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EVALUATION OF DIAGNOSTIC AND PROGNOSTIC ROLE OF CANCER STEM CELLS AND ALDEHYDE DEHYDROGENASE ACTIVITY IN DIFFERENT SALIVARY GLAND TUMORS

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Ph.D Thesis

DEPARTMENT of ORAL PATHOLOGY

GAZI UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

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Dr. ALAA M. SHUİBAT

FARKLI TÜKRÜK BEZİ TÜMÖRLERİNDE KANSER KÖK HÜCRESİ VARLIĞININ VE ALDEHİT DEHİDROGENEZ AKTİVİTESİNİN DİAGNOSTİK VE PROGNOSTİK ROLÜNÜN ARAŞTIRILMASI

(Doktora Tezi)

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GAZİ ÜNİVERSİTESİ
SAĞLIK BİLİMLERİ ENSTİTÜSÜ

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TURKISH ABSTRACT

Tükrük bezi tümörleri farklı organizasyonlar gösteren çeşitli hücrelerden köken almaları nedeniyle değişik histopatolojik ve biyolojik davranışlar gösterirler. Bu özellikleri tükrük bezi tümörlerinin tanı, tedavi ve prognozlarının belirlenmesinde güçlük yaratmaktadır.
Kanser kök hücresi olarak adlandırılan özel bir hücre grubu tümör inişiasyonu, ilaca karşı direnç ve tümör agresifliği ile ilişkilendirilmektedir.Son bulgular, ALDH1, CD44, CD24 ve CD166'nin kanser kök hücreleri için belirleyici olduğunu desteklemiştir. Buradan yola çıkarak bu çalışmada, ALDH1, CD166, CD44 ve CD24'un farklı benign, malign tükürük bezi tümörleri ve normal tükürük bezi dokusundaki dağılımını saptamak ve tümörünlerin klinikopatolojik özellikleri ile korelasyonunu araştırmak amaçlanmıştır. Çalışmaya Gazi Üniversitesi Diş Hekimliği Fakültesi arşivinden elde edilen 24 malign tükürük bezi tümörü(6 mukoepidermoid karsinom, 6 polimorfik düşük dereceli adeno karsinom, 8 adenoid kistik karsinom, 2 karsinom ex-pleomorfik adenom, 2 asinik hücre karsinom), 24 benign tükürük bezi tümörü (21 pleomorfik adenom,3 bazal hücreli adenom) ve 7 normal tükürük bezi dokusu dâhil edilmiştir. Hastaların demografik bilgileri ve yedi yıllık takip süreçleri kaydedilmiştir. Toplam 55parafin blok, immunhistokimyasal olarak ALDH1, CD44, CD24 ve CD166 belirteçleri ile boyanmıştır. Malign tümörlerde izlenen düşük ALDH1 ekspresyon istatistiksel olarak anlamlılık göstermiştir (P0.034).Yüksek dereceli tümörlerde düşük derecelilere göre azalmış ALDH1 ekspresyonu saptanmıştır. Adenoid kistik karsinom (P 0.000) ve bazal hücreli adenomda (P 0.026) görülen ALDH1 ekspresyon yokluğu istatistiksel olarak anlamlı bulunmuştur. Malign tükrük bezi tümörlerinde istatiksel olarak anlamlı yüksek CD166 ekspresyonu saptanmıştır (P 0.002). Metastaz/nüks yapmış ve yüksek dereceli tümörlerde CD166 ekspresyonunda belirgin azalma görülmüştür. CD 44 ekspresyonunun benign ve malign tümörlerde normal tükrük bezi dokusuna göre azaldığı gözlenirken metastaz /nüks yapan tümörlerde metastaz/nüks yapmayan tümörlere göre daha yüksek oranda eksprese olduğu dikkati çekmiştir. Benign tükrük bezi tümörlerinin malign tümörlere oranla daha yüksek CD24 eksprese ettiği gözlenmiştir. Benzer şekilde metastaz/nüks yapan tümörlerde daha yüksek CD24 ekspresyonu saptanmıştır. Yaşla beraber ALDH1 ekspresyonundaki azalma istatistiksel olarak anlamlı bulunmuştur (P 0.007). Sonuç olarak, malign tükrük bezi tümörlerinde benign tömörler ve normal dokulara oranla CD166'nın yüksek ekspresyonu, ALDH1'in ise düşük ekspresyonu izlenmiştir. Bulgular bu iki molekülün tükrük bezi malignansilerinde kök hücreleri tanımlamada belirteç olabileceğini düşündürmektedir. Çalışmanın bulguları bu moleküllerdeki ekspresyon farklılıklarının malign tükürük bezi tümörlerinin prognozu ile ilişkili olabileceği yolundaki literatür bilgilerini desteklemektedir. Adenoid kistik karsinomlarda (Ad CC) izlenen ALDH1 yokluğu bu molekülün Ad CC ayırıcı tanısında potansiyel rolünü tartışmaya açmıştır.

Bölüm Kodu **:** 101.1069

Anahtar Kelimeler **:** tükrük bezi tümör, kanser kök hücre, prognoz, diagnoz.

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Danışman **:** Doç. Dr. Benay Yıldırım

EVALUATION OF DIAGNOSTIC AND PROGNOSTIC ROLE OF CANCER STEM CELLS AND ALDEHYDE DEHYDROGENASE ACTIVITY IN DIFFERENT SALIVARY GLAND TUMORS

(Ph.D Thesis)
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ABSTRACT

Salivary gland tumors exhibit a diverse range of histological appearance because of the various shapes and arrangements of the neoplastic cells which makes the diagnostic and prognostic predictions a real challenge. A subset of cells, tentatively called cancer stem cells (CSCs) have been associated with tumor initiation, drug resistance, and tumor aggressiveness. Recent evidence suggests that enhanced activity of aldehyde dehydrogenase 1 (ALDH 1), CD44, CD24, CD166, as hallmark of cancer stem cells. For that reason this study aims to determine the distribution of the cancer stem cell markers ALDH1, CD166, CD44 and CD24 among different benign, malignant salivary gland tumors as well as normal salivary gland tissues. 24 malignant salivary gland tumors (6 mucoepidermoid carcinoma, 6 polymorphous low grade adenocarcinoma, 8 adenoid cystic carcinoma, 2 carcinoma ex-pleomorphic adenoma, 2 acinic cell carcinoma) 24 benign salivary gland tumors (21 pleomorphic adenoma, 3 basal cell adenoma) and **7** normal salivary gland tissues from the archive of Gazi University Faculty of Dentistry were enrolled in the study. Demographic features and 7 years follow up data of patients were recorded. A total of 55 blocks have been immunohistochemically stained for CSCs markers ALDH1, CD44, CD24, and CD166. ALDH1 expression was down regulated in malignant tumors (P 0.034). Decreased ALDH expression was noted in high grade tumors. The lack of ALDH1 expression in adenoid cystic carcinomas (P 0.000) and basal call adenomas in relation to other tumors (P 0.026) were statistically significant. Malignant SG tumors displayed statistically significant up regulated CD166 expression (P 0.002). Loss of CD166 was determined both in metastasising/recurrent and high grade tumors in comparison to non-metastisizin/non-recurrent and low grade tumors. Diminishing CD44 expression was noted in benign and malignant tumors in descending order while metastisizing/recurrent tumor had higher CD44 expression in comparision to non-metastasizing / non-recurrent tumors. Benign SGT showed higher CD24 expression in comparison to malignant tumors. There was a higher CD24 expression in metastasising /recurrent tumors. Down regulation of ALDH expression by age also showed statistical significance (P 0.007). In conclusion there was a statistically significant up regulation of CD166 and down regulation of ALDH1 expression in malignant salivary gland tumors. This data suggested that these molecules could be useful markers for cancer stem cells in salivary gland tumors. Our results also supported the literature information that variations in expressions of these markers might be correlated with the prognosis of salivary gland tumors. The lack of ALDH1 expression in adenoid cystic carcinoma (Ad CC) suggested the potential role of this molecule as a diagnostic marker in differential diagnosis of Ad CC.

Code of department **:** 101.1069

Keywords **:** Salivary gland tumors, cancer stem cells, prognosis, and diagnosis.

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Dr. ALAA SHUİBAT

DDS. Ph.D

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ABBREVIATIONS

The explanation of abbreviations that were used in this thesis in alphabetical arrangement.

Abbreviation	Meaning
Ac CC	Acinic cell carcinoma
Ad CC	Adenoid cystic carcinoma
ALCAM	Activated Leukocyte Cell Adhesion Molecule
ALDH	Aldehyde Dehydrogenases
BCA	Basal cell adenoma
CSC	Cancer stem cell
EX-MIX	Carcinoma ex pleomorphic adenoma
HA	Hyaluronic acid
MEC	Mucoepidermoid carcinoma
PA	Pleomorphic adenoma
PLGA	Polymorphous low grade adenocarcinoma
SG	Salivary gland
SGT	Salivary gland tumor
SGMN	Salivary Gland Malignant Neoplasm
WHO	World Health Organization

1. INTRODUCTION

Salivary gland tumors (SGTs) are relatively uncommon lesions affecting both major and minor salivary glands. These tumors vary widely in histopathological appearance, which prompted the development of a revised histopathological classification of tumors [1-4].

Due to limited mechanistic understanding of the disease and lack of effective regimens for chemotherapy, surgery is still the main treatment option of these patients. As a consequence, treatment for these tumor is generally accompanied by significant morbidity and debilitating facial disfigurement[5,6].

Recent evidence suggests the existence of a tumorigenic population of cancer cells that demonstrate stem cell-like properties such as self-renewal and multipotency. These cells, termed cancer stem cells (CSC), are able both to initiate and maintain tumor formation and progression [7].

The concept of CSC has been demonstrated in several human cancers including leukemia, brain tumor, breast cancer, prostate cancer, lung cancer, pancreas cancer and colon cancer [8].

