with oral lichen planus who received a mean follow-up of 5 years. ALDH1 may serve as a useful marker for the identification of a high risk of oral potentially malignant lesions progressing to OSCCs (Xu *et al*., 2013).

Specific CSC markers such as ALDH1 and CD44 showed promising results in detection and new therapeutic protocol. ALDH1/CD44 positive cells display high tumor-initiating and radio-resistant properties, and differentially express the Bmi1 gene, which is a promoter of the epithelial–mesenchymal transition (Chen *et al*., 2009).

Solid epithelial tumors are the major cause of cancer deaths and it has now been demonstrated that they are driven by a small subpopulation of malignant stem cells. The ability in identifying cancer stem cells should lead towards a more specific tumor treatment. In fact, by comparing gene expression profiles of cancer stem cells, the bulk tumor cell population, normal stem cells and normal tissue, it may be possible to identify therapeutic targets preferentially attacking cancer stem cells (Al-Hajj *et al*., 2003).

Specific CSC markers such as ALDH1 and CD44 showed promising results in detection and new therapeutic protocol. ALDH1/CD44 positive cells

display high tumor-initiating and radio-resistant properties, and differentially express the Bmi1 gene, which is a promoter of the epithelial–mesenchymal transition (EMT) (Park *et al*., 2003; Chen *et al*., 2009). This concept could be applied in OSMF which is one of the examples of EMT.

The discovery of specific marker sets for cancer stem cells is still in its infancy, and the targeted therapeutic destruction of these cells remains a challenge. Current anticancer treatments usually do not eradicate clones of such cells, and instead do favor the expansion of the cancer stem cell pool, or the selection of resistant clones, or both, which eventually lead to the failure of treatment (Mannelli and Gallo, 2012).

Therapeutic modalities such as surgery, radiation, chemotherapy and combinations of each are used in the management of head and neck cancer disease, but at the same time the scientific community is ongoing throughout the improvement of early detection and prevention of HNSCC. One of the key in determining the treatment failure could be represented by the presence of CSCs that can escape currently therapeutic strategies.

Therefore, clinical implications of cancer stem cells could lead to targeting of CSC tumor population and molecular pathways involved in CSC immortality, to the early detection of CSC from the bulk cell tumor and preventing normal stem cells differentiating into cancer stem cells.

It could be hypothesized that CD44 and ALDH1 might be related with malignant transformation of OSMF and invasiveness of OSCC. Purpose of this study is to identify expression of these markers in OSMF and OSCC, and compare with pathological grades. By doing this research, it can be able to predict the malignant transformation of OSMF and invasiveness of OSCC. Moreover, CSC

therapeutic implications could be used to prevent malignant transformation of OSMF and the further understanding of therapeutic resistance and tumor recurrence would lead to direct CSC therapies alongside traditional therapy in OSCC.

Chapter 2

LITERATURE REVIEW

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Normal cells are constantly subject to signals that dictate whether the cell should divide, differentiate into another cell or die. Cancer cells develop a degree of autonomy from these signals, resulting in uncontrolled growth and proliferation. If this proliferation is allowed to continue and spread, it can be fatal. In fact, almost 90% of cancer-related deaths are due to tumor spreading – a process called metastasis (Hejmadi, 2010).

Oral cancer is one of the major global threats to public health. The development of oral cancer is a tobacco-related multistep and multifocal process involving field cancerization and carcinogenesis. The rationale for molecular-targeted prevention of oral cancer is promising. Biomarkers of genomic instability, including aneuploidy and allelic imbalance, are possible to measure the cancer risk of oral premalignancies. Understanding of the biology of oral carcinogenesis will yield important advances for detecting high-risk patients, monitoring preventive interventions, and assessing cancer risk and pharmacogenomics. In addition, novel chemo-preventive agents based on molecular mechanisms and targets against oral cancers will be derived from studies using appropriate animal carcinogenesis models. New approaches, such as molecular-targeted agents and agent combinations in high-risk oral individuals, are undoubtedly needed to reduce the devastating worldwide consequences of oral malignancy (Tanaka *et al*., 2011).

2.1 Head and neck squamous cell carcinoma (HNSCC)

Head and Neck Squamous Cell Carcinoma (HNSCC) is the eighth and thirteenth most common malignancy in the world for males and females, respectively, with the majority of malignancies of the upper aero-digestive tract being oral squamous cell carcinomas (Warnakulasuriya, 2010).

Despite advances in the understanding and treatment of HNSCC, survival rates have not significantly improved for over 30 years, with the five-year survival rate after diagnosis remaining at 15–50% (Prince *et al*., 2007).

Current treatments for HNSCC can be traumatic, painful, and disfiguring, drastically affecting quality of life. At present, management of HNSCC includes surgical resection and/or combination chemotherapy and radiation therapy. Despite these treatments, the prognosis of HNSCC remains poor due to late stage diagnosis, high rates of primary-site recurrence, and common metastases to loco-regional lymph nodes (Harper *et al*., 2010).

Despite advances in treatment, which have improved quality of life, survival rates have not improve significantly in more than 30 years. Mortality from this disease remains high because of development of metastases and the emergent of therapy-resistance local and regional recurrences. After receiving standard therapy a subset of patients fail to respond the treatment, or their cancer recurs. Nearly 90% of patients with stage I disease can be cured, but more than 10% relapse and die. For more advanced stages, the proportion of recurrences and deaths increases to 30% for stage II, 50% for stage III, and more than 70% for stage IV (Prince *et al*., 2007).

HNSCC are solid tumors consisting of a heterogeneous mixture of cell types. In this unique cellular milieu, not all cells possess the capacity for

spontaneous and indefinite proliferation/regeneration. It is generally thought that in tumor architecture, CSCs are responsible for tumor growth and the differentiated progeny usually contribute to the tumor bulk (Prince *et al.*, 2007).

2.2 Oral squamous cell carcinoma (OSCC)

OSCC is the most frequent malignancy in the mouth, accounting to 95% of all oral malignant lesions. The most affected sites are the tongue, inferior lips and floor of the mouth. The typical demographic profile of OSCC is one of a man in the fifth to eighth decades of life, who is a tobacco chewer and/or a smoker. OSCC is characterized by cellular and subcellular alterations that are associated with a progression towards dedifferentiation and growth. There are several histologically distinct lesions of the oral cavity which have malignant potential. These are leukoplakia, erythroplakia, lichen planus, and submucous fibrosis. These are characterized by a spectrum of chromosomal, genetic, and molecular alterations that they share with each other as well as with the malignant lesions that develop from them (Mithani, 2007).

OSCC has a great predisposition to produce metastasis in lymph nodes. In clinical practice, the treatment plan and prognosis of oral squamous cell carcinoma is mainly based on the TNM (primary tumor, regional lymph node metastasis, and distant metastasis) staging system. Staging aids in planning the course of management. However, TNM system does not provide any information on the biological characteristics and thus an aggressive clinical behavior of the tumor.

2.2.1 Broder's (1920) classification

Accordingly, tumors were graded on the basis of degree of differentiation and keratinization of tumor cells into:

Grade I: Well differentiated tumors – 75-100% of
cells are differentiated

Grade II: Moderately differentiated tumors – 50-75%
of cells are differentiated

Grade III: Poorly differentiated tumors – 25-50% of
cells are differentiated

Grade IV: Anaplastic tumor – 0-25% of cells are
differentiated

2.2.2 Anneroth's (1987) multifactorial grading system

According to this system, three parameters reflecting tumor cell features including keratinization, nuclear pleomorphism, and mitoses were evaluated in the whole thickness of the tumor and each scored. Pattern of invasion, stage of invasion, and lymphoplasmacytic infiltration representing tumor-host relationship were graded in the most invasive margins and scored.

2.2.3 Bryne's (ITF) Invasive Tumor Front grading system

Bryne (1989) presented a hypothesis suggesting that molecular and morphological characteristics at the invasive front area of various squamous cell carcinomas may reflect tumor prognosis better than other parts of the tumor. Several molecular events of importance for tumor spread like gains and losses of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and initiation of angiogenesis occur at the tumor host interface. Several studies have shown that this system is a significantly better predictor of prognosis. All

studies performed so far show that invasive front grading is a valuable supplement to clinical staging, suggesting that it should be introduced into the clinic.

Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. Several studies have indicated that cells at the invasive tumor margins often are different from cells within other parts of various human cancers. Conventional Borders' grading of the whole biopsy had no prognostic value. Invasive cell grading may be of value for treatment planning of oral cancers.

According to this system, number of mitosis and stage of invasion was omitted from the Anneroth's grading system, while the rest of the 4 parameters mentioned above were measured in the deepest invasive margins, and not in the whole thickness of the tumor, and graded similarly. The sum of scores were grouped as follows: 4-8 grade I, 9-12 grade II, 13-16 grade III, and the results were compared in the metastasizing and non-metastasizing groups (Table 1).

2.3 Oral submucous fibrosis

OSMF is a chronic debilitating disease and a premalignant condition of the oral cavity characterized by generalized submucosal fibrosis (Angadi and Hallikerimath, 2011). Most oral squamous cell carcinomas are preceded by clinical premalignant lesions and conditions like oral leukoplakia, erythroplakia, and OSMF. Among these, OSMF, a chronic fibrotic premalignant condition, demonstrates a regional distribution, being particularly prevalent in the Indian subcontinent (Pindborg and Sirsat, 1966; Tilakaratne *et al*., 2006). Scientific literature to date provides firm evidence that areca nut is the major etiological factor for this disease but the exact mechanism of action on the oral tissues remains to be elucidated (Tilakaratne *et al*., 2006; Rajalalitha and Vali, 2005).

The disease is characterized by inflammation and progressive generalized submucosal fibrosis, leading to limitation of mouth opening. It exhibits characteristic histopathologic features that include juxta-epithelial hyalinization and excessive collagen deposition in the connective tissue, secondary to which the epithelium becomes atrophic. This atrophic epithelium is prone to injury by the areca nut extracts that predispose to the development of malignancy (Tilakaratne *et al*., 2006; Rajalalitha and Vali, 2005).

The reported malignant transformation rate of OSMF to OSCC is 7–13% with a long-term follow-up study recording an annual malignant transformation rate of 0.5% (Murti *et al*., 1985). The incidence of this disease is rising in India especially among the younger population due to increased access and improved marketing strategies for the availability of areca nut, which, in turn, predisposes this population to an increased risk for oral cancer (Rajalalitha and Vali, 2005).

It is regarded as a precancerous and potentially malignant condition. The most widely accepted definition of the disease by Pindborg and Sirsat (1966) is one of an insidious, chronic disease that affects any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by, or associated with, formation of vesicles, it is always associated with a juxta-epithelial inflammatory reaction followed by fibro-elastic change of the lamina propria and epithelial atrophy that leads to stiffness of the oral mucosa and causes trismus and an inability to eat.

The definition by the World Health Organization (1980) of a precancerous oral condition: "a generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer" fits well with the characteristics of OSMF. The condition is thought to be multifactorial in origin with a high incidence in people who chew areca-nut and a significant malignant

transformation rate (7–30%) poses global problems for public health. The physical effects, which include a burning sensation in the mucosa and progressive trismus, can also have psychological and social implications for patients (Rajendran, 1994).

