Chapter 7

DISCUSSION

ALDH1 and CD44 function in the carcinogenesis and tumor progression of HNSCC. ALDH1A1 is not expressed in the normal oral mucosa. However, other ALDH isoenzymes, such as ALDH1A3 and ALDH3A1, are found. The expression of the retinoic acid receptors in the oral mucosa underlines the function of ALDH isoenzymes in the normal mucosa. However, ALDH1A1 expression is increased in dysplasia and HNSCC.

It was roughly a decade ago when scientists revisited the view of tumor origination for the case of potentially malignant lesions. Coming from a rather stochastic model, where every single cell of a tumor would be equipped with tumorigenic potential, scientists moved towards a more hierarchical model, with only a subset of cells within malignant tissue those are able to generate tumors. In these model so-called cancer stem cells, CSCs are the founders of tumors. CSCs display to some degree properties of normal stem cells including self-renewal, pluripotency, and limitless proliferation (Cho and Clarke, 2008). The remaining cancer cells that lack these properties are characterized by a restricted or even no growth capacity at all *in vivo*.

Sex and Age distribution of OSMF and OSCC

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%) for OSMF and 29 cases (85.29%) for OSCC were male. Male-female ratio was 3.29:1 for OSMF and 5.8:1 for 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. Male incidence was high in both diseases. Age incidence was older in OSCC than in OSMF.

According to this finding, incidence rate of OSMF was more in male than in female. It may be due to the fact that men are chronic betel quid chewers in Asian continent.

Betel quid chewing is an ancient practice common in many countries of Asia and among migrated communities in Africa, Europe and North America. It enjoys complete social acceptance in many societies and is also popular among women. In its most basic form, betel quid consists of betel leaf (Piper betel), areca nut, the main psychoactive ingredient, and slaked lime (calcium hydroxide). Areca nut is said to be the fourth most commonly used psychoactive substance in the world, after caffeine, nicotine and alcohol. There are a great variety of ingredients and ways of preparing betel quid in different countries. In some, particularly in India, tobacco is added to the quid. In recent years, commercially-manufactured non-perishable forms of betel quid (pan masala or betel quid mixtures and gutka), not containing betel leaf, have been marketed. Within a short period of about 2 decades, this industry has risen in value to several hundred US million dollars. Use of areca nut in any form is not safe for oral health; the use of commercially manufactured forms seems even riskier (Gupta and Ray, 2004)

"Betel quid chewers' oral cancer" is one of the most common malignancies in South and Southeast Asian countries. Oral premalignancies are also very common in betel quid chewers and about 10% of these undergo

malignant transformation. Although education for cessation of the betel quid chewing habit is important, there are few adequate strategies and policies for primary prevention, health promotion and education related to oral cancer control, especially in rural areas. In addition to oral health education, it is also crucial to establish a data-management system as well as monitoring and evaluation systems for oral cancer prevention (Chiba, 2001).

Oral cancer is considered widely to be a form of cancer whose etiology is well understood and which is becoming relatively rare in developed countries. There have been, however, a series of reports indicating that after many years of declining risk, the rates may be rising again in men. National time-series of oral-cancer mortality data available in the World Health Organization's mortality database showed that nineteen out of 24 national datasets demonstrate a similar pattern of increasing cohort-effects for oral cancer in men. The largest increases have occurred in countries of central and Eastern Europe where rates have increased by a factor of from three to 10 within a generation. The cohort-based nature of the changes observed in men suggest that there will be a continuing increase in the absolute numbers of cases of oral cancer to be treated in the coming decades (Macfarlane *et al*., 1994).

OSMF, a precancerous condition of the oral cavity, has been studied by a number of workers in the field. The available epidemiological data showed a clear-cut geographical and ethnic predisposition, which suggested that certain customs/habits prevalent among the population groups in south-east Asia might be possible etiological factors.