Considering the role of CSC in resisting the therapy in other organs, it is possible that this unique sub-population of cells also may be involved in treatment's resistance and aggressiveness of salivary gland tumors. Understanding of CSC in SGT can lead to better understanding of the pathobiology of salivary gland malignancies as well as to development of more effective therapies. Here, we aimed to examine cancer stem cell expression and its correlation with the clincopathological features of SGTs.

In the literature a lot of markers have been used to detect CSC in different organs. ALDH, CD44, CD24, and CD166 have been used extensively for detection of CSC in adenoid tissues like SG and breast [7-9].

The aim of this study was to investigate the distribution of those CSCs in SG tumors and in normal SG tissues, and to analyze their correlation with the clincopathological features of salivary gland tumors.

2. GENERAL INFORMATION

2.1. Salivary Glands

2.1.1. Anatomy

Salivary glands are exocrine organs responsible for the production and secretion of saliva. In humans, there are three paired major salivary glands, located extra orally, and several hundred smaller minor salivary glands, located in the lips, cheeks, tongue, palate, fauces, and retromolar areas. The parotid gland is located subcutaneously, lying over the masseter muscle, just in front of the ear, with a deeper portion extending behind the ramus of the mandible. Its duct, Stensen's duct, runs anteriorly, crossing the masseter muscle and entering the oral cavity at the parotid papilla on the buccal mucosa, opposite the maxillary second molar [1-3].

The submandibular gland is located in the submandibular triangle, below the mylohyoid muscle, with its posterior portion wrapped around the posterior border of the mylohyoid muscle and extending anteriorly for a short distance. Its duct, Wharton's duct, travels anteriorly below the mucosa of the floor of the mouth, opening at the sublingual caruncle [2-4].

The sublingual gland, the smallest of the major glands, is located in the floor of the mouth, medial to the mandible and just above the mylohyoid muscle. Its main duct, Bartholin's duct, opens with the duct of the submandibular gland at the sublingual caruncle. Several smaller ducts of the sublingual gland, the ducts of Rivinus, open separately along the sublingual fold in the floor of the mouth (Figure 2.1) [5, 6].

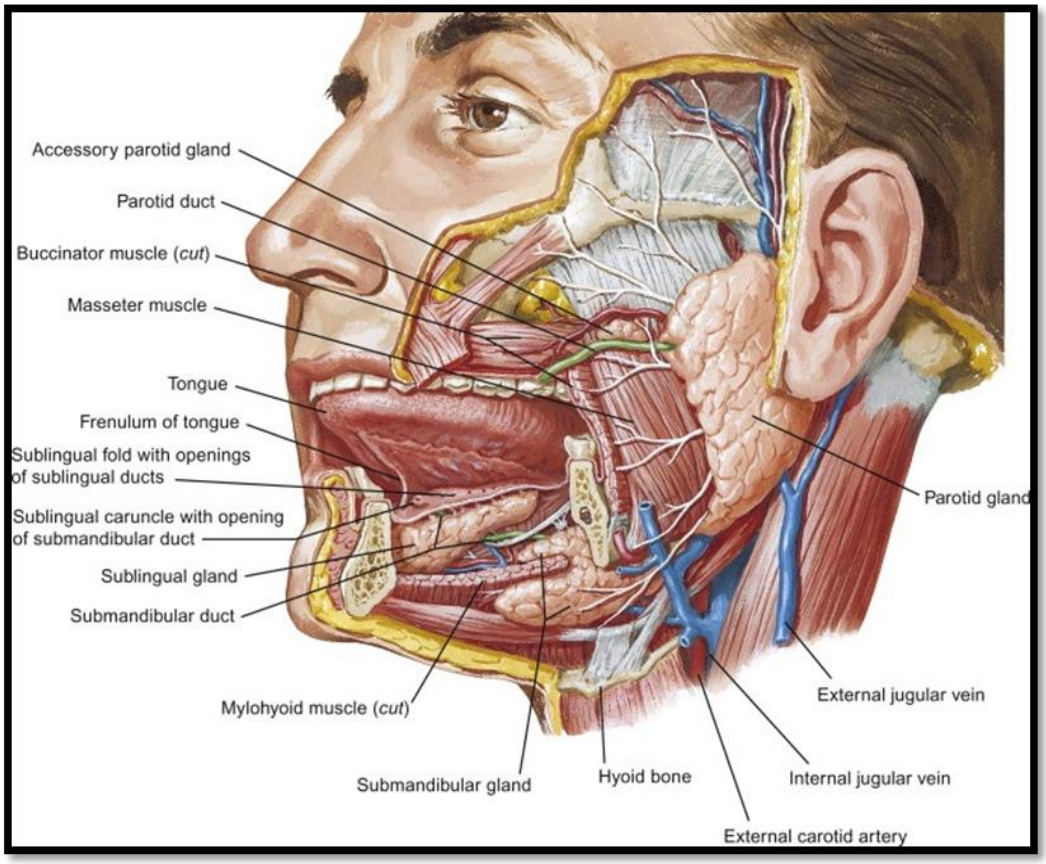


Figure 2.1 Human regional cervical anatomy demonstrates the relative positions of major salivary glands and their ducts (McMinn's Color Atlas of Head and Neck Anatomy, 4th Edition).

2.1.2. Histology

Salivary glands are made up of secretory acini and ducts. There are two types of secretions serous and mucous. The acini can either be serous, mucous, or a mixture of serous and mucous cells. The parotid gland contains only serous secretory end-pieces. The intercalated ducts typically are long and the striated ducts are prominent. The submandibular gland is a mixed gland, with both serous and mucous secretory end-pieces; however, the serous end-pieces predominate [5, 6]. The mucous end-pieces are capped by serous demilune cells. The intercalated ducts also are relatively long and the striated ducts are prominent. The sublingual gland also is a mixed gland, consisting predominantly of mucous end-pieces and serous demilunes; few, if any, serous end-pieces are present. The intercalated ducts are short and relatively few striated ducts are present. The three major salivary glands have a similar anatomic structure: glandular acini/alveoli that connect to intercalated ducts, drain to

intralobular (striated) and then interlobular ducts, and finally merge into excretory ducts that empty into the oral cavity [2, 6].

The minor salivary glands consist of small aggregates of secretory end-pieces and ducts, located in the submucosal layer of the oral mucosa or between muscle fibers of the tongue. The ducts typically open directly into the oral mucosal surface [10]. Most of the minor glands are mucous and some include a serous cell component arranged as occasional demilunes. The only exception is the lingual serous (von Ebner's) gland, located in the posterior part of the tongue. Von Ebner's gland is a pure serous gland and its ducts open into the troughs surrounding the circumvallate papillae and at the rudimentary papillae on the sides of the tongue [10-12].

2.2. Salivary Gland Tumors

Salivary gland tumors represent a diverse group of neoplasm that make up about 3% of all neoplasm of the head and neck [13]. The vast majority of salivary neoplasms are epithelial in origin; rarely the interstitial connective tissue components of the salivary glands give rise to primary neoplasms whose behavior is similar to that of their extra glandular counterparts[13,14].The parotid, submandibular glands and the minor salivary glands of the palate are commonly involved while the sublingual gland is rarely affected. Salivary gland tumors vary widely in histopathological appearance, which prompted the development of a revised classification of tumors [13-15]. Pleomorphic adenoma (PA) is the most common tumor, representing about 60% of cases, followed by mucoepidermoid carcinoma (MEC), which makes about 25% of the epithelial tumors [13-15].

Approximately 64-80% of all primary epithelial salivary gland tumors occur inthe parotid gland mostly in the superficial lobe; minor glands (9-23%) submandibular glands (7-11%) and sublingual glands (1%) follow it in descending order. Benign tumors represent 54-79%, and 21-46% are malignant. The proportion of malignant tumors, however, varies greatly by site. In parotid 15-32% of tumors are malignant, 41-45% in submandibular, 70-90% in sublingual and 50% in minor gland.80%- 90% of tumors that occur in the tongue, floor of mouth, and retromolar areas are malignant [14-16].

The etiological factors for salivary tumors are not clearly defined but viruses, occupation, hormones, nutrition, radiation; life style may play a role in the carcinogenesis of these tumors

[17-19].By contrast with other head and neck cancers, consumption of tobacco or alcohol does not cause an increase in the incidence of malignant salivary gland tumors. Furthermore, chronic inflammation of salivary glands is not clearly defined as a risk factor. Nutrition may be a risk factor; low intake of vitamins A and C correlates with a high incidence of tumors. Irradiation may also be a cause of malignant salivary gland tumors [19]. Therapeutic radiation, particularly of the head and neck region, has been linked with a significantly increased risk of developing salivary gland cancers. Survivors of the atomic bomb explosions in Hiroshima and Nagasaki show an increased relative risk of 3.5 fold for benign, and 11 fold for malignant salivary neoplasms. The risk was directly related to the level of exposure to ionizing radiation. There was a high frequency of both mucoepidermoid carcinomas and Warthin tumors in these patients [20]. Exposure to ultraviolet radiation has also been implicated. There appears to be no excess risk in those exposed to radon, or the microwaves of cellular telephones [12, 20].

2.3. World Health Organization Classification

The 2005 World Health Organization (WHO) classification of salivary gland tumors (SGTs) is complex and comprises 10 benign and 23 malignant entities of epithelial origin. The diversity of epithelial SGTs as well as their rarity and varied morphological aspects often makes diagnosing such neoplasms difficult [15]. Most primary epithelial SGTs occur in the parotid glands; about 10% occur in the submandibular glands, and less than 1% develops in the sublingual glands. Minor glands are involved in 9-23% of SGT cases. Between 54 and 79% of all tumors are benign, and 21 to 46% are malignant. Most SGTs occurring in the sublingual glands are malignant (70-90%). Fifteen to 32% of parotid tumors and about 40% of submandibular lesions are carcinomas [15, 21]. Finally, 50% of minor gland neoplasms are cancers. Notably, SGTs of the tongue, floor of the mouth and retromolar areas are most often malignant. Overall, pleomorphic adenoma is the most frequent SGT, comprising about 50-60% of cases. The second most frequent benign SGT is Warthin tumor. Mucoepidermoid carcinoma is the most common malignant SGT. Histological types vary in frequency according to location. Pleomorphic adenoma, Warthin tumor, and mucoepidermoid carcinoma are commonly found in the parotid glands whereas polymorphous low-grade adenocarcinoma usually arises in minor glands [15, 21-23]. Non epithelial neoplasms are rare, representing about 2-5% of SGTs, they include haemangioma, lymphangioma, schwannoma, neurofibroma, lipoma, sarcoma, lymphoma, and metastatic lesions [15, 23].