2.3.1 Terminology

Schwartz (1952) described a condition in 5 Indian women that he called "atrophia idiopathies (tropica) mucosae oris" (Schwartz presented at the Eleventh International Dental Congress, London, 1952); Schwartz coined the term "submucous fibrosis of the palate and pillars". Other names suggested include "diffuse oral submucous fibrosis", "idiopathic scleroderma of the mouth", "idiopathic palatal fibrosis", and "sclerosing stomatitis". Pindborg and Sirsat used the term "submucous fibrosis" although they suggested that a more appropriate name would be "juxtaepithelial fibrosis". Its premalignant nature was first described by Paymaster in 1956 (Rajendran, 1994).

2.3.2 Clinical presentation

Oral submucous fibrosis is a disease due to a chronic, insidious change in fibro-elasticity, characterized by burning sensation in the oral cavity, blanching, and stiffening of the oral mucosa and oro-pharynx leading to trismus and inability to open the mouth. The symptoms and signs depend on the progression of the lesions and number of affected sites. It is predominantly seen in Indians and other Asians. Once the disease has developed, there is neither regression nor any effective treatment (Angadi and Rekha, 2011).

Clinical presentation depends on the stage of the disease. Initially, most patients present with a burning sensation or intolerance to spicy food, and they may have vesicles, particularly on the palate. Ulceration and dryness of the mouth is later followed by fibrosis of the oral mucosa, which leads to rigidity of

the lips, tongue, and palate, and trismus. Petechiae, in the absence of blood dyscrasias or systemic disorders, are found in about 22% of patients with OSMF, and occur most often on the tongue followed by the labial and buccal mucosa (Rajendran *et al*., 1994).

A useful clinical sign is pain on palpation in the sites where submucosal fibrotic bands are developing, and trismus is caused mostly by fibrosis in the dense tissue around the pterygomandibular raphe. Fibrosis of the Eustachian tube may lead to deafness. When the fibrosis involves the nasopharynx or esophagus, patients may experience referred pain to the ear, a nasal voice, and dysphagia to solids; usually these are features of more advanced disease.

The most obvious clinical signs include blanched, opaque oral mucosa with palpable fibrous bands. Furthermore, the overlying epithelium may become dysplastic and malignant. Restricted mouth opening interferes with examination of the oral mucosa, and makes early diagnosis of cancer a daunting task (Angadi and Rekha, 2011).

2.3.3 Pathogenesis

Data from epidemiological studies provide overwhelming evidence that areca nut is the main etiological factor for OSMF. It is logical to hypothesize that the increased collagen synthesis or reduced collagen degradation is the possible mechanism in the development of the disease. There are numerous biological pathways involved in the above processes and it is likely that the normal regulatory mechanisms are either down regulated or up regulated at different stages of the disease. The copper content of areca nut is high and the possible role of copper as a mediator of fibrosis is supported by the demonstration of the up regulation of lysyl oxidase in OSMF biopsies (Khan *et al*., 2012).

Areca nut is the main etiological factor for OSMF. A clear dose-dependent relationship was observed for both frequency and duration of chewing areca nut (without tobacco) in the development of OSMF. Commercially freeze dried products such as pan masala, Guthka and mawa (areca and lime) have high concentrates of areca nut per chew and appear to cause OSMF more rapidly than by self-prepared conventional betel quid that contain smaller amounts of areca nut. It is logical to hypothesize that the increased collagen synthesis or reduced collagen degradation as possible mechanisms in the development of the disease.

There are numerous biological pathways involved in the above processes and, it is likely that the normal regulatory mechanisms are either down regulated or up regulated at different stages of the disease. Among the chemical constituents, alkaloids from areca nut are the most important biologically whilst tannin may have a synergistic role. These chemicals appear to interfere with the molecular processes of deposition and/or degradation of extracellular matrix molecules such as collagen. *In vitro* studies on human fibroblasts using areca extracts or chemically purified arecoline support the theory of fibroblastic proliferation and increased collagen formation that is also demonstrable histologically in human OSMF tissues. The copper content of areca nut is high and the possible role of copper as a mediator of fibrosis is supported by the demonstration of up regulation of lysyl oxidase in OSMF biopsies. It has been postulated that areca nut may also induce the development of the disease by increased levels of cytokines in the lamina propria. Increased and continuous deposition of extracellular matrix may take place as a result of disruption of the equilibrium between matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMP). Collagen-related genes implicate in the susceptibility and pathogenesis of OSMF (Tilakaratne *et al*., 2006).

2.3.4 Malignant transformation

OSMF has a malignant transformation rate of 7–30%. Pathogenesis is thought to be multifactorial. The carcinogenic effect of tobacco acting in synergy with areca-nut is well known, but the second report on betel quid by IARC identified areca-nut as a "group one carcinogen". Its genotoxic and mutagenic effects are attributed to polyphenols, alkaloids, and areca-nut-specific nitrosamines such as N-nitrosoguvacoline, N-nitrosoguvacine, 3-(N-nitrosomethylamino) propionaldehyde, and 3-(N-nitrosomethylamino) propionitrile. Various studies have been conducted in an attempt to identify molecular markers that could be used to predict malignant change in OSMF. A loss of heterozygosity in 23 "hotspot" loci which alter genes that control the cell cycle has been recognized as an important molecular marker for malignancy in OSMF (Arkeri and Brennan, 2013).

Sixty-six patients with OSMF were followed-up for a period of 17 years (median observation 10 years) in Ernakulum District, Kerala, India. Oral cancer developed in 5 (7.6%) patients. The malignant transformation rate in the same sample was 4.5% over a 15-year observation period (median 8 years). These findings impart a high degree of malignant potential to this condition (Murti *et al*., 1985).

Once the disease has developed, there is neither regression nor any effective treatment. It is considered as a pre-malignant stage of oral cancer, and the reported risk of malignant transformation varies from 2.3-7.6%. The common etiological factor considered for this unremitting disease is use of areca nut; however, the precise mechanism still remains elusive and controversial. A wide range of treatment consisting of drugs, surgical therapy, and physiotherapy have been attempted till date, with varying degrees of benefit, but none of them have

proved to be a cure for this disease. This field remains open for clinical trials and research (Angadi and Rao, 2011).

The clinical hallmark of OSMF is the development of progressive trismus. The latter is a direct consequence of loss of the normal fibro-elasticity of the oral mucosa and replacement of the fibromuscular connective tissue by the deposition of dense collagen. This change in the oral mucosa is etiologically linked to the areca nut chewing habit where the development of OSMF results from the interaction of the mucosa with the chemical constituents of areca. Areca nuts are used as a masticatory substance either alone, in a self-prepared quid or in various commercial preparations known as pan masala and gatka. The habitual usage of these products is rapidly increasing and oral health professionals globally are likely to encounter patients with this disease. The potentially malignant nature of OSMF is well documented and habitual areca nut chewing even in the absence of tobacco is an independent risk factor for oral cancer. Public health education against the areca nut chewing habit is essential to eradicate the deleterious effects of this habit on oral health (Mahomed, 2012).

In submucous fibrosis there is a tendency toward epithelial atrophy associated with hyperothokeratosis and pyknotic changes in the nuclei of the basal-cell layer. Hyperplasia of the epithelium usually associated with hyperparakeratosis was also noticed. A striking feature in this study was the absence of glycogen from most of the Grade III (severe) cases. Vacuolization of prickle-cell layer, increased mitotic activity, and epithelial atypia were also noticed in a few cases (Mani and Singh, 1976).

A useful histological grading in conjunction with the clinical progression of the disease was proposed by Pindborg *et al*., 1966. It is still not clear whether the epithelial atrophy, as reported by various workers, is the aftermath of heavy fibrosis in the underlying connective tissue or is a result of

malnutrition. At least a few hold the view that the epithelium has become stretched and thinned by the changes in the underlying connective tissue. Whatever the cause, it has been stated that atrophic changes in the mucosa predispose to malignant changes in the epithelium (Rajendran, 1994).

It is generally agreed that the pathological alteration in OSMF begins in the lamina propria and the epithelium responds only secondarily to it. On the basis of the histopathological appearances of stained Hematoxylin & Eosin (H & E) sections, the surgical specimens from OSMF can be grouped into four clearly definable stages: very early, early, moderately advanced, and advanced. These stages are based not only on the amounts and nature of the subepithelial collagen, but also on the following criteria taken together:

(a) Presence or absence of edema,

(b) Physical state of the mucosal collagen,

(c) Overall fibroblastic response (number and age of individual

    Cells)

(d) State of the blood vessels, and

(e) Predominant cell type in the inflammatory exudate.

Except for cases which begin with vesicles, the changes in OSMF start in the connective tissue. The histological demonstration of subepithelial vesicles in OSMF should encourage further studies on a possible allergic relationship (Sirsat and Pindborg, 1966).

2.4 Cancer stem cell hypothesis

The cancer stem cell hypothesis posits that tumor growth is driven by a rare subpopulation of cells, designated as CSC. Studies supporting this theory are based in large part on xenotransplantation experiments wherein human cancer cells

are grown in immunocompromised mice and only CSC, often constituting less than 1% of the malignancy, generate tumors (Yoo and Hatfield, 2008).

Most tumors are derived from a single cell that is transformed into a cancer-initiating cell (CIC) that has the capacity to proliferate and form tumors *in vivo*. However, the origin of the cancer stem cell remains elusive. Interestingly, during development and tissue repair the fusion of genetic and cytoplasmic material between cells of different origins is an important physiological process. Such cell fusion and horizontal gene-transfer events have also been linked to several fundamental features of cancer and could be important in the development of the cancer stem cell (Bjerkvig *et al*., 2005).
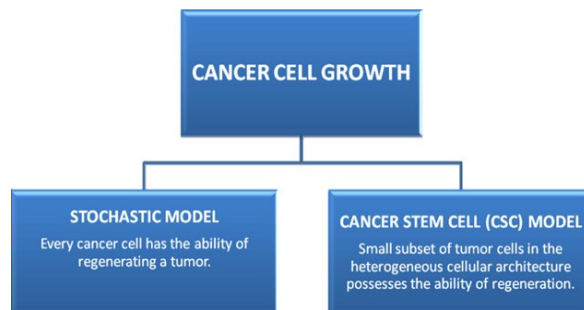
Figure 1. Cancer growth models in tumor development
http://www.elsevier.com/locate/oraloncology