In the present study, mean age of OSCC was higher than that of OSMF. It is logical that time is an important factor to get malignant transformation in the

potentially malignant lesions. Early detection is crucial role to prevent deadly malignancy.

The incidence of oral cancer remains high and is associated with many deaths in both Western and Asian countries. Several risk factors for the development of oral cancer are now well known, including smoking, drinking and consumption of smokeless tobacco products. Genetic predisposition to oral cancer has been found in certain cases but its components are not yet entirely clear. In accordance with the multi-step theory of carcinogenesis, the natural history of oral cancer seems to gradually evolve through transitional precursor lesions from normal epithelium to a full-blown metastatic phenotype. A number of genomic lesions accompany this transformation (Tsantoulis *et al*., 2007).

Expression pattern of CD44 and ALDH1 in OSMF and OSCC

Head and neck squamous cell carcinomas cells are heterogeneous within the same lesion, i.e., their cell hierarchy allows to find subpopulations with exclusive biological behaviors, e.g., tumor growth and metastatic potential (Bhaijee *et al*., 2012). The results of present study showed positive CD44 and ALDH1 expression in both basal and supra-basal cells, which may indicate that the immunohistochemical technique here employed was not appropriate to identify cancer stem cells in HNSCCs based on these markers, as also reported by other authors. Moreover, no specific markers have been established for the identification of cancer stem cell subpopulations in HNSCCs, as is the case with other tumors. Notwithstanding, immunohistochemistry did allow to identify the location of positive cells in different tumor areas (tumor center and invasive front), as well as in the different layers of adjacent non-tumor epithelium and basal and para-basal cell layers of OSMF.

Immunostaining results obtained for the OSCC and non-tumor epithelium from OSMF showed that tumors with ALDH1+ and CD44+ cells also presented positive surrounding epithelial tissues and non-tumor epithelium of OSMF. This followed the concept of field cancerization, according to which carcinogen-induced changes would be present in HNSCC-related non-tumor tissues before morphological alterations can be found. This concept was first proposed by Slaughter *et al.* (1953), and later examined by Califano *et al.* (1996). The finding of ALDH1+ and CD44+ 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.

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. Chi-square test for CD44 and ALDH1 was 37.600 (p= .000) and 14.000 (p= .003) in OSMF, 15.412 (p= .004) and 16.000 (p= .003) in OSCC and CD44+/ALDH1+ in OSMF was .533 (p= .465) and in OSCC 1.059 (p= .303), respectively (Table 4 & 5).

CD44 appears to be present at different stages of Carcinogenesis (Oliveira *et al.*, 1998). At first, CD44 binding to hyaluronic acid in the extracellular matrix stabilizes neoplastic cells in the invasive front, stimulating cell proliferation and consequently the growth of cancer cell islands (Bankfalvi *et al.*, 2002; Gergolios *et al.*, 2006). Single-cell invasion shows a strongly positive expression of CD44 around the entire plasma membrane (Oliveira *et al.*, 1998). Subsequently, the loss of CD44 expression suggests a second stage of tumor

progression, in which cells lose their cell–cell adhesion, facilitating mobility and consequently the invasion and migration of neoplastic cells (Carinci *et al*., 2002).

The expression of specific CD44 variants may be reduced in tumors with a worse prognosis and high tumor grade, but overall CD44 expression is not (Sato *et al*., 2000; Stoll *et al*., 1999; Wang *et al*., 2009).

In the present study, ALDH1 and CD44 immunostaining results were higher in non-tumor epithelium of OSMF, suggesting that these markers could be used as an instrument to predict higher or lower risk of malignant transformation. Moreover, in the non-tumor epithelial portions of OSMF, CD44 and ALDH1 was seen in 80% and 56.67%; in tumor portion and non-tumor portion of OSCC, CD44 and ALDH1 was seen in 58.82% and 55.88% respectively. This finding indicates the presence of cells with a highly tumorigenic potential even in cancer-free zones.