Table 2.1 2005 WHO classification of epithelial SGTs

Malignant epithelial tumors:	**Benign epithelial tumors:**
Acinic cell carcinoma	Pleomorphic adenoma
Mucoepidermoid carcinoma	Myoepithelioma
Polymorphous low-grade adenocarcinoma	Basal cell adenoma
Adenoid cystic carcinoma	Warthin tumor
Oncocytic carcinoma	Oncocytoma
Mucinous adenocarcinoma	Canalicular adenoma
Low-grade cribriform cystadenocarcinoma	Sebaceous adenoma
Cystadenocarcinoma	Lymphadenoma
Malignant sebaceous tumors	Ductal papilloma
Basal cell adenocarcinoma	Cystadenoma
Epithelial-myoepithelial carcinoma	
Sialoblastoma	
Clear cell carcinoma, not otherwise specified	
Salivary duct carcinoma	
Adenocarcinoma, not otherwise specified	
Myoepithelial carcinoma	
Carcinoma ex pleomorphic adenoma	
Carcinosarcoma	
Metastasizing pleomorphic adenoma	
Squamous cell carcinoma	
Small cell carcinoma	
Large cell carcinoma	
Lymphoepithelial carcinoma	

As seen in the WHO classification; SGTs comprised of 10 benign and 23 malignant entities. Here in this thesis only the tumors that are included in the study will be described.

2.3.1. Pleomorphic adenoma (Mixed tumor)

The mixed tumor is the most common tumor of the major and minor salivary glands. The parotid gland accounts for approximately 85% of these tumors, whereas the submandibular gland and the intraoral minor salivary glands account for 8% and 7%, respectively. Mixed tumors occur at any age, favor males slightly more than females, and are most prevalent in the fourth through sixth decades of life. They constitute approximately 50% of all intraoral minor salivary gland tumors. Generally, they are mobile except when they occur in the hard palate. They appear as firm, painless swellings and, in the vast majority of cases do not cause ulceration of the overlying mucosa. The palate is the most common intraoral site, followed by the upper lip and buccal mucosa [14, 23].

The histogenesis of pleomorphic adenoma (PA) or mixed tumor, relates to a dual proliferation of cells with ductal or myoepithelial features in a stroma of mucoid, myxoid, and, less commonly, chondroid quality. This separates it from monomorphic adenomas composed of only one cell type and a more homogeneous or less varied stroma. The myoepithelial-differentiated cell assumes an important role in determining the overall composition and appearance of mixed tumors. A range of cell types and microscopic patterns are seen in mixed tumors those composed almost completely of epithelial cells at one end of a spectrum and those composed almost completely of myoepithelial cells at the other end. Between these two extremes, less well developed cells with features of both myoepithelial and ductal elements may be seen. Alternatively, it has been theorized that rather than simultaneous proliferation of neoplastic epithelial and myoepithelial cells, a single cell with the potential to differentiate toward either epithelial or myoepithelial cells may be responsible for these tumors [14, 24].

Microscopically, mixed tumors demonstrate a wide spectrum of histologic features. The pleomorphic patterns and the variable ratios of ductal to myoepithelial cells are responsible for the synonym pleomorphic adenoma figure (2.2). Approximately one third of mixed tumors show an almost equal ratio of epithelial and mesenchymal elements (believed to be derived from myoepithelial-differentiated cells). The epithelial component may appear as ducts, tubules, ribbons, and solid sheets and the mesenchymal component may appear as myxoid, hyalinized connective tissue. Infrequently, fat, cartilage, and/or bone may be seen [24, 25].

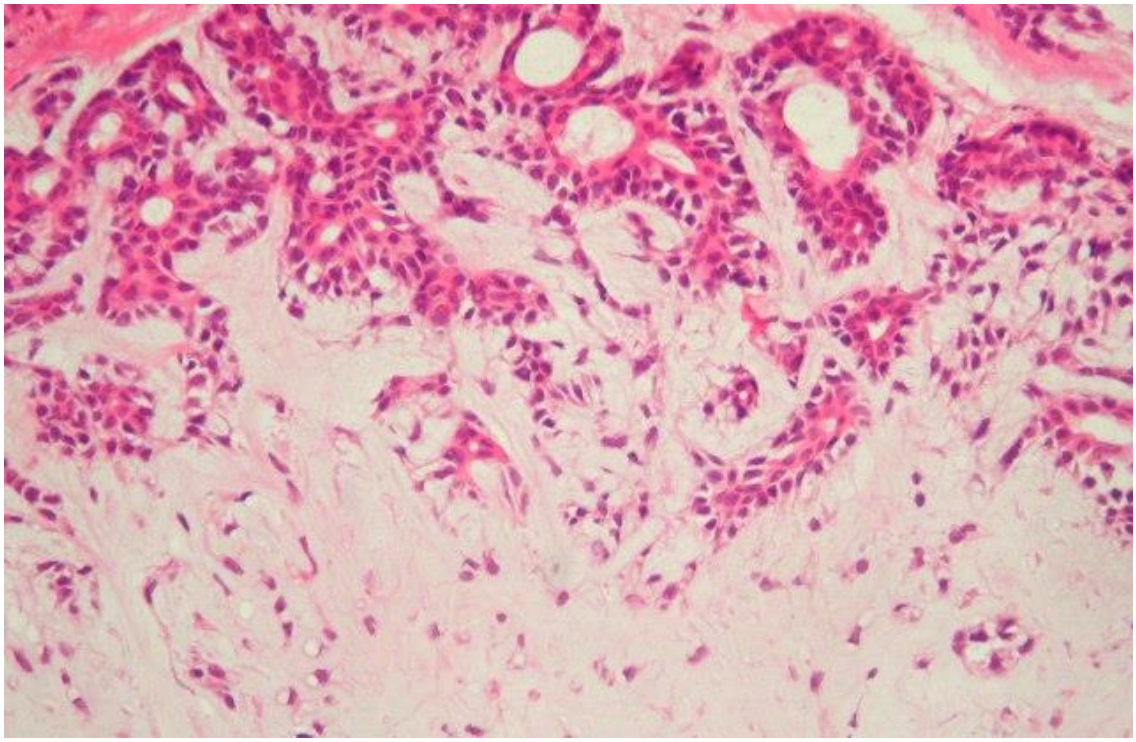


Figure 2.2 Histology of pleomorphic adenoma: duct like and tubular epithelial tumor component in myxoid connective tissue (H&E X200).

Myoepithelial cells may appear as plasmacytoid cells or spindled cells with an immunoprofile showing co-expression of cytokeratin markers, vimentin, variable positivity for S-100 protein, calponin, alpha-smooth muscle actin, and muscle-specific actin [14, 26,27].

2.3.2. Basal cell adenomas

Basal cell adenomas constitute approximately 1% to 2% of all salivary gland adenomas. About 70% are found within the parotid followed by the submandibular gland. In minor salivary glands, most occur in the upper lip, followed in frequency by adenomas in the palate, buccal mucosa, and lower lip[14]. Basal cell adenomas are generally slow growing, solitary, and painless. The lesions tend to be clinically distinct and firm on palpation, but they can be multifocal and multinodular. The age range of patients is between 35 and 80 years, with a mean age of approximately 60 years. A distinct male predilection is noted. The membranous adenoma (dermal analog tumor) variant occurs in the parotid gland in more than 90% of cases, with no cases reported in the intraoral minor glands. These lesions vary from 1 to 5 cm in greatest dimension and generally present as an asymptomatic swelling. Several patients with this particular finding in the parotid gland have presented with synchronous or

metachronous adnexal cutaneous tumors, including dermal cylindroma, trichoepithelioma, and eccrinespiradenoma[14, 28].

In the solid variety of basal cell adenoma, islands or sheets of isomorphic basaloid cells often show peripheral palisading, with individual cells at the periphery appearing cuboidal to low columnar in profile (figure 2.3).The trabecular-tubular form of basal cell adenoma exhibits trabecular cords of epithelial cells or tubular epithelial elements. Membranous adenoma grows in a nodular fashion with variable-sized islands of tumor tissue surrounded by a thick periodic acid-Schiff positive hyaline membrane. Eosinophilic hyaline material is also noted in droplet form within the intercellular areas of the tumor islands. Membranous adenomas may also contain foci of normal salivary gland, giving the erroneous impression of invasiveness and necessitating separation from adenoid cystic carcinoma [14, 28, 29].