2.4.1 Stochastic model and Cancer stem cell model

The stochastic model of tumor formation suggests that any cell may acquire a mutation leading to clonal expansion. This clone may then acquire further mutations which eventually lead to tumor formation. The cancer stem cell model suggests that the cancer stem cells have gained the capacity to proliferate and are responsible for generating the bulk of the tumor. These cancer stem cells make up only a small population of the tumor.
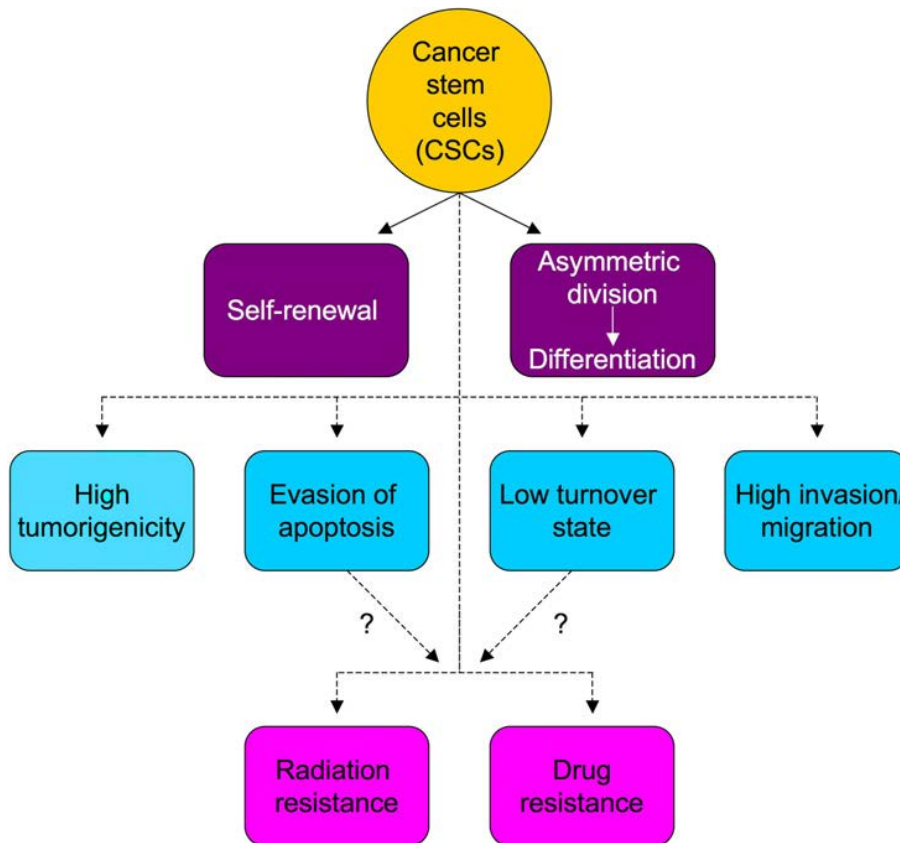
Figure 2. Cancer stem cell model http://www.elsevier.com/locate/oraloncology (Proposed biological properties of HNSCC CSCs. HNSCC CSCs are defined primarily by their capacities for self-renewal and differentiation. They also can possess several additional CSC traits (high tumorigenicity, low-turnover, high invasion/migration, evasion of apoptosis), some of which may contribute to their resistance to chemo and radiotherapies.)

There has been increasing evidence suggesting the existence of stem cells in many different types of cancers, including blood cancers such as leukemia, and solid cancers including breast, brain, colon, pancreatic, head and neck cancers, and other types of carcinomas (Prince *et al*., 2007). This has been facilitated through identification of cell surface markers, using fluorescent-activated cell sorting (FACS) techniques and *in vivo* transplantation of potential cancer stem cell populations into immunocompromised mice. Therefore, it has become increasingly

important to identify and isolate these types of cells in tumors and develop strategies to specifically target them in order to more effectively eradicate the tumor.

The identification and characterization of cancer stem cells might lead to more effective treatments for some cancers by focusing therapy on the most malignant cells. To achieve this goal it will be necessary to determine which cancers follow a cancer stem cell model and which do not, to address technical issues related to tumorigenesis assays, and to test the extent to which cancer cell heterogeneity arises from genetic versus epigenetic differences (Shackleton *et al*., 2009).

The cancer stem cell theory elucidates not only the issue of tumors initiation and development, tumor's ability to metastasize and reoccur, but also the ineffectiveness of conventional cancer therapy. Stem cell properties are self-renewal, heterogeneity, and resistance to apoptosis. The 'niche' hypothesis is presented, and mechanisms of division, differentiation, self-renewal and signaling pathway regulation are explained. Epigenetic alterations and mutations of genes responsible for signal transmission may promote the formation of cancer stem cells (Gil *et al*., 2008).

Much has been made of the idea that asymmetric cell division is a defining characteristic of stem cells that enables them to simultaneously perpetuate themselves (self-renew) and generate differentiated progeny. Yet many stem cells can divide symmetrically, particularly when they are expanding in number during development or after injury. Thus, asymmetric division is not necessary for stem-cell identity but rather is a tool that stem cells can use to maintain appropriate numbers of progeny. The facultative use of symmetric or asymmetric divisions by stem cells may be a key adaptation that is crucial for adult regenerative capacity (Morrison and Kimble, 2006).

The maintenance and repair of many adult tissues are ensured by stem cells (SCs), which reside at the top of the cellular hierarchy of these tissues. Functional assays, such as *in vitro* clonogenic assays, transplantation and in vivo lineage tracing, have been used to assess the renewing and differentiation potential of normal SCs. Similar strategies have suggested that solid tumors may also be hierarchically organized and contain CSCs that sustain tumor growth and relapse after therapy (Beck and Blanpain, 2013).

Why are tumors heterogeneous, in terms of cell phenotype and proliferative potential, even in cases in which all cells are derived from a single clone? Ongoing mutagenesis can partially explain this heterogeneity, but it also seems that some tumors arise from small populations of 'cancer stem cells' that give rise to phenotypically diverse cancer cells, with less proliferative potential. These cancer stem cells are likely to arise from mutations that dysregulate normal stem-cell self-renewal. Using this information, it might be possible to devise more effective therapies (Pardal *et al*., 2003).

CSC biology is a rapidly developing field within cancer research. CSCs are postulated to be a unique cell population exclusively capable of infinite self-renewal, multi-lineage differentiation and with ability to evade conventional cytotoxic cancer therapy. These traits distinguish CSCs from their more differentiated counterparts, which possess only limited or no potential for self-renewal and tumor initiation. Therefore, CSCs would be the driving motor of malignant growth and therapy resistance. Accordingly, successful cancer treatment would need to eliminate this highly potent group of cells, since even small residual numbers would suffice to recapitulate the disease after therapy. Putative CSCs has been identified in a broad range of human malignancies and several cell surface markers have been associated with their stem cell phenotype. Despite all efforts, a pure CSC population has not been isolated and often in vitro clonogenic and in

vivo tumorigenic potential is found in several cell populations with occasionally contradictory surface marker signatures (Fábián *et al.*, 2013).

Until the last century, infectious diseases were the leading cause of human mortality. Therefore, current medical reasoning is profoundly influenced by views that originated from medical microbiology. The notion that cancer growth is sustained by a sub-population of particular cells, the cancer stem cells, is highly reminiscent of the germ theory of disease as exemplified by Koch's postulates in the XIXth century. However, accumulating data underscore the importance of cell-cell interactions and tumor environment. Hence it is essential to critically review the basic tenets of the cancer stem cell concept on the light of their relationships with Koch's postulates. Shifting the pathogenic element from a special cellular entity (cancer stem cell or microorganism) to a "pathogenic field" could be critical for curing both cancer and drug-resistant infectious diseases (Garcion *et al.*, 2009).

Although monoclonal in origin, most tumors appear to contain a heterogeneous population of cancer cells. This observation is traditionally explained by postulating variations in tumor microenvironment and coexistence of multiple genetic sub-clones, created by progressive and divergent accumulation of independent somatic mutations. An additional explanation, however, envisages human tumors not as mere monoclonal expansions of transformed cells, but rather as complex tridimensional tissues where cancer cells become functionally heterogeneous as a result of differentiation. According to this second scenario, tumors act as caricatures of their corresponding normal tissues and are sustained in their growth by a pathological counterpart of normal adult stem cells, cancer stem cells. This model, first developed in human myeloid leukemias, is today being extended to solid tumors, such as breast and brain cancer (Dalerba *et al.*, 2007).

CSCs can be operationally defined as a subset of neoplastic cells which are responsible for the growth and re-growth of primary and metastatic

tumors. Although the existence of perpetually dividing cells is a logical necessity to explain the malignant properties of human tumors, experimental data supporting their existence have only recently been obtained. New knowledge in basic stem cell biology and the availability of several cell surface markers for the definition and isolation of small subsets of immature cells coupled to the use of the classical model of xenotransplantation in immune deficient mice has identified putative CSCs in several solid tumors such as mammary, colon, brain, pancreas, prostate, melanoma and others. However, the theory must be considered as still in its infancy, since tumors grown in mice only partially recapitulate the biology of human cells. In addition, whether the "transformed" cell is the neoplastic counterpart of a normal stem cell or whether complete malignant behavior can occur in a more differentiated cell has still to be demonstrated. In spite of these difficulties, the CSC hypothesis could be of clinical relevance, especially in the definition of new ways to assay drug sensitivity of primary human tumors (Vezzoni and Parmiani, 2008).

Tumors are being increasingly perceived as abnormal organs that, in many respects, recapitulate the outgrowth and differentiation patterns of normal tissues. In line with this idea is the observation that only a small fraction of tumor cells is capable of initiating a new tumor. Because of the features that these cells share with somatic stem cells, they have been termed CSC. Normal stem cells reside in a "stem cell niche" that maintains them in a stem-like state. CSCs also rely on a similar niche, dubbed the "CSC niche," which controls their self-renewal and differentiation. Moreover, CSCs can be generated by the microenvironment through induction of CSC features in more differentiated tumor cells. In addition to a role in CSC maintenance, the microenvironment is hypothesized to be involved in metastasis by induction of the epithelial-mesenchymal transition, leading to dissemination and invasion of tumor cells. The localization of secondary tumors

also seems to be orchestrated by the microenvironment, which is suggested to form a pre-metastatic niche. Thus, the microenvironment seems to be of crucial importance for primary tumor growth as well as metastasis formation. Combined with its role in the protection of CSCs against genotoxic insults, these data strongly put forward the niche as an important target for novel therapies (Borovski *et al*., 2011).

Solid tumors are an enormous cancer burden and a major therapeutic challenge. The CSC hypothesis provides an attractive cellular mechanism to account for the therapeutic refractoriness and dormant behavior exhibited by many of these tumors. There is increasing evidence that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of CSCs. The clinical relevance of CSCs remains a fundamental issue but preliminary findings indicate that specific targeting may be possible (Visvader and Lindeman, 2008).

2.4.2 The biology of cancer stem cells

Stem cell biology has come of age. Unequivocal proof that stem cells exist in the hematopoietic system has given way to the prospective isolation of several tissue-specific stem and progenitor cells, the initial delineation of their properties and expressed genetic programs, and the beginnings of their utility in regenerative medicine. Perhaps the most important and useful property of stem cells is that of self-renewal. Through this property, striking parallels can be found between stem cells and cancer cells: tumors may often originate from the transformation of normal stem cells, similar signaling pathways may regulate self-renewal in stem cells and cancer cells, and cancer cells may include 'cancer stem cells' - rare cells with indefinite potential for self-renewal that drive tumorigenesis (Reya *et al*., 2001).