Role of Hyaluronan and CD44 in Oral cancer

A number of studies have aimed at identifying the specific molecules expressed in HNSCC that correlate with invasive behavior. Among such candidates are hyaluronan (HA) (Knudson *et al*., 2002; Toole *et al*., 2008) and its major cell surface receptor, CD44. HA is a major component of the extracellular matrix component and is significantly enriched in many types of tumors (Knudson *et al*., 2002; Toole *et al*., 2008). HA binds to its specific cell surface receptor, CD44, a multifunctional transmembrane glycoprotein expressed in many cells and tissues including HNSCC cells and carcinoma tissues (Turley *et al*., 2002; Assimakopoulos *et al*., 2002; Carinci *et al*., 2001). CD44 is often expressed as a variety of variant isoforms, generated by an alternative splicing mechanism (Screaton *et al*., 1992). The expression of certain CD44 variant (CD44v) isoforms

is known to be associated with head and neck cancer progression (Turley *et al.*, 2002; Assimakopoulos *et al.*, 2002; Carinci *et al.*, 2001).

CD44 denotes a family of glycoproteins that are often overexpressed in a variety of human solid neoplasms including head and neck cancers. HA, the major glycosaminoglycan found in the extracellular matrix of mammalian tissues, is now considered to be a physiologically relevant ligand for CD44 (also known as a hyaluronan receptor) in many cell types including head and neck tumor cells (Knudson *et al.*, 2002; Toole *et al.*, 2008). Most of malignant solid tumors display high levels of HA (Toole *et al.*, 2008). HA has been suggested to play an important role in tumor angiogenesis and invasion. Specifically, the large HA polymers is thought to provide a hydrated micro-niche that facilitates the invasion of tumor cells into the extracellular matrix materials (Toole *et al.*, 2008), whereas the smaller HA fragments (large HA degraded by hyaluronidases) promote angiogenesis and tumor neovascularization. The invasive potential can be further enhanced by HACD44 interaction during tumor progression. Both HA and CD44 appear to be overexpressed at the sites of tumor attachment and are known to be involved in tumor cell-specific properties (*e.g.* tumor cell growth, migration, and invasion) (Turley *et al.*, 2002).

The stem cell niche provides a regulatory microenvironment for cells as diverse as totipotent embryonic stem cells to cancer stem cells (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.

CD44 is a central component in the cross-talk between ASCs and CSCs and their respective niches. The complexity of CD44 splicing and the number of isoforms generated reflect the diversity of CD44 function in the niches of many stem cell types, including ESCs, HSCs, MSCs, tissue ASCs and CSCs. CD44 binds HA to mediate self-renewal, retention of peri-cellular matrix, proliferation, differentiation and/or homing to bone marrow. CD44/HA interactions are also significant in preserving the integrity of the stem cell genome by decreasing DNA damage, increasing DNA repair and enhancing cell survival under conditions of oxidative stress. CD44/integrin/E-selectin interactions promote TEM and invasion of MSCs into bone marrow. Moreover, CD44 interacts with other ECM components, including heparin sulfate and a range of growth factor ligands, to promote CD44/ligand/RTK complex formation and signal transduction. These signaling pathways regulate matrix production and assembly, development, cell migration, homing and preparation of the (pre)metastatic site. Of particular note is that CD44/ ligand/RTK signaling modulates microRNA expression to regulate promoter methylation status and gene expression. Through this mechanism, CD44 participates in reprogramming cells to exhibit a more stem cell-like phenotype. Therefore, reprogramming cancer cells to exhibit a CSC phenotype could be an elemental mechanism in promoting tumor progression and chemo-resistance.

CD44 is an integral membrane glycoprotein that has diverse functions in cell-cell and cell-substrate interactions. It has been suggested that it may be a determinant of metastatic and invasive behavior in carcinomas. There was no significant difference of CD44 expression between *in situ* and invasive carcinomas. However, a strong difference of reaction between carcinomas and the other cases was observed. CD44 expression was statistically higher in dysplastic lesions than the cases of keratosis. CD44 expression may be involved in the multiple

mechanism of the development and progression of laryngeal lesions and may help to predict the risk of transformation of the benign or precancerous lesions to cancer (Ioachim, 1999).