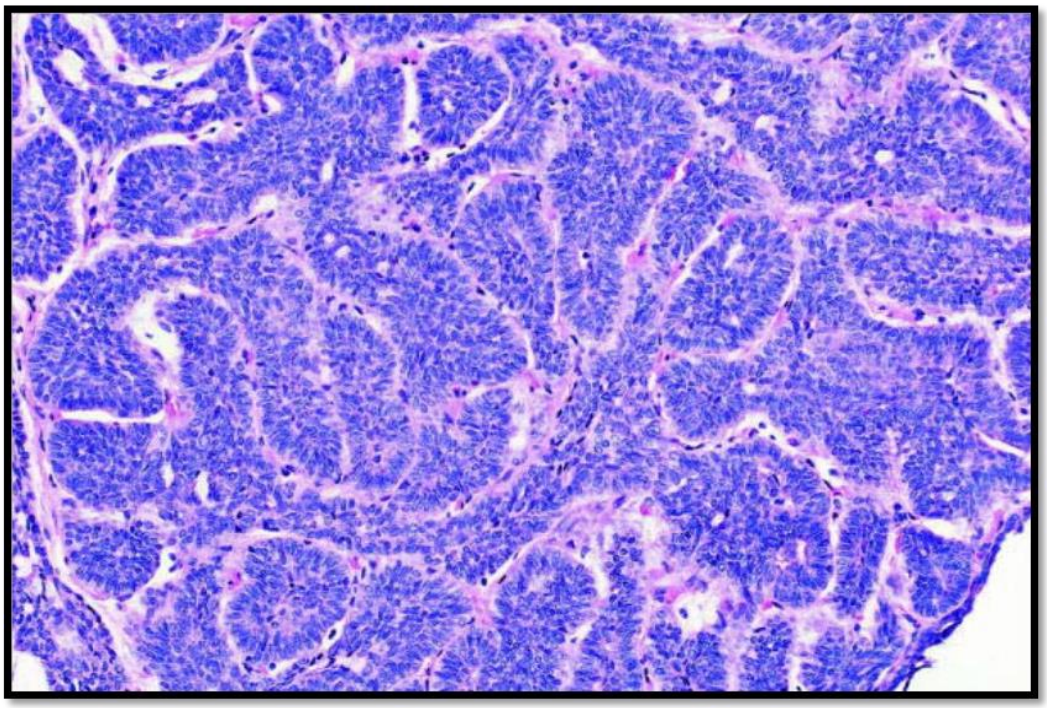


Figure 2.3 Histology of basal cell adenoma, solid pattern (H&E X200).

2.3.3. Carcinoma ex pleomorphic adenoma

Carcinoma ex pleomorphic adenomatumor represents an epithelial malignancy arising in a preexisting mixed tumor in which such remnants may be identified. When metastatic disease occurs, only the malignant component metastasizes. Carcinoma ex pleomorphic adenoma is an exceedingly rare neoplasm of the minor salivary gland. Prognostic parameters are recurrence, capsular invasion, and metastasis [30]. Patients present with rapid growth and/or

ulceration of a known, untreated PA. The mass is usually painless but about one third of patients have pain or facial nerve paralysis. The lesion may be fixed to underlying soft tissues [30,31]. Similarly to PA, carcinoma ex PA mainly occurs in the parotid gland; it usually develops a decade later compared to PA. It may result from accumulation of genetic alterations in long-standing tumors. Indeed, the risk of malignant transformation increases with time. The malignant component may totally replace the benign portion of the tumor figure (2.4). It may correspond to poorly differentiated adenocarcinoma, undifferentiated carcinoma or any other type of epithelial malignancy [23, 32].

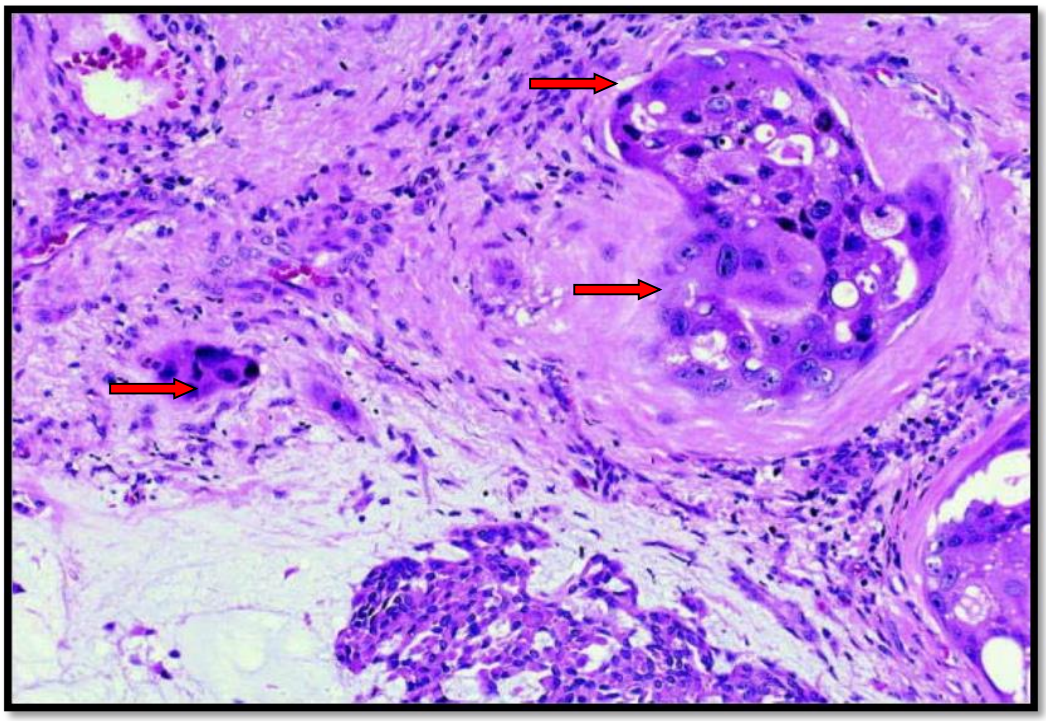


Figure 2.4 Histology of carcinoma ex-pleomorphic adenoma. The arrows point malignant component of tumor (H&E X400).

2.3.4. Mucoepidermoid carcinoma

Mucoepidermoid carcinoma (MEC) is an epithelial malignancy characterized by mucous, intermediate and (non-keratinizing/ squamous-like) epidermoid cells, with additional clear and oncocytic populations. The proportions of the different cell types and their architectural configuration including cyst formation vary between tumors and within any individual neoplasm. It is the most common primary salivary gland malignancy worldwide which can occur at any age (range 3-95 years, mean 46 years) with a slight female predominance [33]. About 53% of cases have been reported from the major glands, but MEC is also frequent in the palate and other minor glands [14].

Microscopic examination shows variable proportions of the three main cell types, but in most tumors intermediate cells predominate. While mucous and squamous-like cells are relatively easy to characterize, intermediate cells range from small basal type cells to larger round or polygonal cells, often with clear cytoplasm. All cell types can show degrees of nuclear pleomorphism and mitotic activity.

The stroma is variable, but can be fibrous and hyalinized; a lymphoid reaction is often prominent with germinal centre formation. Histological variants include clear cell predominant [33], oncocytic [34], sebaceous [35], as well as sclerosing MEC [36,37] Figure (2.5).

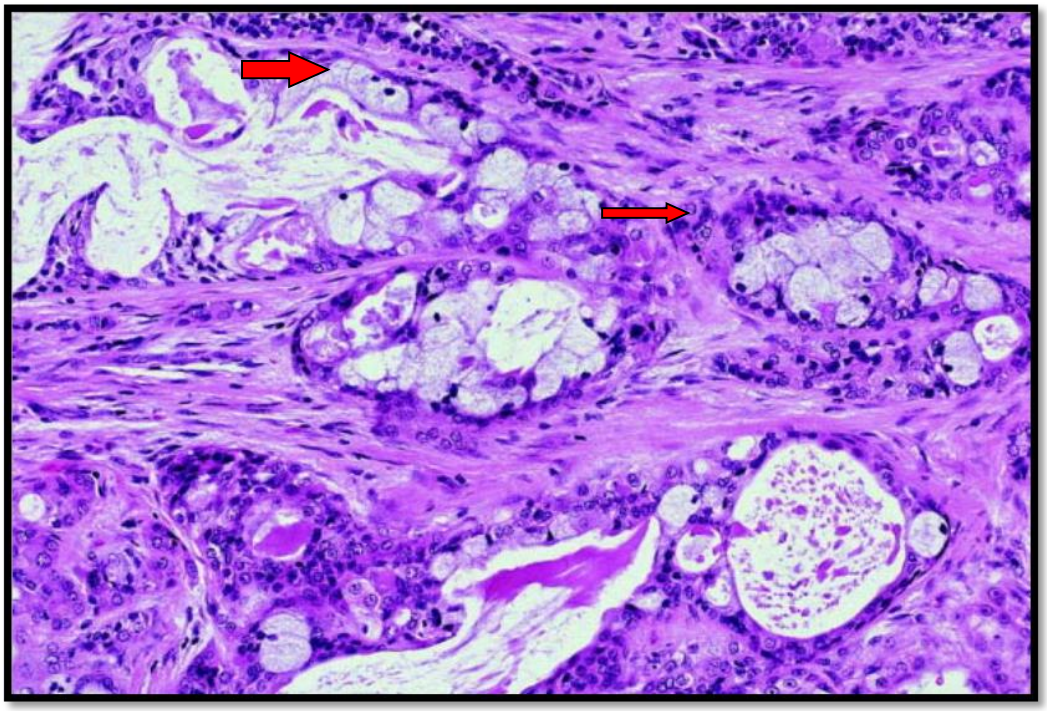


Figure 2.5 Histology of mucoepidermoid carcinoma. Intermediate (thin arrow) and mucous cells (thick arrow) (H&E X400).

2.3.5. Polymorphous low-grade adenocarcinoma

Polymorphous low-grade adenocarcinoma (PLGA) was first reported in 1983 by two different groups under the terms lobular carcinoma of salivary glands and terminal duct carcinoma[14]. Today the term polymorphous low-grade adenocarcinoma is the accepted term for this entity. It has been segregated from other salivary tumors because of its distinctive clinical, histomorphologic, and behavioral aspects. This tumor is generally considered to be a low-grade malignancy with a relatively indolent course and low risk of

recurrence and metastasis. The putative origin of the polymorphous low-grade adenocarcinoma is believed to be from reserve cells in the most proximal portion of the salivary duct. Myoepithelial differentiated cells appear in this neoplasm, but only in slight to moderate numbers [14, 38].

This neoplasm occurs in the fifth through eighth decades of life with no gender predilection. It accounts for 26% of all salivary carcinomas, with over 70% occurring in patients between the ages of 50 and 70 with a mean age of 59 years and appears almost exclusively in minor salivary glands, the palate being the most frequently reported site. Polymorphous low-grade adenocarcinomas typically present as firm, elevated, no ulcerated nodular swellings that are usually non tender. A wide range in size has been noted, but most are between 1 and 4cm in diameter. The slow growth rate is evidenced by the long duration—many months to years—[39].Neurologic symptoms are usually not reported in association with this tumor. Metastasis to local nodes is present at the time of diagnosis in approximately 10% of patients. Rare instances of lung metastasis have been reported [40].