Cancers originally develop from normal cells that gain the ability to proliferate aberrantly and eventually turn malignant. These cancerous cells then

grow clonally into tumors and eventually have the potential to metastasize. A central question in cancer biology is which cells can be transformed to form tumors? The presence of cancer stem cells has the exclusive ability to regenerate tumors. These cancer stem cells share many characteristics with normal stem cells, including self-renewal and differentiation. With the growing evidence that cancer stem cells exist in a wide array of tumors, it is becoming increasingly important to understand the molecular mechanisms that regulate self-renewal and differentiation because corruption of genes involved in these pathways likely participates in tumor growth. This new paradigm of oncogenesis has been validated in a growing list of tumors. Studies of normal and cancer stem cells from the same tissue have shed light on the ontogeny of tumors. That signaling pathways such as Bmi1 and Wnt have similar effects in normal and cancer stem cell self-renewal suggests that common molecular pathways regulate both populations. Understanding the biology of cancer stem cells will contribute to the identification of molecular targets important for future therapies (Lobo *et al*., 2007).

Much has been made of the idea that asymmetric cell division is a defining characteristic of stem cells that enables them to simultaneously perpetuate themselves (self-renew) and generate differentiated progeny. Yet many stem cells can divide symmetrically, particularly when they are expanding in number during development or after injury. Thus, asymmetric division is not necessary for stem-cell identity but rather is a tool that stem cells can use to maintain appropriate numbers of progeny. The facultative use of symmetric or asymmetric divisions by stem cells may be a key adaptation that is crucial for adult regenerative capacity (Morrison and Kimble, 2006).

The maintenance and repair of many adult tissues are ensured by SCs, which reside at the top of the cellular hierarchy of these tissues. Functional assays, such as *in vitro* clonogenic assays, transplantation and *in vivo* lineage tracing, have

been used to assess the renewing and differentiation potential of normal SCs. Similar strategies have suggested that solid tumors may also be hierarchically organized and contain CSCs that sustain tumor growth and relapse after therapy (Beck and Blanpain, 2013).

Since the cancer stem cell concept has been widely accepted, several strategies have been proposed to attack CSC. Accordingly, stem cell markers are now preferred therapeutic targets. However, the problem of tumor specificity has not disappeared but shifted to another question: how can cancer stem cells be distinguished from normal stem cells, or more specifically, how do CSC markers differ from normal stem cell markers? A hypothesis is proposed which might help to solve this problem in at least a subgroup of stem cell markers. Glycosylation may provide the key (Karsten and Goletz, 2013).

Solid tumors are an enormous cancer burden and a major therapeutic challenge. The CSC hypothesis provides an attractive cellular mechanism to account for the therapeutic refractoriness and dormant behavior exhibited by many of these tumors. There is increasing evidence that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of CSCs. Direct evidence for the CSC hypothesis has recently emerged from mouse models of epithelial tumorigenesis, although alternative models of heterogeneity also seem to apply. The clinical relevance of CSCs remains a fundamental issue but preliminary findings indicate that specific targeting may be possible (Visvader and Lindeman, 2008).

Genetic analyses have shaped much of our understanding of cancer. However, it is becoming increasingly clear that cancer cells display features of normal tissue organization, where CSCs can drive tumor growth. Although often considered as mutually exclusive models to describe tumor heterogeneity, the genetic and CSC models of cancer can be harmonized by considering the role of

genetic diversity and non-genetic influences in contributing to tumor heterogeneity (Kreso *et al.*, 2014).

2.4.3 Definition of CSC
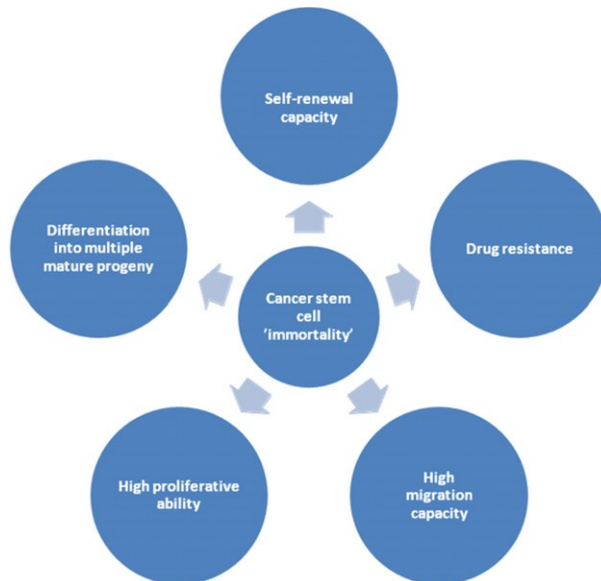
Figure 3. Characteristic of cancer stem cell

http://www.elsevier.com/locate/oraloncology

Scientists have tried for decades to understand cancer development in the context of therapeutic strategies. The realization that cancers may rely on "cancer stem cells" that share the self-renewal feature of normal stem cells has changed the perspective with regard to new approaches for treating the disease. One of the differences between normal stem cells and cancer stem cells is their degree of dependence on the stem cell niche, a specialized microenvironment in which stem cells reside. The stem cell niche in adult somatic tissues plays an essential role in maintaining stem cells or preventing tumorigenesis by providing primarily inhibitory signals for both proliferation and differentiation. However, the niche also provides transient signals for stem cell division to support ongoing tissue regeneration. The balance between proliferation-inhibiting and proliferation-

promoting signals is the key to homeostatic regulation of stem cell maintenance versus tissue regeneration.

Loss of the niche can lead to loss of stem cells, indicating the reliance of stem cells on niche signals. Therefore, cancer stem cells may arise from an intrinsic mutation, leading to self-sufficient cell proliferation, and/or may also involve deregulation or alteration of the niche by dominant proliferation-promoting signals. Furthermore, the molecular machinery used by normal stem cells for homing to or mobilizing from the niche may be "hijacked" by cancer stem cells for invasion and metastasis (Li and Neaves, 2006).

The existence of a stem cell niche, or physiological microenvironment, consisting of specialized cells that directly and indirectly participate in stem cell regulation has been verified for mammalian adult stem cells in the intestinal, neural, epidermal, and hematopoietic systems. In light of these findings, it has been proposed that a "cancer stem cell niche" also exists and that interactions with this tumor niche may specify a self-renewing population of tumor cells (Sneddon and Werb, 2007).

Phenotypic and functional heterogeneity arise among cancer cells within the same tumor as a consequence of genetic change, environmental differences and reversible changes in cell properties. Some cancers also contain a hierarchy in which tumorigenic cancer stem cells differentiate into non-tumorigenic progeny. However, it remains unclear what fraction of cancers follow the stem-cell model and what clinical behaviors the model explains. Studies using lineage tracing and deep sequencing could have implications for the cancer stem-cell model and may help to determine the extent to which it accounts for therapy resistance and disease progression (Meacham and Morrison, 2013).

The niche is the environment in which stem cells reside and is responsible for the maintenance of unique stem cell properties such as self-renewal

and an undifferentiated state. The heterogeneous populations which constitute a niche include both stem cells and surrounding differentiated cells. This network of heterogeneity is responsible for the control of the necessary pathways that function in determining stem cell fate. The concept that cancer stem cells, a subpopulation of cells responsible for tumor initiation and formation, reside in their own unique niche is quickly evolving and it is of importance to understand and identify the processes occurring within this environment. The necessary intrinsic pathways that are utilized by this cancer stem cell population to maintain both self-renewal and the ability to differentiate are believed to be a result of the environment where cancer stem cells reside. The ability of a specific cancer stem cell niche to provide the environment in which this population can flourish is a critical aspect of cancer biology that mandates intense investigation. Homeostatic processes such as inflammation, epithelial to mesenchymal transition, hypoxia and angiogenesis contribute to the maintenance and control of cancer stem cell fate by providing the appropriate signals within the microenvironment. It is necessary to understand the key processes occurring within this highly specialized cancer stem cell niche to identify potential therapeutic targets that can serve as the basis for development of more effective anticancer treatments (Cabarcas *et al*., 2011).

2.4.4 Signaling pathways in cancer stem cells

Wnt signals are transduced to the canonical pathway for cell fate determination, and to the non-canonical pathway for control of cell movement and tissue polarity. Anti-Wnt1 and anti-Wnt2 monoclonal antibodies show in vitro effects in cancer treatment. After the optimization, derivatives of small-molecule compound and human monoclonal antibody targeted to the Wnt signaling pathway could be used in cancer medicine (Katoh and Katoh, 2007).

The Hedgehog (Hh) pathway has been implicated in a wide variety of human tumors, and early clinical trials with pathway antagonists have validated Hh signaling as a bona fide anticancer target. Despite these encouraging results, several issues surrounding the basic biology of the Hh pathway in human cancers remain unclear. These include the influence of specific oncogenic events on Hh signal transduction, the precise mode of Hh signaling (i.e., autocrine or paracrine) that occurs within human tumors, and the best means to inhibit aberrant pathway activity in the clinical setting. The CSC hypothesis may explain a number of clinical phenomena, such as unchecked self-renewal and the development of metastatic disease, and to some extent, the Hh signaling pathway has been implicated in all of these processes. Therefore, Hh pathway inhibitors may also represent some of the first agents to formally examine the CSC hypothesis in the clinical setting. The diverse nature of Hh signaling in human cancers suggests that disease-specific factors must be carefully considered to identify the optimal use of novel pathway inhibitors (Merchant and Matsui, 2010).

The Hippo signaling pathway, consisting of a highly conserved kinase cascade and downstream transcription coactivators, plays a key role in tissue homeostasis and organ size control by regulating tissue-specific stem cells. Moreover, this pathway plays a prominent role in tissue repair and regeneration. Dysregulation of the Hippo pathway is associated with cancer development. Recent studies have revealed a complex network of upstream inputs, including cell density, mechanical sensation, and G-protein-coupled receptor (GPCR) signaling, that modulate Hippo pathway activity (Mo *et al*., 2014).

The Wnt signaling pathways have been conserved throughout evolution and regulate cell proliferation, morphology, motility, and fate during embryonic development. These pathways also play important roles throughout adult life to maintain homeostasis of tissues including skin, blood, intestine, and

brain by regulating somatic stem cells and their niches. Aberrant regulation of the Wnt pathway leads to neoplastic proliferation in these same tissues. It has been suggested that Wnt signaling is also involved in the regulation of CSC, because there are many similarities in the signaling pathways that regulate normal adult stem cells and CSC (Takahashi-Yanaga and Kahn, 2010).

Transitions between epithelial and mesenchymal states have crucial roles in embryonic development. Emerging data suggest a role for these processes in regulating cellular plasticity in normal adult tissues and in tumors, where they can generate multiple, distinct cellular subpopulations contributing to intra-tumoral heterogeneity. Some of these subpopulations may exhibit more differentiated features, whereas others have characteristics of stem cells. Owing to the importance of these tumor-associated phenotypes in metastasis and cancer-related mortality, targeting the products of such cellular plasticity is an attractive but challenging approach that is likely to lead to improved clinical management of cancer patients (Polyak and Weinberg, 2009)

2.5 Cancer stem cell markers

Understanding the role of cancer stem cells in tumor initiation and progression became a major focus in stem cell biology and in cancer research. CD44+CD24- stem cell phenotype is associated with basal-type breast cancers in human patients; in particular BRCA1 inherited cancers, but does not correlate with clinical outcome. These very interesting findings cautions indicate in translating cancer stem cell research into clinical practice depend on how thorough and rigorous in characterizing these cells (Dontu, 2008).