In the present study, CD44 was expressed in 80% of OSMF and 58.82% of OSCC with intensity of strong staining 60% and 23.53%, respectively. This observation confirmed a more general function of CD44 on OSMF as well as OSCC. One explanation was CD44 a ubiquitously expressed family of cell surface adhesion molecules involved in cell-cell and cell-matrix interactions. 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 (Goodison and Tarin., 1999).

Correlation of markers with lymphocytes in epithelial-connective tissue junction of OSMF and lymphoplasmacytic infiltrations around the tumor cells of OSCC.

CD44 is an adhesion protein expressed on inflammatory and vascular cells. CD44 supports the adhesion of activated lymphocytes to endothelium and smooth muscle cells. Furthermore, ligation of CD44 induces activation of both inflammatory and vascular cells (Cuff *et al*., 2001). In the present study, CD44 expression was higher in a scanty concentration of lymphocytes in epithelial-connective junction in OSMF cases, as well as it was the same event in OSCC cases.

CSCs are considered as the "seeds" in tumor development, metastasis and recurrence. Despite the various immunosurveillance mechanisms in the host, CSCs may possess the phenotypic and functional properties to evade host immunosurveillance and immune-mediated rejection in immunologically intact

individuals. The mechanisms of CSC recognition and their consequent destruction are actively disturbed by various processes, including altered immunogenicity of CSCs, production of TSC-derived regulatory molecules, and interaction of CSCs with tumor-infiltrating immune cells. In addition to these CSC-mediated mechanisms, the diverse mesenchymal cells and cytokines in the tumor microenvironment are contributed to CSC immune escape (Qi *et al*., 2012).

Carcinomas are composed of neoplastic epithelial cells, which form the heart of the tumor, as well as a variety of mesenchymal cell types and extracellular matrix components that comprise the tumor stroma, often termed its microenvironment. The normal counterparts of some stromal cells are thought to limit tumor growth, while tumor-associated stromal cells have been convincingly shown to actively promote tumor progression via complex heterotypic interactions with the nearby carcinoma cells. More recent advances have revealed that tumor-host interactions extend well beyond the local tissue microenvironment (i.e., interactions between the neoplastic cells and the nearby stroma) and that tumors not only respond to, but actively perturb host organs at distant anatomic sites. This indicates that many aspects of tumor biology can only be explained by a detailed understanding of both local and systemic interactions (McAllister and Weinberg, 2010).

In the present study, CD44 and ALDH1 proteins are detected in the decreased local immune response in premalignant lesion and oral malignancy. It could be suggested that these markers actively participate in plasticity and heterogeneity of microenvironment in the absence of immune response.

Themeli *et al*. (2013) made a research to generate tumor-targeted human T-lymphocytes from induced pluripotent stem cells for cancer therapy. Progress in adoptive T-cell therapy for cancer and infectious diseases is hampered by the lack of readily available, antigen-specific, human T lymphocytes.

Pluripotent stem cells could provide an unlimited source of T lymphocytes, but the therapeutic potential of human pluripotent stem cell-derived lymphoid cells generated to date remains uncertain. They combine induced pluripotent stem cell (iPSC) and chimeric antigen receptor (CAR) technologies to generate human T cells targeted to CD19, an antigen expressed by malignant B cells, in tissue culture. These iPSC-derived, CAR-expressing T cells display a phenotype resembling that of innate γδ T cells. Similar to CAR-transduced, peripheral blood γδ T cells, the iPSC-derived T cells potently inhibit tumor growth in a xenograft model. This approach of generating therapeutic human T cells 'in the dish' may be useful for cancer immunotherapy and other medical applications (Themeli *et al*., 2013).