Absence of encapsulation together with infiltrating streams of cells and a general lobular morphology characterize this group of low-grade adenocarcinomas. Infiltration into the surrounding salivary gland and connective tissue is evident at low-power examination. In cases involving the hard palate or jaw bone, extension into surrounding or adjacent bone may be noted. A wide range of histomorphologic patterns between and within individual tumors is characteristic. In most areas the tumor is composed of a homogeneous population of cells with prominent, bland, often vesicular nuclei and minimal cytoplasm [14,41] (Figure 2.6).

These cells are arranged in lobules, as well as in solid nests. Tubules lined by a single layer of cells are also typical of this tumor. Cribriform structures bearing a resemblance to adenoid cystic carcinoma may also be seen. Tumor cells, often spindled, are also arranged in trabeculae and narrow cords. Striking patterns in which concentric arrangements of individual cells appear around blood vessels and nerves may be noted. Perineural growth around small nerve twigs is evident in a majority of cases but appears to have no clinical relevance. Nuclear atypia, necrosis, and mitotic figures are absent. The stroma may contain areas of mucoid quality and hyalinization [14, 42].

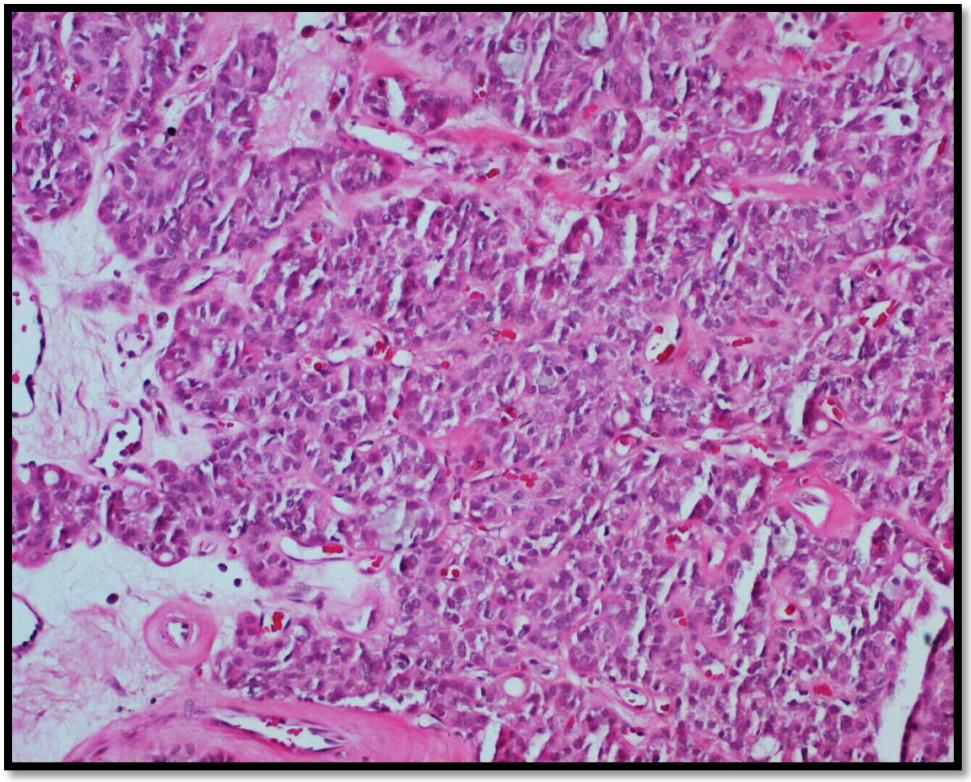


Figure 2.6. Histology of Polymorphous low-grade adenocarcinoma. Homogeneous population of cells with prominent, bland, often vesicular nuclei and minimal cytoplasm. (H&E X200).

2.3.6. Adenoid cystic carcinoma

Adenoid cystic carcinoma (Ad CC) manifests as a slowly growing mass often accompanied by pain and in some cases, facial paralysis [23]. Adenoid cystic carcinoma is a high-grade malignancy that has a fair 5-year survival rate but a dismal 15-year survival rate. It is composed of duct-type epithelial cells and myoepithelial cells in variable patterns. Typically showing little cellular atypia and only rare mitotic figures, it pursues an unrelenting course that defies most therapeutic measures [14].This lesion accounts for approximately 23% of all salivary gland carcinomas. Approximately 50% to 70% of all reported cases of adenoid cystic carcinoma occur in minor salivary glands of the head and neck, chiefly of the palate. In the major salivary glands the parotid gland is most often affected. Most patients with adenoid cystic carcinoma are in the fifth through seventh decades of life, and there is no gender predilection [14, 43]. In the major salivary glands the clinical appearance is usually a slowly growing unilobular mass that is firm on palpation, although with occasional pain or tenderness. These lesions are generally characterized by a slow growth rate; they are often present for several years before the patient seeks treatment. Facial nerve weakness or

paralysis may occasionally be the initial presenting symptom, especially in late-stage lesions [23, 44].

Bone invasion occurs often, initially without radiographic changes, because of infiltration through marrow spaces. Distant spread to the lungs is more common than metastasis to regional lymph nodes. It typically invades perineural spaces, leading to extension of neoplasm well beyond the primary mass [45]. A common feature of intraoral lesions, particularly those arising on the palate, is ulceration of the overlying mucosa, point often used to help distinguish this lesion clinically from the more common benign mixed tumor [14].

Adenoid cystic carcinoma is an epithelial malignancy composed of ductal epithelial and myoepithelial cells variably arranged in tubular, cribriform figure (2.7), and solid patterns. These cells are Small bland myoepithelial cells with scant cytoplasm and dark compact angular nuclei surround pseudoglandular spaces with PAS+ excess basement membrane material and mucin.The cribriform pattern, which is the most common, is characterized by nests of cells containing small, circular cyst-like spaces. The solid pattern is associated with a poor prognosis compared to the tubular and cribriform architecture. Neural invasion is a hallmark of this entity, and often extends beyond the main tumor mass. Infiltration of adjacent soft tissues is also characteristic of adenoid cystic carcinoma [14, 23, 44].

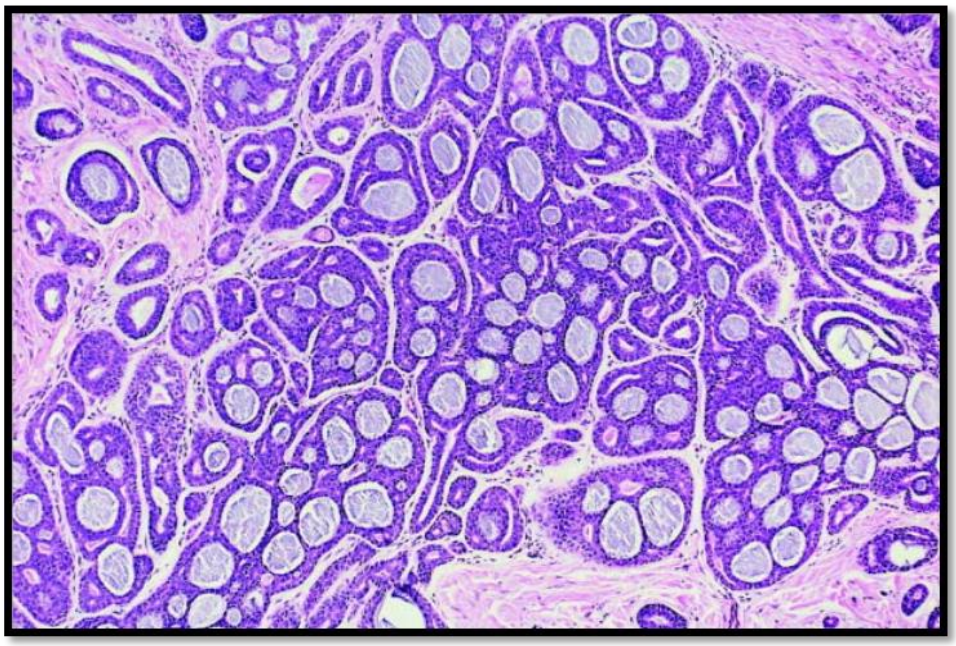


Figure 2.7 Histology of adenoid cystic carcinoma**,** cribriform pattern: nests of cells containing small, circular cyst-like spaces (H&E X200).

2.3.7. Acinic cell carcinoma

Acinic cell carcinoma (Ac CC) occurs predominantly in the major salivary glands, especially the parotid. The putative origin of acinic cell carcinoma is from the intercalated duct reserve cell, although there is reason to believe that the acinic cell itself retains the potential for neoplastic transformation [14].

Acinic cell carcinoma is found in all age groups, including children, with the peak incidence noted within the fifth and sixth decades of life. There appears to be no gender predilection. This lesion accounts for 14% of all parotid gland tumors and 9% of the total of salivary gland carcinomas of all sites[46]. An unusual feature is the frequency of bilateral parotid gland involvement in approximately 3% of cases. Most cases develop within the superficial lobe and inferior pole of the parotid gland (approximately 80%). Fewer cases have been reported within the submandibular gland (4%) and intraoral minor salivary glands (17%). Within the oral cavity most cases occur in the palate and buccal mucosa. Acinic cell carcinoma usually presents as a slow-growing lesion less than 3cm in diameter. Although it is not indicative of the prognosis, pain is a common presenting symptom [14, 46].

Acinic cell carcinoma typically grows in a solid pattern, although one third of lesions show a microcystic growth pattern Papillary and follicular patterns may also be seen. Hemosiderin is often found, and there is little stromal tissue. Tumor cells are uniform and well differentiated. They often contain cytoplasmic PAS-positive, diastase digestion resistant granules similar to those found in normal acinic cells [14, 46-48] (Figures 2 .8).