Cancer stem cells are thought to be a critical subpopulation in tumor development, progression, metastasis and recurrence, and the identification of these cells is an initial step in understanding their role in oncogenesis and in seeking valuable markers for diagnosis or development of targeting therapeutics (Lingala *et al.*, 2010).

The lineages assumed by stem cells during hematopoiesis can be identified by the pattern of protein markers present on the surface of cells at different stages of differentiation. Specific antibodies directed at these markers have facilitated the isolation of hematopoietic stem cells by flow cytometry. Similarly, stem cells in solid organs also can be identified using cell surface markers. In addition, solid tumors have recently been found to contain small proportions of cells that are capable of proliferation, self-renewal, and differentiation into the various cell types seen in the bulk tumor. Of particular concern, these tumor-initiating cells (termed cancer stem cells when multi-potency and self-renewal have been demonstrated) often display characteristics of treatment resistance, particularly to ionizing radiation. Because multiple markers, typically examined on single cells using flow cytometry, are used routinely to identify the subpopulation of tumor-initiating cells, and because the number of these cells is small, the challenge remains to detect them in clinical samples and to determine their ability to predict outcome and/or response to treatment, the hallmarks of established biomarkers (Woodward and Sulman, 2008).

Small populations within an increasing array of solid tumors, labeled cancer stem cells (CSC) or tumor-initiating cells (TIC), have the ability to differentiate, self-renew, and replicate the original tumor in vivo. To date, these cells have been distinguished from the bulk-tumor population by the expression pattern of cell-surface proteins (e.g., CD24, CD44, and CD133) and cellular activities, such as the efflux of Hoechst dye or aldehyde dehydrogenase activity.

Recent data have shown that these markers are inducible by exposure to anticancer agents; this finding highlights not only the potential fluidity of the CSC compartment, but also the functionality of these markers. The involvement of CD44 in invasion, adhesion, and metastasis, or the role of CD24 in modulation of src, FAK, and GLI1 are examples of these relevant roles (Keysar and Jimeno, 2010).

Specific targeting of cancer stem cells has proved to be a significant challenge due to the commonality of many markers between normal and cancer stem cells. However, research in the area of cancer biomarkers is slowly, but steadily, progressing (Natarajan and FitzGerald, 2007).

2.5.1 CD44

The CD44 proteins form a ubiquitously expressed family of cell surface adhesion molecules involved in cell-cell and cell-matrix interactions. The multiple protein isoforms are encoded by a single gene by alternative splicing and are further modified by a range of post-translational modifications. CD44 proteins are single chain molecules comprising an N-terminal extracellular domain, a membrane proximal region, a transmembrane domain, and a cytoplasmic tail. The CD44 gene has only been detected in higher organisms and the amino acid sequence of most of the molecule is highly conserved between mammalian species. The principal ligand of CD44 is hyaluronic acid, an integral component of the extracellular matrix. Other CD44 ligands include osteopontin, serglycin, collagens, fibronectin, and laminin. The major physiological role of CD44 is to maintain organ and tissue structure via cell-cell and cell-matrix adhesion, but certain variant isoforms can also mediate lymphocyte activation and homing, and the presentation of chemical factors and hormones. Increased interest has been directed at the characterization of this molecule since it was observed that expression of multiple

CD44 isoforms is greatly upregulated in neoplasia. CD44, particularly its variants, may be useful as a diagnostic or prognostic marker of malignancy and, in at least some human cancers; it may be a potential target for cancer therapy (Goodison *et al.*, 1999).

CD44 was once thought to simply be a transmembrane adhesion molecule that also played a role in the metabolism of its principal ligand hyaluronan. Additional functions for CD44 include its capacity to mediate inflammatory cell function and tumor growth and metastasis. It has also become evident that intricate posttranslational modifications of CD44 regulate the affinity of the receptor for its ligands. CD44 exists in three phases, as a transmembrane receptor, as an integral component of the matrix, and as a soluble protein found in body fluids, each with biologically significant functions of which some are shared and some distinct. CD44 represents a model for understanding posttranslational processing and its emerging role as a general mechanism for regulating cell behavior (Cichy and Puré, 2003).

CD44 is a multistructural and multifunctional cell surface molecule involved in cell proliferation, cell differentiation, cell migration, angiogenesis, presentation of cytokines, chemokines, and growth factors to the corresponding receptors, and docking of proteases at the cell membrane, as well as in signaling for cell survival. All these biological properties are essential to the physiological activities of normal cells, but they are also associated with the pathologic activities of cancer cells. Experiments in animals have shown that targeting of CD44 by antibodies, antisense, and CD44-soluble proteins markedly reduces the malignant activities of various neoplasms, stressing the therapeutic potential of anti-CD44 agents. Furthermore, because alternative splicing and posttranslational modifications generate many different CD44 sequences, including, perhaps, tumor-specific sequences, the production of anti-CD44 tumor-specific agents may be a

realistic therapeutic approach. However, in many cancers (renal cancer and non-Hodgkin's lymphomas are exceptions), a high level of CD44 expression is not always associated with an unfavorable outcome. On the contrary, in some neoplasms CD44 upregulation is associated with a favorable outcome. Even worse, in many cases different research grows analyzing the same neoplastic disease reached contradictory conclusions regarding the correlation between CD44 expression and disease prognosis, possibly due to differences in methodology. These problems must be resolved before applying anti-CD44 therapy to human cancers (Naor *et al*., 2002).

Members of the CD44 family of transmembrane glycoproteins, in particular CD44v6 isoforms, were shown to be metastatic determinants of rat pancreatic tumor cells back in the early 1990s. Furthermore, the expression of several CD44 proteins correlates with aggressive stages of various human cancers. Because of the frequent and homogeneous expression of CD44v6 isoforms in squamous cell carcinoma, antibodies recognizing these proteins were used in clinical trials for patients suffering from HNSCC. Although the phase I clinical trials looked promising, the studies were abruptly ended after the death of a patient. Despite the termination of the trials, CD44 certainly remains a valid target for anti-cancer therapy. Alternative strategies targeting CD44 functions are the binding to hyaluronan (HA), the collaboration with osteopontin and the contribution of CD44 isoforms to receptor tyrosine kinase (RTKs) activation. These new attempts led to the development of peptides that interfere for example with HA binding and that might be used to induce apoptosis in mammary carcinoma or to prevent homing of leukemia stem cells. Other peptides block RTK activation and thereby inhibit tumor angiogenesis and metastatic spread (Orian-Rousseau, 2010).

2.5.2 ALDH1

ALDH1 has been shown to play a role in the early differentiation of stem cells in some human malignancies. Whether cancer stem cells occur in ALDH1-associated cervical cancer is not known (Yao *et al*., 2011).

OSCC is caused by high-risk (HR) human papillomavirus (HPV) or alcohol and tobacco abuse. ALDH1 is a confirmed marker for cancer stem-like cells (CSCs) of OSCC responsible for therapy resistance, recurrence and metastasis. ALDH1^{+} CSCs are detectable in OSCC and metastases. ALDH1 high-grade OSCC exhibits a more aggressive phenotype characterized by higher nodal classification and lower differentiation. This suggests a subpopulation contained in the ALDH1-positive OSCC cell pool able to complete the metastatic cascade and subsequently enriching in metastasis independent of tumor etiology and ALDH1 content (Qian *et al*., 2013).

During vertebrate embryogenesis retinoic acid (RA) synthesis must be spatiotemporally regulated in order to appropriately stimulate various retinoid signaling pathways. Various forms of mammalian ALDH have been shown to oxidize the vitamin A precursor retinal to RA *in vitro*. Raldh2 and Aldh1 control distinct retinoid signaling pathways by stimulating high and low RA biosynthetic activities, respectively, in various trunk and cranial tissues (Haselbeck *et al*., 1999).

The major cytosolic aldehyde dehydrogenase isozyme (ALDH1) exhibits strong activity for oxidation of retinal to retinoic acid, while the major mitochondrial ALDH2 and the stomach cytosolic ALDH3 have no such activity. Major physiological substrate of human ALDH1 is retinal and that its primary biological role is generation of retinoic acid resulting in modulation of cell differentiation including hormone-mediated development (Yoshida *et al*., 1992).

ALDH1 and CD44 act as important biomarkers in several solid tumors. ALDH1 was found to be a predictor of postoperative recurrence and

prognosis in esophageal SCC, and CD44 might be a predictor of recurrence and prognosis. ALDH1 expression might affect the treatment strategy for ESCC (Minato *et al.*, 2012).

CD44 and ALDH1 are considered putative markers of highly tumorigenic cells (i.e., cancer stem-like cells) in HNSCCs. This small subset of cells is believed to be the primary responsible for tumor initiation and progression. ALDH1 immunostaining in the invasive front and in adjacent non-tumor epithelium may help identify tumors with a more aggressive behavior, potentially contributing to improving treatment customization and the monitoring of patients with head and neck cancer (Hildebrand *et al.*, 2014).

2.6 Cancer stem cell markers and Fibrosis

The stem cell niche provides a regulatory microenvironment for cells as diverse as totipotent embryonic stem cells to CSCs which exhibit stem cell-like characteristics and have the capability of regenerating the bulk of tumor cells while maintaining self-renewal potential. The transmembrane glycoprotein CD44 is a common component of the stem cell niche and exists as a standard isoform (CD44s) and a range of variant isoforms (CD44v) generated though alternative splicing. CD44 modulates signal transduction through post-translational modifications as well as interactions with hyaluronan, extracellular matrix molecules and growth factors and their cognate receptor tyrosine kinases. Determining the role of CD44 and CD44v in normal stem cell, CSC and (pre)metastatic niches and elucidating their unique functions could provide tools and therapeutic strategies for treating diseases as diverse as fibrosis during injury repair to cancer progression (Williams *et al.*, 2013).

The regenerative potential of skeletal muscle declines with age and this impairment is associated with an increase in tissue fibrosis. The Wnt signaling

pathway may play a critical role in tissue-specific stem cell aging and an increase in tissue fibrosis with age (Brack *et al.*, 2007).

Vascular endothelial cells can demonstrate considerable plasticity to generate other cell types during embryonic development and disease progression. This process occurs through a cell differentiation mechanism known as endothelial-mesenchymal transition (EndMT). The generation of mesenchymal cells from endothelium is a crucial step in endothelial cell differentiation to several lineages including fibroblasts, myofibroblasts, mural cells, osteoblasts, chondrocytes, and adipocytes. Such differentiation patterns have been observed in systems of cardiac development, fibrosis, diabetic nephropathy, heterotopic ossification and cancer (Medici and Kalluri, 2012).

Cancer is increasingly being viewed as a stem cell disease, both in its propagation by a minority of cells with stem-cell-like properties and in its possible derivation from normal tissue stem cells. But stem cell activity is tightly controlled, raising the question of how normal regulation might be subverted in carcinogenesis. The long-known association between cancer and chronic tissue injury, and the more recently appreciated roles of Hedgehog and Wnt signaling pathways in tissue regeneration, stem cell renewal and cancer growth together suggest that carcinogenesis proceeds by misappropriating homeostatic mechanisms that govern tissue repair and stem cell self-renewal (Beachy *et al.*, 2004).