In the present study, decreased tumor-host immune reaction revealed the prominent expression of tumor stem cell markers. Cancer chemotherapy should be used to eliminate the cancer stem cell niche.

Different views upon CD44 as Oral CSC marker

Mărgăritescu *et al*., (2012) stated that CD44 has limited utility in identifying oral CSCs, while CD117 and CD133 expression appears to be limited more in identifying mesenchymal stem cells.

However Bahar *et al*., (1997) suggested that alteration in CD44v6 may occur as an early event in primary oral SCC development, as well as in premalignant severe epithelial dysplasia. It can thus, be used as a molecular progression marker when screening for oral cancer. CD44v6 expression was clearly downregulated in most cases of severe premalignant lesions as well as in most of the SCCs. The staining pattern and intensity varied according to the degree of dysplasia and to the degree of differentiation of the SCCs. Premalignant severe epithelial dysplasia cases with early features of invasion, not yet developed into SCC, showed distinctly downregulated expression of CD44v6 protein whereas

hyperplastic and benign epithelial lesions (papilloma) expressed positive staining patterns comparable to those of the normal counterparts (Bahar *et al.*, 1997).

CD44 is up-regulated during the development of ovarian carcinomas but is subsequently down-regulated during their progression, resulting in aggressive behavior and an unfavorable prognosis (Saegusa *et al.*, 1999).

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 is expressed in the basal layer of the normal oral mucosa (Uhlen *et al.*, 2010; Mack B and Gires, 2008; Sterz *et al.*, 2010; Richard & Pillai, 2010). As a consequence, CD44 was overexpressed in many of the examined OSMF and OSCC tumors in the present study. CD44 was located entirely throughout the oral epithelium of submucous fibrosis and the cell nest or peripheral to the stroma of OSCC cases. CD44 can be linked to the EMT in OSMF and linked to the invasion of OSCC.

In the present study, CD44 and/or ALDH1 were expressed in 24 (80%) of OSMF samples. CD44 was more often expressed in extensive areas, but ALDH1A1 more frequently represented to stain localized areas of epithelial cells in OSMF samples. 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.

Indeed, CD44+ populations exhibit heterogeneity in expression of other CSC markers, proliferation, and tumor formation/propagation and can be

subdivided using additional markers such as ALDH1 and c-Met to enhance tumorigenicity and stemness (Krishnamurthy *et al*., 2010; Sun and Wang, 2011).

Role of CD44 in fibrosis and in tumor invasion in OSCC

The principal cells implicated as a source of extracellular matrix in areas of fibrosis are fibroblasts. Accumulation of connective tissue matrix is secondary to factors such as cytokines and growth factors. Increased bFGF expression in early stages of the disease was explainable to an initial injury phase because of areca consumption, followed by cellular activation by chemotactic cytokines and other growth factors with eventual fibrosis occurring as a result of molecular alteration at the cellular level (Bishen *et al*., 2008).

Increased levels of colligin in OSMF may contribute to the deposition of collagen and consequent increased fibrosis in the oral submucosa in OSMF lesions (Kaur *et al*., 2001).

Li *et al*. (2011) studied in a case of idiopathic pulmonary fibrosis (IPF). Tissue fibrosis is a major cause of morbidity, and IPF is a terminal illness characterized by unremitting matrix deposition in the lung. The mechanisms that control progressive fibrosis are unknown. Myofibroblasts accumulate at sites of tissue remodeling and produce extracellular matrix components such as collagen and hyaluronan (HA) that ultimately compromise organ function. Targeted overexpression of HAS2 (HA synthase 2) by myofibroblasts produced an aggressive phenotype leading to severe lung fibrosis and death after bleomycin-induced injury. Fibroblasts isolated from transgenic mice overexpressing HAS2 showed a greater capacity to invade matrix. Conditional deletion of HAS2 in mesenchymal cells abrogated the invasive fibroblast phenotype, impeded myofibroblast accumulation, and inhibited the development of lung fibrosis. Both

the invasive phenotype and the progressive fibrosis were inhibited in the absence of CD44. Treatment with a blocking antibody to CD44 reduced lung fibrosis in mice in vivo. Finally, fibroblasts isolated from patients with IPF exhibited an invasive phenotype that was also dependent on HAS2 and CD44. Understanding the mechanisms leading to an invasive fibroblast phenotype could lead to novel approaches to the treatment of disorders characterized by severe tissue fibrosis (Li *et al*., 2011).