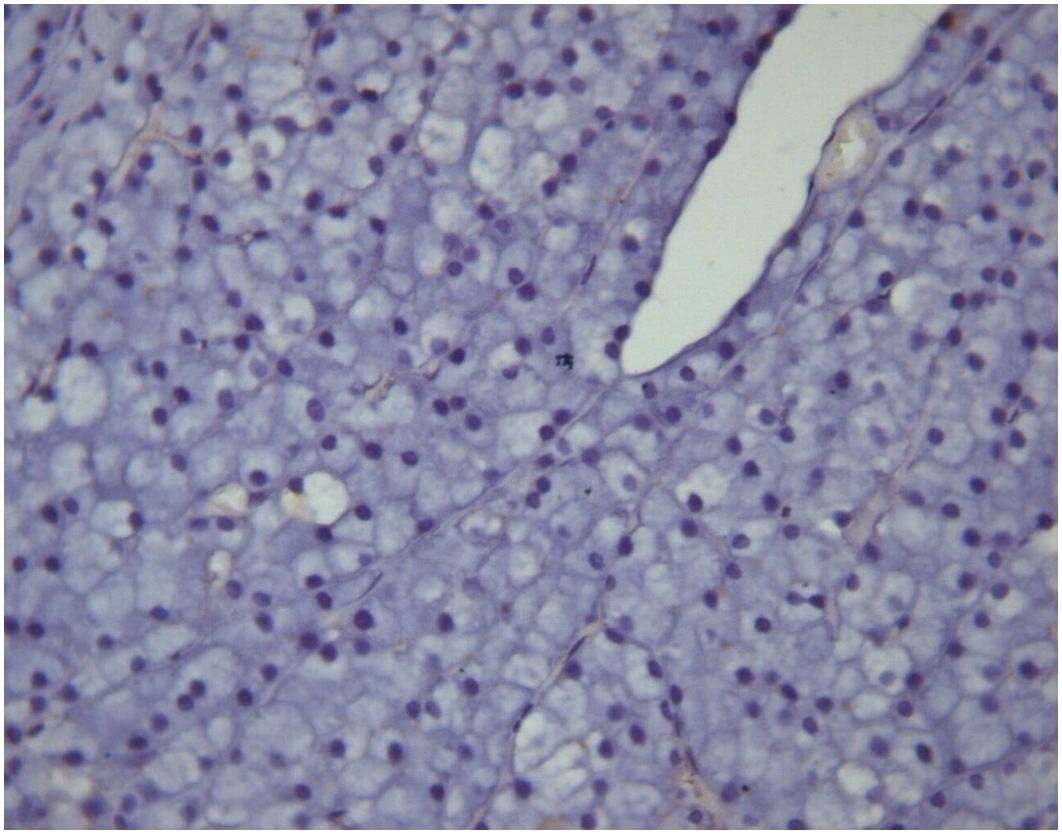


Figure 2.8. Histology of acinic cell carcinoma, solid pattern (H&E X400).

2.4. Cancer Stem Cell (CSC)

2.4.1. Definition

Cancer stem cells are cancer cells found within tumors or hematological cancers that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. CSCs are therefore tumorigenic , perhaps in contrast to other non-tumorigenic cancer cells. CSCs may generate tumors[49, 50]. It can be argued that while all cells within a tumor are equal, at any given time only a small fraction of cells is in an appropriate state or stimulated by appropriate external signals to form a new tumor. Alternatively, it can be reasoned that there is a predetermined population of cells with the "cancer stem cell" phenotype, enabling this cell to perpetuate the tumor, while other cells of the same tumor are incapable of self-renewal [51].

The tumor stem cell is the original cell of the tumor, responsible for tumorigenesis, tumor differentiation, tumor maintenance, and also for tumor spread and relapse. This is analogous to a stem cell being the original cell of an organ, responsible for organogenesis and organ maintenance [52].

2.4.2. Characteristics of the CSC

Cancer stem cells have, similar to stem cells, a variety of specific properties:

i. Unlimited self-renewal is the central function of tumor stem cells allowing the maintenance of the tumor stem-cell pool by symmetric cell division. The regulation of this function demands tightly coordinated pathways. For some tumor types, pathological changes in those pathways have already been shown [52].
ii. Differentiation is the capability of stem cells to produce specific tissue types and to maintain tissue homeostasis by asymmetric cell division. Analogously, the aberrant differentiation of tumor stems cell leads to formation of the heterogeneous bulk tumor [53].
iii. Self-preservation of tumor stem cells is achieved by strategies including activation of antiapoptotic pathways, increased activity of membrane transporters, and enhanced DNA-repair activity [53].
iv. Tumor stem cells have the ability to induce a phenotypic copy of the original tumor after transplantation [53].

2.4.3. Historical background of CSC

The theory of neoplastic disease, originating from developmentally undifferentiated stem cells, was first proposed by the cytological work of pathologist Julius Cohnheim in 1867 [54-56].His interpretation of karyotypic chromosomal differences between epithelial and mesenchymal tumors contributed to the characterization and understanding of tumor metastasis, which led to the belief that neoplasia, was a ‘stem cell disorder’. During this era, one of the more prominent proposals came from Boveri's hypothesis: ‘oncogeny by chromosomal mutation’ [55, 56]. The idea favored chromosomal number normalization and tumor evolution through accumulation of precise mutations and selective growth. Boveri's hypothesis led to the reemergence of the ‘stem-line concept’ as proposed by O. Winge in 1930 [56]. In the early 1950s Klein and coworkers developed a new model based on serial transplantation in vivo that could give rise to primary tumors and metastasis [57].

To prove the latter would require prospective isolation of this population. Indeed, this was done by John Dick’s group, who demonstrated that cells capable of establishing a human acute myeloid leukemia phenotype in a recipient mouse were isolated only within the cell

fraction expected to contain the hematopoietic stem cells, defined by the CD34+ CD38− phenotype [58, 59]. Further, these cells could be passed from animal to animal and maintain the AML phenotype [60], confirming the property of self-renewal. Thus, it was demonstrated for the first time that there are cells within the tumor which have properties similar to stem cells, i.e. the capacity to reconstitute the tumor when transplanted into an appropriate recipient (differentiation) through several rounds of transplantation (self-renewal).

Similar approach has led to the identification of subpopulations of tumor cells with stem cell properties within breast tumors [61], gliomas [52], melanoma [62], prostate cancer[63] and osteosarcoma [64]. These observations have led to the "cancer stem cell hypothesis" which postulates that within a tumor, a small proportion of cells with unlimited proliferative capacity drive tumour growth [65].

2.4.4. Markers for cancer stem cell

Aldehyde dehydrogenase 1

Aldehyde dehydrogenase 1 (ALDH1) belongs to the aldehyde dehydrogenase superfamily which is responsible for the oxidation of aldehydes to their corresponding carboxylic acids [66, 67]. ALDHs metabolize endogenous and exogenous aldehydes and thereby mitigate oxidative/electrophilic stress in prokaryotic and eukaryotic organisms. Many ALDHs in evolutionarily distant and seemingly unrelated species perform similar functions, including protection against a variety of environmental stressors such as dehydration and ultraviolet radiation. The ability to act as an "aldehyde scavenger" during lipid peroxidation is another ostensibly universal ALDH function found across species. Mutations in various ALDHs are associated with a variety of pathological conditions in humans, highlighting the fundamental importance of these enzymes in physiological and pathological processes [67, 68].
Only recently, scholars have found that high ALDH1 activity can be used to identify and isolate CSCs. ALDH1 was first used as marker of cancer stem cells in hematopoietic cells [68]. It was also studied in solid tumors like breast cancer [69], head and neck squamous carcinoma [70], lung cancer [71, 72], prostate cancer [73], cervical cancer [74] and colorectal cancer [75]. Extremely limited data about ALDH are available in salivary gland tumors. Sreerama et al. documented elevated level of class 3 ALDH in Warthin tumor and mucoepidermoid carcinoma of human parotid gland [76]. In another study Sun et al.

indicated that ALDH^{high} adenoid cystic carcinoma (Ad CC) cells possessed enhanced invasive potential *in vitro* and highly metastatic capability *in vivo*[77].

CD44

CD44 is a ubiquitous multistructural and multifunctional cell surface adhesion molecule involved in cell-cell and cell-matrix interactions. Twenty exons are involved in the genomic organization of this molecule. The first five and the last ***5*** exons are constant, whereas the 10 exons located between these regions are subjected to alternative splicing, resulting in the generation of a variable region. [78, 79]. CD44 has been identified as a marker of CSC in breast [61], colorectal[80], and pancreatic[81], head and neck squamous cell carcinomas [82]. Altered CD44 expression is reported in many types of malignancy, particularly in association with invasion and metastasis [83]. Contradictory results have been published on the role of the CD44 family in carcinogenesis and metastatic spread of squamous cell carcinomas of the head and neck, including oral cancer. The main stream of results indicates a predominant loss of certain CD44 isoforms during the stepwise course of oral carcinogenesis, with heterogeneous expression in invasive carcinomas [82, 83].

Fonseca et al characterized the distribution of CD44 isoforms (CD44 v3–v6) by immunohistochemistry and immunoelectron microscopy in human salivary gland tissue. They proposed that CD44 has a role in the regulation of growth and renewal of normal SG, and on the morphogenesis of salivary neoplasms[84].

In SG tumors limited studies are available in the literature. Saove et al have presented some evidence that the analysis of isolated CD44 immunoexpression could give prognostic information associated with clincopathological features of salivary gland malignant neoplasms.

CD166 (ALCAM)

The activated leukocyte adhesion molecule (ALCAM) is a highly conserved 110-kD multidomain transmembrane type 1 glycoprotein of the immunoglobulin superfamily. ALCAM plays a role in the development of different tissues during embryogenesis and in adults, and it functions via homotypic and heterotypic interactions between the cells [85, 86].