Epithelial-mesenchymal transition (EMT) is a developmental process in which epithelial cells acquire the motile, migratory properties of mesenchymal cells. Induction of EMT stimulates cultured breast cells to adopt characteristics of stem cells (Radisky and LaBarge, 2008).

Rapid advances in the CSC field have provided cause for optimism for the development of more reliable cancer therapies in the future. Strategies aimed at efficient targeting of CSCs are becoming important for monitoring the

progress of cancer therapy and for evaluating new therapeutic approaches (Klonisch *et al*., 2008).

Chapter 3

RESEARCH HYPOTHESIS

Immunohistochemical expression of CD44 and ALDH1 might be related with malignant transformation of oral submucous fibrosis and invasiveness of oral squamous cell carcinoma.

Chapter 4

AIM AND OBJECTIVES

4.1 Aim

The aim of this study was to identify immunohistochemical expression of CD44 and Aldehyde dehydrogenase 1 on oral submucous fibrosis and oral squamous cell carcinoma.

4.2. Objectives

4.2.1 To identify and compare CD44 and ALDH1 expression in OSMFs and OSCCs.

4.2.2 To analyze the association between CD44 and ALDH1 expression and histological grade in OSMFs.

4.2.3 To analyze the association between CD44 and ALDH1 expression and histological grade of OSCCs.

Chapter 5

MATERIALS AND METHODS

5.1 Study design

The present study was a cross-sectional hospital and laboratory-based descriptive and analytical study.

5.2 Place of study

1. Department of Oral Medicine, University of Dental Medicine, Yangon

2. Department of Oral Surgery, University of Dental Medicine, Yangon

3. Pathology Division, Department of Medical Research (Lower Myanmar), Yangon

4. Department of Laboratory, Faculty of Dentistry, Thammasat University, Bangkok, Thailand

5.3 Duration of study

This study was carried out within two years starting from January 2014 to December 2015.

5.4 Study populations

Total study populations were 30 cases of histopathologically proven OSMFs, 34 cases of OSCCs and 15 cases of normal persons.

5.5 Selection of subjects

5.5.1. Inclusion criteria

(1) Histologically proven biopsy tissues of OSMFs, OSCCs and normal cases were selected starting from January 2014 to December 2015.

(2) Archival FFPE tissues of OSMFs and OSCCs with clinical summary from Department of Oral Medicine, Yangon were selected for this study.

(3) Normal patients who came to UDM for last molar extraction and minor mucosal surgery (e.g. Mucocele) without history of tobacco habits were selected for normal control study.

(4) OSMFs and OSCCs with history of tobacco habits at least two years duration were selected for this study.

5.5.2 Exclusion criteria

(1) Cases of OSMFs and OSCCs unless the patient agreed to participate in this study were excluded.

(2) Cases of OSMFs and OSCCs without history of tobacco habits were excluded.

(3) Normal cases with history of tobacco habits were excluded.

5.6 Sampling method and sample size

According to sample size determination in designing and conducting health systems research projects, by Varkevisser *et al*., (2003), International Development Research Center, required sample size is 30 specimens in OSMFs and 30 specimens in OSCCs.

5.7 Materials

5.7.1 Laboratory equipment for H&E stain

    (1) Automatic tissue processor

    (2) Paraffin wax oven

    (3) Tissue cassettes

    (4) Rotary microtome and blades

    (5) Paraffin section mounting bath

    (6) Ordinary glass slides

    (7) Coplain jar

    (8) Staining rack

    (9) Cover slips, 24x24 mm, 0.13-0.15mm thick

    (10) Hot plate

    (11) Timer

    (12) Balance- Chyo balance

    (13) Measuring cylinder 1000 cc, 100 cc

    (14) Ordinary microscope (binocular) Olympus, Japan

5.7.2 Reagents for H&E stain

    (1) 10% buffered neutral formalin solution

    (2) Graded ethyl alcohol- 80%, 90%, 95%, absolute alcohol

    (3) Chloroform

    (4) Paraffin wax

    (5) Xylene

    (6) DPX mounting media

    (7) Distilled water

(8) Harris Hematoxylin

5.7.3 Materials for IHC study

5.7.3.1 Laboratory equipment for IHC staining method

(1) Autoclave

(2) Moist chamber

(3) Salinized slides

   Pre-cleaned 76x26 mm, 0.9-1.2mm thickness

(4) Matsunami new marker pen

(5) Staining jars and staining trough

(6) Blotting paper

(7) Timer

(8) Balance- Chyo balance

(9) Litmus paper   pH 6-8

(10)   Beaker

(11)   Measuring cylinders   1000 cc, 100 cc

(12)   Automated pipettes

(13)   Ordinary light microscope (binocular) Olympus, Japan

5.7.3.2 Reagents for IHC stain

(1) Xylene

(2) Ethanol 100%, 90%, 80%

(3) Buffers: Phosphate buffer saline (PBS) p H 7.6

(4) 3% Hydrogen peroxide

(5) DAB substrate solution, 3, 3-diaminoazobenzine tetra hydrochloride

5.7.3.3 Primary antibodies

(1) Mouse anti-human CD44 (Miltenyi Biotec Inc. Aubum, CA)

(2) Primary goat-anti-human ALDH1A1 from Santa Cruz

5.7.3.4 Counterstains

(1) 4′,6-diamidino-2-phenylindole (DAPI) in mounting medium (from Vector Laboratories, Inc. Burlingame, CA)
(2) H&E staining

5.7.3.5 Blocking antibody

(1) Normal goat IgG

5.8 Methodology

Thirty-four OSCC specimens and thirty OSMFs specimens in paraffin-embedded tissue sections were provided through the Oral Pathology Department, University of Dental Medicine, Yangon. The human subject protocol was approved by the Institutional Ethical Board according to the NIH guidelines. All specimens provided by the Oral Pathology Department were recoded. In these specimens, OSCC differentiations were determined by Oral Pathologist as well, moderate and poorly differentiation. Histopathologic grading of OSMF will be determined according to the Pindborg and Sirsat (1966) classification.

As for control study, fifteen cases of oral mucosal lesions of patients who came to UDM for surgery apart from OSMFs and OSCCs without tobacco and alcohol habits were chosen.

Following approval of the institutional review board, formalin-fixed paraffin-embedded (FFPE) tissue blocks (64 cases); OSMF (30) and OSCC (34) were retrieved. Fifteen tissue blocks of normal oral mucosa obtained from gingival and vestibular mucosa after extraction of impacted teeth were used as controls. H&E stained sections were reviewed and diagnosis confirmed by oral pathologist.

OSMF tissue sections were further subdivided into (1) very early, (2) early, (3) moderately advanced, and (4) advanced, using criteria of Pindborg and Sirsat (1966). The OSCC cases were graded as Bryne's ITF classification. These cases of OSMF and OSCC along with normal oral mucosa were studied for IHC expression of CD44 and ALDH1.

5.8.1 IHC staining

Paraffin-embedded sections were deparaffinized with xylene and a series of concentrations of ethanol, blocked with 20% IgG, and stained with ALDH1 from Santa Cruz Biotechnologies (Santa Cruz, CA) and mouse anti-human CD44 from (Miltenyi Biotec Inc. Aubum, CA). Sections were counterstained with $4'$, 6-diamidino-2-phenylindole (DAPI) in mounting medium (from Vector Laboratories, Inc. Burlingame, CA) demonstrating nuclei. Negative controls were stained without primary antibodies. Stained sections were examined under microscope. Positive rates for CD44 and ALDH1 were counted in all OSCCs and OSMFs sections.

5.9 Operational and working definition

(1) Squamous cell carcinoma

SCC is a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and or the presence of intercellular bridges.

(2) Oral submucous fibrosis

Oral submucous fibrosis (OSMF) is a chronic debilitating disease and a premalignant condition of the oral cavity characterized by generalized submucosal fibrosis.

(3) Histological grading criteria for OSMF (Pindborg and Sirsat, 1966)

Very early stage (grade I)

Presence of fine fibrillar collagen with marked edema

Extensive fibroblastic response

Blood vessels are normal or dilated and congested

Inflammatory cells usually neutrophils and occasional eosinophils

Early stage (grade II)

Early hyalinization in the juxta- epithelial region

Plump young fibroblasts in moderate numbers

Blood vessels are dilated and congested

Inflammatory cells are lymphocytes, eosinophils and occasional plasma cells

Moderately advanced stage (grade III)

Collagen is moderately hyalinized

Fibroblastic response is less evident consisting of only adult fibrocytes

Blood vessels are normal or constricted

Inflammatory cells are lymphocytes and plasma cells with occasional eosinophil

Advanced stage (grade IV)

Collagen is completely hyalinized

Hyalinized areas are devoid of fibroblasts

Blood vessels are completely obliterated

Inflammatory cells are lymphocytes and plasma cells

(4)Histological grading criteria for OSCC (Bryne's invasive tumor front grading system)

Four parameters mentioned below were measured in the deepest invasive margins of OSCCs, and not in the whole thickness of the tumor, and graded as Grade I, II and III.

Table 1. Bryne's (ITF) Invasive Tumor Front grading system

Morphologic parameter	Points			
	1	2	3	4
Degree of keratinization	>50% cells keratinized	20-50% cells keratinized	5-20% cells keratinized	0-5% cells keratinized
Nuclear pleomorphism	0-1	2-3	4-5	>5
Pattern of invasion	Pushing, well-delineated infiltrating borders	Infiltrating, solid cords, bands and/or strands	Small groups or cords of infiltrating cells	Marked and widespread cellular dissemination in small groups and/or in single cells
Lympho-plasmacytic infiltration	Marked	Moderate	Slight	None

The sum of scores was grouped as follows:

Grade I 4-8

Grade II 9-12

Grade III 13-16.

(5)Tobacco habit

Tobacco habit was defined in this study as usage of betel quid chewing at least 6/day or usage of smoking tobacco or smokeless tobacco at least 2 years duration in the past.

5.10 Data analysis

Data analysis was done by using SPSS version 19, IBM product. Quantitative data was expressed as means±SD, and the difference in positive rates of CD44 and ALDH1 in OSMF specimens or OSCC specimens were analyzed by using Kruskal-Wallis and Chi-square test. A p-value of less than 0.05 was considered as statistically significant.

Chapter 6

RESULTS

Table 2. Age and sex distribution of OSMF and OSCC (%)

Age group	OSMF (n=30)		OSCC (n=34)		Total (n=64)	
	M	**F**	**M**	**F**	**M**	**F**
10-20	6(20)	-	-	-	6(9.36)	-
21-30	14(46.67)	5(16.67)	1(2.94)	-	15(23.44)	5(7.81)
31-40	2(6.67)	1(3.33)	8(23.53)	-	10(15.63)	1(1.56)
41-50	1(3.33)	1(3.33)	8(23.53)	2(5.88)	9(14.06)	3(4.69)
51-60	-	-	7(20.59)	-	7(10.94)	-
61-70	-	-	4(11.76)	3(8.82)	4(6.25)	3(4.69)
>71	-	-	1(2.94)	-	1(1.56)	-
Total	23(76.67)	7(23.33)	29(85.29)	5(14.7)	52(81.24)	12(18.75)

Table 3. Statistics for age

	OSMF	**OSCC**	**Remark**
Mean age	26.80	50.26	
SD	7.618	11.177	
Chi-square test	13.067	10.000	
df	16	21	
Asymp. Sig.	.668	.979	Not significant

SD= Standard Deviation

Age and sex distribution of OSMF and OSCC (Table 2 & 3)

In the present study, 30 cases of OSMF and 34 cases of OSCC were chosen for the immunohistochemical expression of CD44 and ALDH1. Among them, 23 cases (76.67%) in OSMF and 29 cases (85.29%) in OSCC were male. Male-female ratio was 3.29:1 in OSMF and 5.8:1 in OSCC. Mean age for OSMF was 26.80 and standard deviation was 7.618 while mean age for OSCC was 50.26 and standard deviation was 11.177. Prevalence rate of both diseases was higher in male than female. Mean age was older in OSCC than in OSMF.