Fibroblast proliferation is an early feature of progressive tissue fibrosis and is largely regulated by the cytokine transforming growth factor-β1 (TGF-β1). In the oral mucosa, fibroblasts have a unique phenotype and demonstrate healing with no fibrosis/scarring. Whereas dermal fibroblasts proliferate in response to TGF-β1, oral fibroblasts have an anti-proliferative response to this cytokine. Hyaluronan (HA) was directly linked to this TGF-β1-dependent response. Epidermal growth factor (EGF) and its receptor (EGFR) are essential for TGF-β1/HA/CD44-dependent proliferation. Increased HA levels promote EGFR and CD44 coupling, potentiating signal transduction through the MAPK/ERK pathway. Thus, in a HA-rich environment, late ERK1/2 activation results from EGFR/CD44 coupling and leads to a proliferative response to TGF-β1. In comparison, in a non-HA-rich environment, only early ERK1/2 activation occurs, and this is associated with an anti-proliferative response to TGF-β1. HA facilitates TGF-β1-dependent fibroblast proliferation through promoting interaction between CD44 and EGFR, which then promotes specific MAPK/ERK activation, inducing cellular proliferation (Meran *et al*., 2011).

Cell-adhesion molecules, once believed to function primarily in tethering cells to extracellular ligands, are now recognized as having broader functions in cellular signaling cascades. The CD44 transmembrane glycoprotein family adds new aspects to these roles by participating in signal-transduction

processes--not only by establishing specific transmembrane complexes, but also by organizing signaling cascades through association with the actin cytoskeleton. CD44 and its associated partner proteins monitor changes in the extracellular matrix that influence cell growth, survival and differentiation (Ponta *et al*., 2003).

CD44 was once thought to simply be a transmembrane adhesion molecule that also played a role in the metabolism of its principal ligand hyaluronan. Investigations of CD44 have established additional functions for CD44, including 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).

In the present study, expression of CD44 and ALDH1 markers in OSMF was directly associated with increased fibroblast cells. It was suggested that anti-CD44 and anti-ALDH1 should be directed in the treatment of OSMF.

Role of ALDH1 in OSMF and OSCC

In the present study, ALDH1 was detected 56.67% of OSMF and 55.88% of OSCC with strong intensity of 16.67% and 20.59%, respectively. This observation also confirmed the important role of ALDH1 in both premalignant and malignant oral lesions. This result was consistent with the Chen's finding in 2010. ALDH1-A1 is expressed in HNSCC and its expression pattern implies that ALDH1-A1 might be a marker for metastatic HNSCC (Chen *et al*., 2010).

The ALDH family is cytosolic isoenzyme responsible for oxidizing intracellular aldehydes, thus contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation. Murine and human hematopoietic and neural stem cells have high ALDH activity.

Increased ALDH1 activity has been found in stem cell populations in human multiple myeloma, acute myeloid leukemia, and brain and breast cancers. Therefore, ALDH1 activity might be usable as a common marker for both normal and malignant stem cell populations.

ALDH1 expression has been reported in some lung cancer cell lines, and the increased expression of ALDH1 could result from cigarette smoking and contribute to malignant transformation of lung cells (Patel *et al*., 2008).