Since its discovery CD166 expression has been regarded as a cause of tumour progression and metastasis in a subset of tumors; such as cutaneous melanoma, prostate carcinoma, breast cancer, colorectal carcinoma, bladder cancer, gynecologic, pancreatic and esophageal squamous cell cancer. However previous studies addressing ALCAM's role in cancer have yielded conflicting results. Existing reports are paradoxical, with ALCAM gene expression being highly up regulated in some cancers and greatly down regulated in others. Furthermore depending on the tumor cell type, ALCAM expression has been reported to be both positively and negatively correlated with cancer progression and metastasis in the literature [86-87].

Concerning CD166 expression in salivary gland tumours, Azadeh et al. found that CD166 expression in malignant salivary gland tumours (MEC and Ad CC) was significantly higher than that of benign salivary gland tumors (PA), and higher in PA than normal salivary gland tissue. They also found that CD166 expression was significantly higher in high grade tumors compared to low grade ones [88].

CD24

CD24 is a mucin-like adhesion molecule expressed by neutrophils, prelymphocytes and a large variety of solid tumors. Functionally, CD24 enhances the metastatic potential of malignant-cells, because it has been identified as a ligand of P-selectin, an adhesion receptor on activated endothelial cells and platelets [89].The CD24 protein is expressed in keratinocytes, renal tubules, regeneratin-g muscles and the developing brain and pancreas [89,90]. It may be involve as a regulator factor for the control of cell proliferation, cell adhesion and apoptosis. However, the expression and physiological function of CD24 in human malignancies has not yet been completely elucidated [89].In human carcinomas, there is evidence that CD24 expression is related to prognosis [91] and may contribute to metastasizing tumor cells [89-91], but the relationship between CD24 and prognosis is not completely understood.

Soave et al. has shown positive CD24 expression in 9 malignant salivary gland tumors. The analysis of the CD24 positive cells in SGMN showed an association between CD24 expression, and clinical stage. These results suggested that CD24 may correlate with advance stage of SGMN. On the other hand Ma and colleagues reported that metastatic adenoid cystic carcinoma cell lines lack CD24 expression[92,93].

3. MATERIALS AND METHODS

3.1. Tissue Samples

Formalin-fixed, paraffin-embedded tissues of 24 malignant tumors (8 adenoid cystic carcinomas, 6 mucoepidermoid carcinomas, 2 acinic cell carcinomas, 2 carcinoma ex pleomorphic adenoma and 6 polymorphous low grade adenocarcinoma), 24 benign tumors (21 pleomorphic adenomas, 3 basal cell adenoma) and 7 normal salivary glands tissues were obtained from the archive of Gazi University Faculty of Dentistry Department of Oral Pathology between the period of 2004-2014. The normal salivary glands have been isolated from intact salivary gland tissues of mucocele, sialadenitis, or from reactive gingival tissues that contain minor salivary glands.

The medical files were analyzed to record information on age, gender, tumor evolution, histological classification, tumor recurrences and metastasis. Five malignant cases have 3 years of follow up, while the other14 SG tumors have an average of 7 years of follow up. We couldn't reach the clinical information of 5 patients. The age 60 years was determined as reference age [96].

Hematoxylin and eosin (H&E) stained slides from 55 cases were revised to confirm the histopathological diagnosis, grade, andalso to detect the most representative tumor areas.

3.2. Immunohistochemical Staining

All 55 samples were fixed in 4% neutral formalin and embedded in paraffin. Immunohistochemical studies were performed on 4-μm-thick serial sections of the tissue specimens, mounted on glass slides coated with aminoalkylsilane. Sections were dewaxed in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersing the slides in 10% hydrogen peroxide for 10 minutes at room temperature and rinsing with distilled water. The sections were steamed in a citrate buffer for 25 minutes, allowed to cool, and then immersed in a protein-blocking solution. Then the primary antibodies (ALDH1, CD44, and CD166) (mouse monoclonal antibody, Novocastra^{TM} Lyophilized Newcastle upon Tyne, United Kingdom) were diluted to 1:100 while CD24 (mouse monoclonal antibody, Novocastra^{TM} Lyophilized Newcastle upon Tyne, United

Kingdom) was diluted to 1:150 and slides were incubated in primer antibodies for 1 hour in humidified chamber at room temperature.The sections were washed with phosphate-buffered saline and incubated in secondary antibodies (Invitrogen, Carlsbad USA) for 10 minute. Sections were then washed with phosphate-buffered saline and incubated with diminobenzidine (Thermo Scientific^{TM} USA) according to the manufacturer's recommendations. Sections were counterstained with Mayer's Hematoxylin, dehydrated in grade series of alcohols. For positive control human tonsil tissue was used for CD24 and CD44, skin tissue for CD166 and lung adenocarcinoma for ALDH1. Negative controls were prepared by omitting the primary antibody.

In accordance with protocols devised by Honeth and colleagues CD44 staining was detected in membrane [93]. CD24 staining was detected in membrane, cytoplasm and nucleus [93, 94]. ALDH1 expression was detected in cytoplasm[69] while CD166 was detected in both membrane and cytoplasm[94]. Immunohistochemical analysis of the sections was performed without knowledge of the patient's diagnosis or clinical status. All tumor fields were evaluated by Olympus BX51 light microscopy at x400 magnification.

Scoring for CD44 and CD24 was considered as follows: 0, 0–10% of positive tumor cells; 1+, 10–25% of positive tumor cells; 2+, 25–50% of positive tumor cells; 3+, more than 50% of positive tumor cells. For CD44, the cases classified as 0 were considered negative, whereas 1+, 2+ and 3+ were established as positive cases. For CD24, the cases were divided into negative, when considered 0 or 1+, or in positive cases,andas positive when classified as 2+ or 3+ [93]. Immunohistochemical staining of ALDH1 was classified as positive when more than 1% of tumor cells showed clear cytoplasmic positivity [94]. For CD166 sections were scored for ratio (1 = <10%; 2 = 10–50%; 3 = >50%) and intensity (1 = weak; 2 = moderate; 3 = strong) and the sum of ratio and intensity have been calculated. The cases with score 3 or more were considered positive [95].

CSC markers expression in different cell types have been recorded and scored depending of the intensity and ratio as ++,+ as well.

3.3. Statistical Analysis

The statistical analysis was performed by the commercially available SPSS (SOFTWARE PACKAGE STATISTICAL ANALYSIS) Statistics 16 software for Windows (SPSS Inc.,

Chicago, IL. USA). Statistical significant differences were determined by **Fisher's exact test** (x^2 test) for correlation analysis. A P of ≤ 0.05 was considered to indicate statistical significance.

4. RESULTS

4.1. Demographic and Clinical Data

A total of 55 patients (28 men 51% and 27 women 49%) were included in the study. Patient's age ranged from 22 to 86 years with mean age of 49 (±). The male mean age was 52, 3 (±) years while the female mean age was 44.1 (±) years.

The distribution of neoplasms were as follow : mucoepidermoid carcinoma 6 cases (12.5%), polymorphous low grade adenocarcinoma 6 cases (12.5%), adenoid cystic carcinoma 8 cases (17%), acinic cell carcinoma 2 cases (4%), carcinoma ex-pleomorphic adenoma 2 cases (4%), basal cell adenoma 3 cases (6.25%), pleomorphic adenoma 21 cases (44 %), and 7 normal salivary gland tissueas control.

Age and gender distribution of patients on the basis of tumor type is given in table number (4.1).

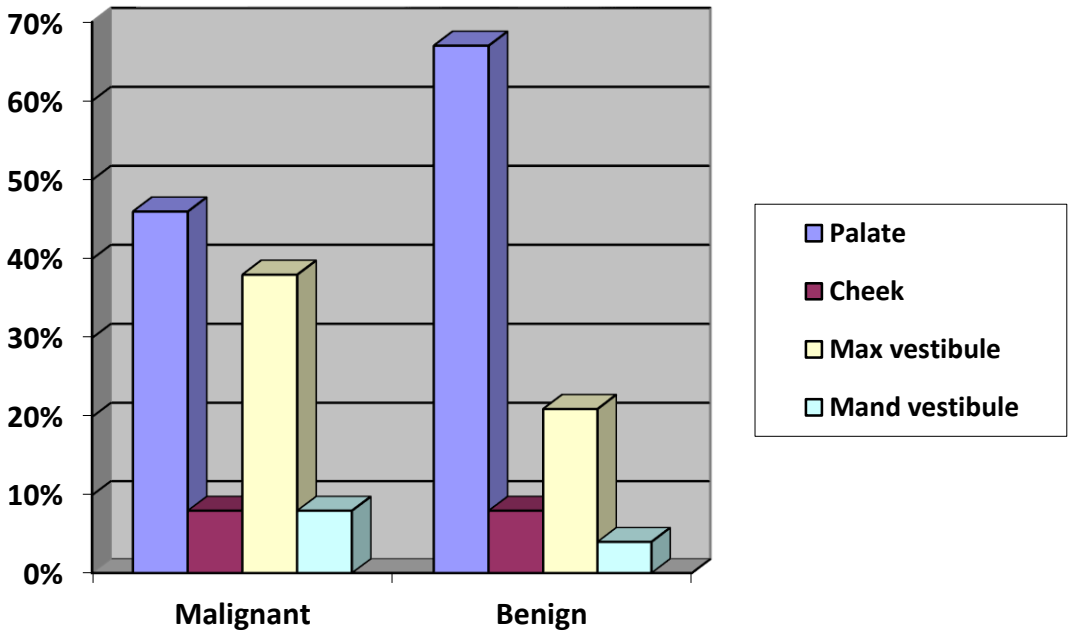
Table 4.1. Demographic data of the neoplasms

	MALE MEAN AGE (n= 28)	FEMALE MEAN AGE (n= 27)	GENERAL MEAN AGE (n= 55)	Percentage Of Male (n=28)	Percentage Of Female (n= 27)
MALIGNANT TUMORS (n= 24)					
MEC (n=6)	64	40	52	35%	65%
PLGA (n=6)	54	54,5	54,25	17%	83%
Ad CC (n=8)	59,5	67,5	63,5	50%	50%
Ac CC (n=2)	50	50	50	0	100%
EX-MIX (n=2)	42	72	57	50%	50%
BENIGN TUMORS(n= 24)					
BCA (n=3)	75	0	37,5	100%	0
PA (n=21)	40	42	41	65%	35%
CONTROL NORMAL SG (n= 7)					
	42	27	34,5	42%	58%

Anatomic localization of tumors were as follow : the most common localization of MEC was the palate, for PLGA were the palate and maxillary posterior vestibule, for Ad CC was the maxillary posterior vestibule, for Ac CC was the cheeks, for EX-MIX were the palate and mandibular posterior vestibule, for BCA was maxillary posterior vestibule and for PA was the palate. Localizations of tumors are summarized in table number (4.2) and graphic (4.1).