Table 4. IHC staining of CD44 and ALDH1 in OSMF, OSCC and control cases

	CD44	**ALDH1**	**Co-expression**
OSMF (n=30)	80.00% (n=24)	56.67% (n=17)	56.67% (n=17)
OSCC (n=34)	58.82% (n=20)	55.88% (n=19)	41.18% (n=14)
Normal (n=15)	6.67% (n=1)	6.67% (n=1)	0.00% (n=0)

Table 5. Test statistics of CD44 and ALDH1 in OSMF and OSCC

	OSMF		OSCC		CD44+/ALDH1 +	
	CD44	ALDH1	CD44	ALDH1	OSMF	OSCC
Chi-square	37.600	14.000	15.412	16.000	.533	1.059
df	5	3	4	4	1	1
Asymp. Sig.	.000*	.003*	.004*	.003*	.465**	.303**

*= Significant   **=Not significant

General expression of CD44 and ALDH1 in OSMF, OSCC and control cases (Table 4 & 5)

In the present study, general expression of CD44 was 80% in OSMF and 58.82% in OSCC, while general expression of ALDH1 was 56.67% in OSMF and 55.88% in OSCC. In control cases, these markers were seen only 6.67%. Co-expression of CD44 and ALDH1 was 56.67% in OSMF, 41.18% in OSCC and 0% in control cases. In OSMF, Chi-square test for CD44 was 37.600 (p= .000), ALDH1 was 14.000 (p= .300) and co-expression was .533 (p= .465). In OSCC, Chi-square test for CD44 was 15.412 (p= .004), ALDH1 was 16.000 (p= .003) and co-expression was 1.059 (p= .303).

Table 6. CD44 and ALDH1 expression in OSMF cases (n = 30)

Expression	CD44 and/or ALDH1 expression in OSMF cases, n (%)			
	CD44+	ALDH1+	CD44+/ALDH1+	CD44-/ALDH1-
General expression	24 (80)	17 (56.67)	17 (56.67)	6 (20)
Score 4 CD44+	20 (83.33)	14 (82.35)	14 (82.35)	-
Score 3 CD44+	2 (8.33)	2 (11.76)	2 (11.76)	-
Score 2 CD44+	2 (8.33)	1 (5.88)	1 (5.88)	-
Score 1 CD44-	-	-	-	6 (100)
Score 4 ALDH1+	4 (16.67)	4 (23.53)	4 (23.53)	-
Score 3 ALDH1+	1 (4.17)	1 (5.88)	1 (5.88)	-
Score 2 ALDH1+	12 (50)	12 (70.59)	12 (70.59)	-
Score 1 ALDH1-	7 (29.17)	-	-	6 (100)

Area distribution score

Score – 1   None

Score – 2 Focal/localized areas

Score – 3 Diffused areas

Score – 4 Extensive areas

CD44 and/or ALDH1 were expressed in 24 (80%) of OSMF samples. CD44 was more often expressed in extensive areas, but ALDH1 more frequently represented to stain localized areas of epithelial cells in OSMF samples.

Table 7. CD44 and ALDH1 expression in OSCC cases (n = 34)

Expression	CD44 and/or ALDH1 expression in OSCC cases, n (%)			
	CD44+	ALDH1+	CD44+/ALDH1+	CD44-/ALDH1-
General expression	20 (58.82)	19 (55.88)	14 (41.18)	9 (26.47)
Score 4 CD44+	8 (40)	7 (36.84)	7 (50)	-
Score 3 CD44+	3 (15)	2 (10.53)	2 (14.29)	-
Score 2 CD44+	9 (45)	5 (26.32)	5 (35.71)	-
Score 1 CD44-	-	5 (26.32)	-	9 (100)
Score 4 ALDH1+	-	-	-	-
Score 3 ALDH1+	6 (30)	7 (36.84)	6 (42.86)	-
Score 2 ALDH1+	8 (40)	12 (63.16)	8 (57.14)	-
Score 1 ALDH1-	6 (30)	-	-	9 (100)

Area distribution score

Score – 1 None

Score – 2 Focal/localized areas

Score – 3 Diffused areas

Score – 4 Extensive areas

CD44 and/or ALDH1 were expressed in 25 (73.53%) of OSCC samples. Percentages of CD44 and ALDH1 expressions were nearly the same in studied OSCC samples. However, CD44 was more often expressed in extensive areas, whereas ALDH1 more frequently represented to stain a localized area of tumor cell population.

In CD44+OSMF cases, 83.33% (20/24) showed extensive area expression (Table 6). In CD44+OSCC cases, 40% (8/20) showed extensive area expression (Table 7).

In ALDH1+OSMF cases, 23.53% (4/17) were detected extensive area expression (Table 6). In ALDH1+OSCC cases, extensive area expression was not detected and only diffused and localized area expression was detected (Table 7).

Table 8. Staining intensity in OSMF (n =30)

Marker	Staining intensity of CD44 and ALDH1 in OSMF cases, n (%)			
	Score 1	Score 2	Score 3	Score 4
CD44	6 (20%)	2 (6.67%)	4 (13.33%)	18 (60%)
ALDH1	13 (43.33%)	12 (40%)	5 (16.67%)	-

Intensity score for IHC

Score – 1 No staining

Score – 2 Mild staining

Score – 3 Moderate staining

Score – 4 Strong staining

Remark: In OSMF, staining intensity of CD44 was high in strong intensity and ALDH1 was high in mild to moderate staining.

Table 9. Staining intensity in OSCC (n = 34)

Marker	Staining intensity of CD44 and ALDH1 in OSCC cases, n (%)			
	Score 1	**Score 2**	**Score 3**	**Score 4**
CD44	14 (41.18%)	11 (32.35%)	1 (2.94%)	8 (23.53%)
ALDH1	15 (44.18%)	12 (35.29%)	7 (20.59%)	-

Intensity score for IHC

Score – 1 No staining

Score – 2 Mild staining

Score – 3 Moderate staining

Score – 4 Strong staining

Remark: In OSCC, staining intensity of both protein markers was likely to be the same.

For staining intensity, CD44 revealed strong staining about 60% in OSMF and 23.53% in OSCC. ALDH1 showed only moderate staining, 16.67% in OSMF and 20.59% in OSCC (Table 8 & 9).

Table 10. Evaluation of patient data and expression of CD44

and ALDH1 in OSMF cases

Patient data	n	Evaluation of patient data and pathological variance in OSMF cases, n (%)					
		CD44+		ALDH1+		CD44+/ ALDH1+	CD44- /ALDH1-
		(+)	(-)	(+)	(-)		
Gender							
Male	23	18 (78.26)	5 (21.74)	14 (60.87)	9 (39.13)	14 (60.87)	5 (21.74)
Female	7	6 (85.71)	1 (14.29)	3 (42.86)	4 (57.14)	3 (42.86)	1 (14.29)
Age							
≤30 year	25	19 (76)	6 (24)	15 (60)	10 (40)	15 (60)	6 (24)
31-60 year	5	5 (100)	-	2 (40)	3 (60)	2 (40)	-
>60 year	-	-	-	-	-	-	-
Duration							
<6 m	23	18 (78.26)	5 (21.74)	12 (52.17)	11 (47.83)	12 (52.17)	5 (21.74)
6-12 m	6	5 (83.33)	1 (16.67)	4 (66.67)	2 (33.33)	4 (66.67)	1 (16.67)
>12 m	1	1 (100)	-	1 (100)	-	1 (100)	-

OSMF was more prevalent in male due to their habit of betel quid. According to the present study, male <30 year of age was high prevalence rate as well as positivity of both markers. Positive cells were directly proportionate to duration of disease symptoms.

Table 11. Evaluation of pathological variance and expression

of CD44 and ALDH1 in OSMF Cases (n = 30)

Pathologic variance	+/-	n	Evaluation of patient data and pathological variance in OSMF cases, n (%)					
			CD44+		ALDH1+		CD44+/ ALDH1+	CD44- /ALDH1-
			(+)	(-)	(+)	(-)		
Lymphocytes in ECJ	P	12	8 (66.67)	4 (33.33)	6 (50)	6 (50)	6 (50)	4 (33.33)
	N	18	16 (88.89)	2 (11.11)	11 (61.11)	7 (38.89)	11 (61.11)	2 (11.11)
Hyalinization	P	29	23 (79.31)	6 (20.69)	17 (58.62)	12 (41.38)	17 (58.62)	6 (20.69)
	N	1	1 (100)	-	-	1 (100)	-	-
Compressed vessel	P	23	17 (73.91)	6 (26.09)	12 (52.17)	11 (47.83)	12 (52.17)	6 (26.09)
	N	7	7 (100)	-	5 (71.43)	2 (28.57)	5 (71.43)	-
Fibroblasts	P	8	7 (87.5)	1 (12.5)	6 (75)	2 (25)	6 (75)	1 (12.5)
	N	22	17 (77.27)	5 (22.73)	11 (50)	11 (50)	11 (50)	5 (22.27)
Lesser vessel	P	5	5 (100)	-	2 (40)	3 (60)	2 (40)	1 (20)
	N	25	19 (76)	6 (24)	15 (60)	10 (40)	15 (60)	5 (20)
Muscle atrophy	P	10	10 (100)	-	7 (70)	3 (30)	7 (70)	-
	N	20	14 (70)	6 (30)	10 (50)	10 (50)	10 (50)	6 (30)

(P = Positive, N = Negative)

Increased CD44 and ALDH1 positive cells are detected in scanty lymphocytic infiltration in epithelial-connective junction of OSMF cases. CD44+cells are associated with numerous fibroblast cell counts and muscle atrophy.

Table 12. Evaluation of patient data and expression of CD44

and ALDH1 in OSCC cases

	n	**Evaluation of patient data and pathological variance in OSCC cases, n (%)**					
		CD44+		**ALDH1+**		**CD44+/ ALDH1+**	**CD44- /ALDH1-**
		(+)	(-)	(+)	(-)		
Gender							
Male	29	16 (55.17)	13 (44.83)	15 (51.72)	14 (48.28)	10 (34.48)	8 (27.59)
Female	5	4 (80)	1 (20)	4 (80)	1 (20)	4 (80)	1 (20)
Age							
≤30 year	1	1 (100)	-	-	1 (100)	-	-
31-60 year	25	12 (48)	13 (52)	14 (56)	11 (44)	10 (40)	8 (32)
>60 year	8	7 (87.5)	1 (12.5)	5 (62.5)	3 (37.5)	4 (50)	1 (12.5)
Duration							
<6 m	33	19 (57.58)	14 (42.42)	18 (54.55)	15 (45.45)	13 (39.39)	9 (27.27)
6-12 m	1	1 (100)	-	1 (100)	-	1 (100)	-
>12 m	-	-	-	-	-	-	-

In OSCC, CD44+ and ALDH1+ are more evident in female, with increasing age.