The aldehyde oxidative function of the aldehyde dehydrogenase family of enzymes participates in retinoic acid biosynthesis and is thus innately linked to the regulation of squamous epithelial differentiation (Douville *et al*., 2009). High ALDH1 activity has been detected in some normal stem cell populations, particularly hematopoietic progenitor cells (Kastan *et al*., 1990), and subsequently used to isolate CSC candidates in different cancers, including SCCs.

ALDH1 is a CSCs marker and that its presence strongly correlates with tumor malignancy as well as self-renewal properties of stem cells in different tumors, including breast cancer, hepatoma, colon cancer, and lung cancer (Ginestier *et al*., 2007).

ALDH1, a detoxifying enzyme, is a stem-like cell marker. ALDH1 was expressed in some of the astrocytoma but was not detected in normal brain tissues. The proportion of ALDH1-expressing cells was positively correlated with the pathological grade of the astrocytoma, but not with patient age, sex or tumor

size. ALDH1 is expressed in astrocytoma, and that its expression is correlated with pathological grade and patient survival (Liu *et al*., 2012).

Normal stem cells and cancer stem cells (CSCs) share similar properties, in that both have the capacity to self-renew and differentiate into multiple cell types. In both the normal stem cell and cancer stem cell fields, there has been a great need for a universal marker that can effectively identify and isolate these rare populations of cells in order to characterize them and use this information for research and therapeutic purposes. Currently, it would appear that certain isoenzymes of the aldehyde dehydrogenase (ALDH) superfamily may be able to fulfill this role as a marker for both normal and cancer stem cells. ALDH has been identified as an important enzyme in the protection of normal hematopoietic stem cells, and is now also widely used as a marker to identify and isolate various types of normal stem cells and CSCs. In addition, emerging evidence suggests that ALDH1 is not only a marker for stem cells, but may also play important functional roles related to self-protection, differentiation, and expansion (Ma and Allan, 2011).

Role of CD44 and ALDH1 as CSC markers in oral lesions

Multifactorial conditions underlie progression of potentially malignant oral lesions (PMOL) to oral squamous cell carcinoma (OSCC) and there is currently need for better prediction of malignant transformation. The hypothesized existence of cancer stem cells in dysplastic oral tissues provides the potential for more informed assessment of PMOL progression. Stain intensity scores for ALDH1 and CD44 were greater for OSMF and OSCC than normal control tissues.

The intensity of ALDH1 immunostaining correlated with increased oral epithelial disease severity (Abdul *et al*., 2013).

ALDH1A1 and CD44 are common CSC markers in HNSCC (Chen *et al.*, 2009 and Visus *et al.*, 2007). Corresponding to the hierarchical tumor model, CSCs exist in every tumor (Brabletz *et al.*, 2005 and 2012).

In the present study, the ALDH1 and CD44 proteins were expressed in the majority of tumors and majority of OSMF. Consequently, ALDH1 and CD44 could also be expressed in the CSCs of these tumors. As a CSC marker, CD44 was more often expressed in the tumors than ALDH1. However, a lot of OSCC expressed CD44 in almost all tumor cells and all surface epithelium of OSMFs showed CD44 positivity. To identify CSCs, the markers must isolate CSCs from the tumor bulk. ALDH1 was a better marker to define a subpopulation of tumor cells. Finally, the two markers were not sufficient to isolate the CSCs from the bulk of tumor cells and bulk of premalignant lesions. Further CSC markers should be used to define and isolate the CSC population.

In addition, ALDH1 and CD44 expression did not completely overlap. In the majority of OSMF and OSCC, ALDH1+/CD44+, ALDH1+/CD44- and ALDH1-/CD44+ populations were observed. This observation may indicate that different CSC populations could exist within one tumor. For HNSCC, this theory of different CSC phenotypes was first suggested by Biddle *et al.,* 2011.

CD44 has been reported to be involved with tumor growth and metastasis and has also been implicated as a CSC marker in head and neck squamous cell cancer (HNSCC). Pan-CD44 and CD44-v6 expression were both correlated with 5-year OS rate of patients with laryngeal and pharyngolaryngeal cancer. CD44 is related to worse T category, N category, tumor grade and prognosis, in pharyngeal and laryngeal cancer, but no clear association was revealed between CD44 expression and oral cancer (Chen *et al.*, 2014).

In the present study, CD44 expression was more frequent in premalignant lesion, OSMF, than in OSCC. It could be suggested that loss of

CD44 expression in OSMF can be considered an early event in carcinogenesis and a marker of major alterations of CD44 expression in the derived tumor tissue.

Relationship of pathological variance and expression of markers

In OSMF, both markers tend to be seen higher positivity rate in accordance with scanty lymphocyte amount in ECJ and muscle atrophy. In OSCC, they were also associated with scanty lymphoplasmacytic infiltration around the tumor cells and Bryne's tumor classification grade III.

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. Qian *et al*., (2013) studied associations between HR-HPV/p16, CSC frequency and clinic-pathological parameters in patients with metastatic OSCC were investigated. 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).

In the present study, CD44+ and ALDH1+ cells were detectable in Bryne's grade III tumor classification. This finding was compatible and consistent with Qian *et al*., (2013) study.

Adhesive interactions between receptors on vascular endothelial cells (EC) and circulating leukocytes are pivotal in regulating leukocyte extravasation.

Although primary adhesion of lymphocytes to EC has been primarily attributed to the selectin family of receptors, CD44 can also mediate this function when activated to bind its ligand hyaluronan (HA). Triggering through the T cell receptor induces activated CD44 and CD44-dependent primary adhesion in both human and mouse lymphocytes, and the interaction can mediate the extravasation of activated T cells into an inflamed site. Lymphocytes capable of CD44/HA-dependent primary adhesion are found in peripheral blood of some rheumatologic patients, and their presence is associated with concurrent symptomatic or active disease. Thus, circulating T cells bearing activated CD44 may represent a pathogenically important subpopulation of activated cells that is elevated under conditions of chronic inflammation. Together, these data add to the selectin and immunoglobulin gene families a new receptor/ ligand pair and their potential physiological role; i.e., antigen-specific T cell activation together with local vascular inflammation permits the CD44/HA interaction and subsequent T cell extravasation (Siegelman *et al*., 1999).

According to this finding, correlation between CD44 and lymphocytic infiltration was important role in inflammatory process, tumor initiation and tumor metastasis.

In the present study, the ALDH1 and CD44 proteins were expressed in the majority of OSCCs and majority of OSMFs. Consequently, ALDH1 and CD44 could also be expressed in the CSCs of these tumors. As a CSC marker, CD44 was more often expressed in the tumors than ALDH1. However, a lot of OSCCs expressed CD44 in almost all tumor cells and all surface epithelium of OSMFs expressed more CD44. To identify CSCs, the markers must isolate CSCs from the tumor bulk. ALDH1 was a better marker to define a subpopulation of tumor cells. Finally, the two markers were not sufficient to isolate the CSCs from

the bulk of tumor cells and bulk of premalignant lesions. Further CSC markers should be used to define and isolate the CSC population.

In addition, ALDH1A1 und CD44 expression did not completely overlap. In the majority of tumors ALDH1+/CD44+, ALDH1+/CD44-and ALDH1-/CD44+ populations were observed. This observation may indicate that different CSC populations could exist within one tumor. This finding favored and followed the theory of different CSC phenotypes in HNSCC.

In conclusion, CD44 and ALDH1 appear to be important factors in carcinogenesis and tumor progression in OSMF and OSCC. It could be suggested that loss of CD44 expression in OSMF can be considered an early event in carcinogenesis and a marker of major alterations of CD44 expression in premalignant lesions. Positivity of both markers was directly associated with Bryne's tumor grade and inversely related with tumor-host immune reaction. ALDH1 and CD44 may be expressed in the CSCs of most examined tumors and OSMF. However, these markers are not sufficient to precisely isolate the CSC subpopulation from the tumor bulk.