Table 13. Evaluation of pathological variance and expression

of CD44 and ALDH1A1 in OSCC cases

Pathological variance	n	Evaluation of patient data and pathological variance in OSCC cases, n (%)				CD44+/ ALDH1+	CD44- /ALDH1-
		CD44+		ALDH1+			
		(+)	(-)	(+)	(-)		
Keratinization							
>50%	6	5 (83.33)	1 (16.67)	4 (66.67)	2 (33.33)	4 (66.67)	1 (16.67)
20-50%	14	7 (50)	7 (50)	8 (19.51)	6 (42.86)	4 (28.57)	3 (21.43)
5-20%	13	7 (53.85)	6 (46.15)	6 (46.15)	7 (53.85)	5 (38.46)	5 (38.46)
0-5%	1	1 (100)	-	1 (100)	-	1 (100)	-
Nuclear Polymorphism							
Little	1	1 (100)	-	-	1 (100)	-	-
Moderately abundant	19	11 (57.89)	8 (42.11)	13 (68.42)	6 (31.58)	8 (42.11)	3 (15.79)
Abundant	13	7 (53.85)	6 (46.15)	5 (38.46)	8 (61.54)	5 (38.46)	6 (46.15)
Extreme	1	1 (100)	-	1 (100)	-	1 (100)	-
Mitosis							
0-1	14	6 (42.86)	8 (57.14)	5 (35.71)	9 (64.29)	1 (7.14)	6 (42.86)
2-3	17	12 (70.59)	5 (29.41)	11 (64.71)	6 (35.29)	11 (64.71)	3 (17.65)
4-5	3	2 (66.67)	1 (33.33)	3 (100)	-	2 (66.67)	-
>5	-	-	-	-	-	-	-
Pattern of Invasion							
Score 1	6	5 (83.33)	1 (16.67)	5 (83.33)	1 (16.67)	4 (66.67)	-
Score 2	2	2 (100)	-	-	2 (100)	-	-
Score 3	12	5 (41.67)	7 (58.33)	6 (50)	6 (50)	4 (33.33)	3 (25)
Score 4	14	8 (57.14)	6 (42.86)	8 (57.14)	6 (42.86)	6 (42.86)	6 (42.86)
Lympho-plasmacyte*							
Marked	9	4 (44.44)	5 (55.56)	4 (44.44)	5 (55.56)	2 (22.22)	3 (33.33)
Moderate	10	5 (50)	5 (50)	3 (30)	7 (70)	2 (20)	3 (30)
Slight	14	11 (78.57)	3 (21.43)	11 (78.57)	3 (21.43)	10 (71.43)	3 (21.43)
Non-	1	-	1 (100)	1 (100)	-	-	-
Bryne's Classification							
Grade I (4-8)	13	9 (69.23)	4 (30.77)	8 (61.54)	5 (38.46)	6 (46.15)	2 (15.38)
Grade II	15	6 (40)	9 (60)	7 (46.67)	8 (53.33)	4 (26.67)	6 (40)

(9-12)							
Grade III (13-16)	6	5 (83.33)	1 (16.67)	4 (66.67)	2 (33.33)	4 (66.67)	1 (16.67)

*In slight lymphoplasmacytic infiltration, positivity of both markers is higher.

Table 14. Expression of CD44 and ALDH1 in OSMF and OSCC cases (%)

Patient data	CD44+		ALDH1+		CD44+/ALDH1+		CD44-/ALDH1-	
	OSMF	OSCC	OSMF	OSCC	OSMF	OSCC	OSMF	OSCC
General*	80	58.82	56.67	55.88	56.67	41.18	20	26.47
Gender								
Male	78.6	55.17	60.87	51.72	60.87	34.48	21.74	27.59
Female	85.71	80	42.86	80	42.86	80	14.29	20
Age								
≤30 y	76	100	60	--	60	--	24	--
31-60 y	100	48	40	56	40	40	--	32
>60 y	--	87.5	--	62.5	--	50	--	12.5
Duration**								
<6 m	78.26	57.58	52.17	54.55	52.17	39.39	21.74	27.27
6-12 m	83.33	100	66.67	100	66.67	100	16.67	--
>12 m	100	--	100	--	100	--	--	--

* CD44+OSMF were greater than CD44+OSCC. ALDH+ was nearly the same in two diseases.

** Positive rates of markers were directly associated with duration of disease.

General expression of CD44 and ALDH1 in OSMF case

The majority of OSMF cases (24/30; 80%) expressed a minimum of one protein (Table 6). Only six samples (20%) expressed neither CD44 nor ALDH1. CD44 expression was detected in 80% (24/30) of studied OSMF cases. In the CD44+ OSMF cases, 83.33% (20/24) showed extensive area of protein expression, two cases (8.33%) revealed diffused area of protein expression and the remaining two cases (8.33%) was seen localized area of protein expression. CD44 expression intensity was scaled within OSMF cells (Table 8). Sixty % (18/30) of the CD44+ OSMF expression for the receptor was strong (+++), 13.33% (4/30) the CD44 expression was moderate (++) and 6.67% (2/30) of samples the CD44 level was weak (+). Epithelial cells were solely CD44+ or CD44-expressing epithelial cells were located in the oral lining epithelium (Fig.16 - 21).

ALDH1 expression was detected in 56.67% (17/30) of the studied OSMF samples (Table 6). In the ALDH1+ OSMF cases, 23.53% (4/17) showed extensive area of protein expression and only one case (5.88%) was detected diffused area of expression. Majority of ALDH1+ OSMF cases (12/17; 70.59%) revealed localized area of protein expression. None of the ALDH1+ OSMFs this enzyme was expressed strongly in the majority of epithelial cells (Fig.28-31). Five cases (16.67%) were expressed moderate intensity and majority of ALDH1+ OMFS cases (12/30; 40%) was evident with mild intensity of protein expression (Table 8). ALDH1-expressing cells were found to be singular or in groups. More often ALDH1+ OSMF cell groups were located deeper areas of connective tissues.

More than half of the analyzed OSMFs (17/30; 56.67%) expressed co-staining of CD44 and ALDH1 (Table 6). In these OSMF cases the expression pattern of CD44 and ALDH1 intersected, but did not completely overlap. In CD44+/ALDH1+ OSMF cases, CD44 showed mainly extensive area expression

(14/17; 82.35%) and ALDH1 showed localized area expression (12/17; 70.59%) (Table 6). High staining intensity of CD44 was seen in 60% of OSMF cases and ALDH1 was detected 56.67% of OSMF cases with mild and moderate intensity (Table 8).

General expression of CD44 and ALDH1 in OSCC cases

The majority of OSCC cases (25/34; 73.53%) expressed a minimum of one protein (Table 7). Percentage of CD44+ OSCC and ALDH1+ OSCC was nearly the same, 20/34 (58.82%) and 19/34 (55.88%) respectively. Only nine samples (26.47%) expressed neither CD44 nor ALDH1. CD44+ OSCC cases showed extensive area as well as localized area of expression nearly the same amount cases, 8/20 (40%) was for the former and 9/20 (45%) was detected as localized expression. Three cases (15%) showed diffused expression. CD44 expression intensity was scaled within OSCC cells (Table 9). In OSCC, 23.53% (8/34) showed CD44 expression for the receptor was strong (+++), 2.94% (1/34) was moderate (++) and 32.35% (11/34) of samples the CD44 level was weak (+) (Fig.22-25).

ALDH1 expression was detected in 55.88% (19/34) of the studied OSCC samples (Table 7). In the ALDH1+ OSCC cases, seven cases (36.84%) showed diffused area of expression and 12 cases (63.16%) revealed localized area. Staining intensity for ALDH1 was slightly faint. ALDH1-expressing cells were found to be singular, in groups or throughout the entirely cell nest (Fig.32-35). Staining intensity of both markers in OSCC cases was frequently the same (Table 9).

CD44+/ALDH1+ OSCC cases are detected in 41.18% (14/34) of studied OSCC samples (Table 7). In these cases the expression pattern of CD44 and ALDH1 intersected, but did not completely overlap.   In

CD44+/ALDH1+OSCC 14 cases, CD44 showed (7/14) 50% extensive area expression while ALDH1 was detected localized area expression eight cases (57.14%) (Table 9).

Analysis of CD44 and ALDH1A1 expression in OSMF patients

CD44 was more extensively and strongly expressed in OSMF cases than ALDH1. Numbers of ALDH1+ OSMF cases and CD44+/ALDH1+ OSMF cases were the same amount. Both proteins were prominent in male patients and age range was more significant in ≤30 years of age. Protein expressions were more prominent in duration of signs and symptoms ≤6 months (Table 10). As the pathological variance, both proteins showed nearly the same expression in association with lymphocytic infiltration in epithelial connective tissue junction. However, CD44-/ALDH1- OSMF cases are more prominent in lymphocytic infiltration. Lymphocytic infiltration was favored for negative expression of both markers. Hyalinization showed no significant effect on expression pattern. Fibroblasts gave rise to positivity of CSCs. Amount of fibroblasts was inversely associated with CD44-/ALDH- cells. Lesser blood vessel was favored for decreased expression of CSCs. In muscle atrophy cases, amounts of CD44+/ALDH1+ cells are significantly higher (Table 11).

Analysis of CD44 and ALDH1 expression in OSCC patients

CD44 and ALDH1 were detected nearly the same amount of OSCC cases. Male was predominant than female and age range that markers were prominently detected in OSCC was in 31-60 years of age and <6 months duration of signs and symptoms (Table 12). ALDH1+ cells were high in 20-50% keratinization and small groups or cords of invasive front. Moderate amount of nuclear polymorphism was positively associated with CD44 and ALDH1. CD44

was higher in cases of 2-3 mitosis. In OSCC, lymphoplasmacytic infiltration is inversely related with CD44+/ALDH1+ cells, that is, in marked lymphoplasmacytic infiltration, expression of CD44 and ALDH1 was low and in slight lymphoplasmacytic infiltration, expression rate of markers was high. In Bryne's classification, positivity of CD44 marker was significantly high in Grade III oral squamous cell carcinoma stage and ALDH1 marker was relatively high in Grade I and II OSCC stages (Table 13).

Comparative analysis of expression of CD44 and ALDH1 in OSMF and OSCC cases

Generally, expression rate of CD44 and ALDH1 protein was higher in OSMF than in OSCC. Especially, CD44 was significantly higher in OSMF (80%) than in OSCC (58.82%). In male, expression rate was higher in OSMF, but in female, it was directed to OSCC. OSMF cases were prominently seen <30 years of age, OSCC cases were evident in 31-60 years of age groups and its highest rate was seen in >60 years of age groups. Expression rates of both markers in two entities were directly proportionate to duration of disease signs and symptoms (Table 14).