Table 4.2. Anatomic localization of the tumors

	PALATE **n=26**	**CHEEK** **n=4**	**MAXILLA VESTİBULE** **n=14**	**MANDIBLE VESTIBULE** **n=4**	**TOTAL NUMBER OF CASES** **n= 48**
MALIGNANT TUMORS(n=24)					
MEC	**4**	**0**	**0**	**2**	**6**
PLGA	**3**	**0**	**3**	**0**	**6**
Ad CC	**2**	**0**	**6**	**0**	**8**
Ac CC	**0**	**2**	**0**	**0**	**2**
EX-MIX	**1**	**0**	**0**	**1**	**2**
BENIGN TUMORS(n=24)					
BCA	**0**	**0**	**2**	**1**	**3**
PA	**16**	**2**	**3**	**0**	**21**



Graphic 4.1. Localization of malignant and benign tumors

Loco-regional metastases and recurrence developed in 6 patients (12, 5 %). A total of 4 recurrence cases (2 cases of PLGA, 1 MEC, 1 Ad CC), and 2 loco-regional metastasis (1 MEC and one Ad CC) were recorded in mean follow up time of 7 years.

4.2. The Expression of CSC Markers

All four markers were expressed in normal salivary gland tissue but their staining ratio and intensity showed discrepancy. In general ductal cells more widely expressed CSC markers. Serous acinar cells had expressed CD44 more extensively, while mucous acinar cells showed ALDH1 and CD166 expression more. CD24 expression didn't show any difference on the basis of acinar cell type (Figure 4.1).

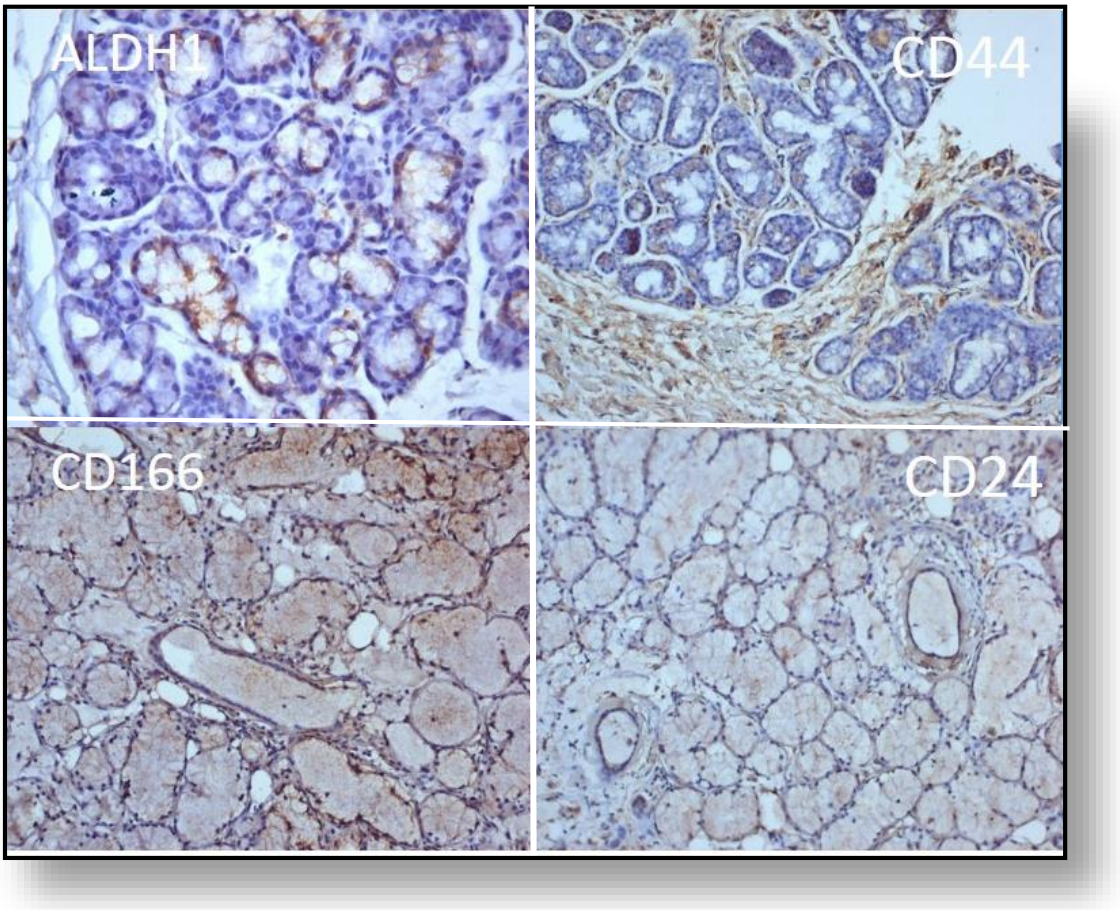


Figure 4.1. The expression of CSC markers in normal salivary gland (DAB x400). The expression of CSC markers are summarized in the tables and figures for each tumor type

MEC:

ALDH expression was higher in mucous cells in comparison to epidermoid and intermediate cells. CD44 showed higher expression in epidermoid cells. There was not any expression difference for CD166 and CD24.

Table 4. 3. The expression of CSC markers in MEC

Marker	**ALDH**	**CD44**	**CD24**	**CD166**
Number and ratio of positive tumors (n =6)	(n =6) 100 %	(n =3) 50 %	(n =3) 50%	(n =6) 100%
Target cells	Epidermoid + Mucous ++ Intermediate +	Epidermoid ++ Mucous + Intermediate +	Epidermoid+ Mucous + Intermediate+	Epidermoid+ Mucous + Intermediate+

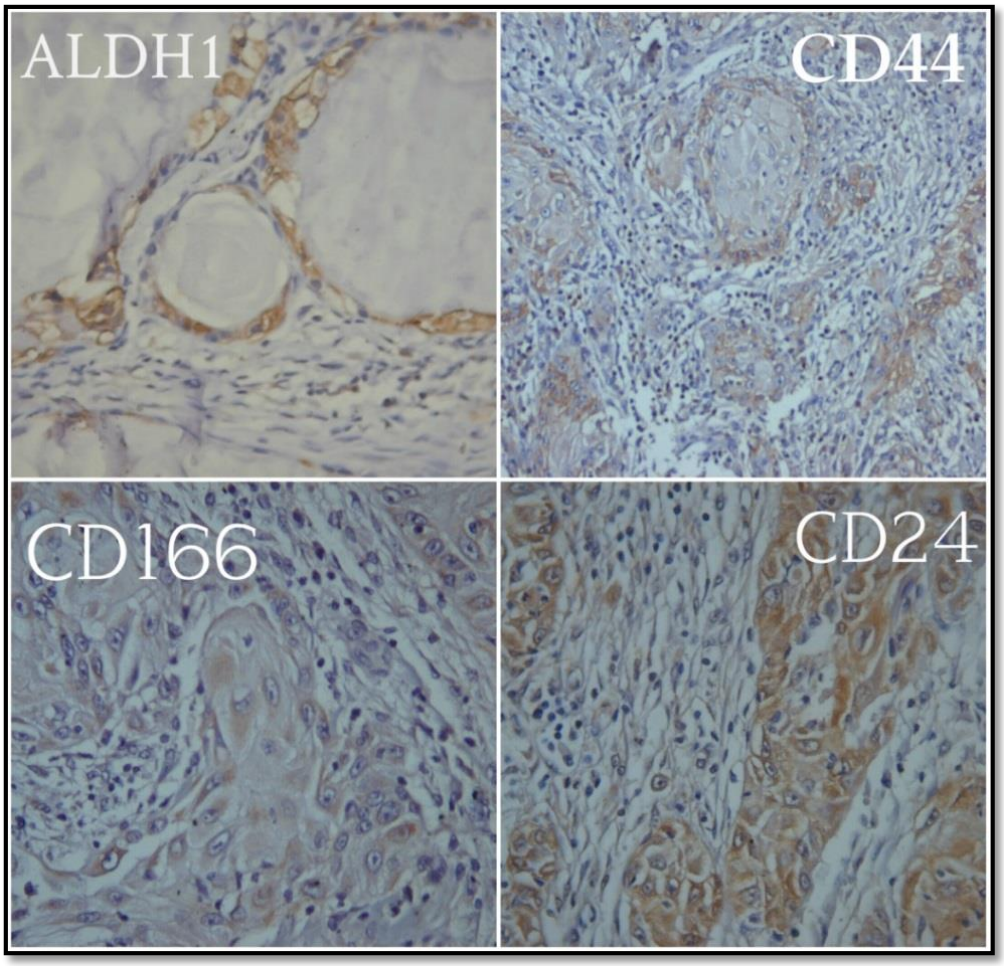


Figure 4.2 The expression of CSC markers in MEC (DAB X400).

PLGA:

Tumor cells showed similar expression for all the CSC markers.

Table 4.4. The expression of CSC markers in PLGA

Marker	**ALDH**	**CD44**	**CD24**	**CD166**
Number and ratio of positive tumors (n=6)	(n =4) 67 %	(n =5) 83 %	(n =3) 50 %	(n =5) 83 %
Target Cells	Epithelial tumor cells	Epithelial tumor cells	Epithelial tumor cells	Epithelial tumor cells

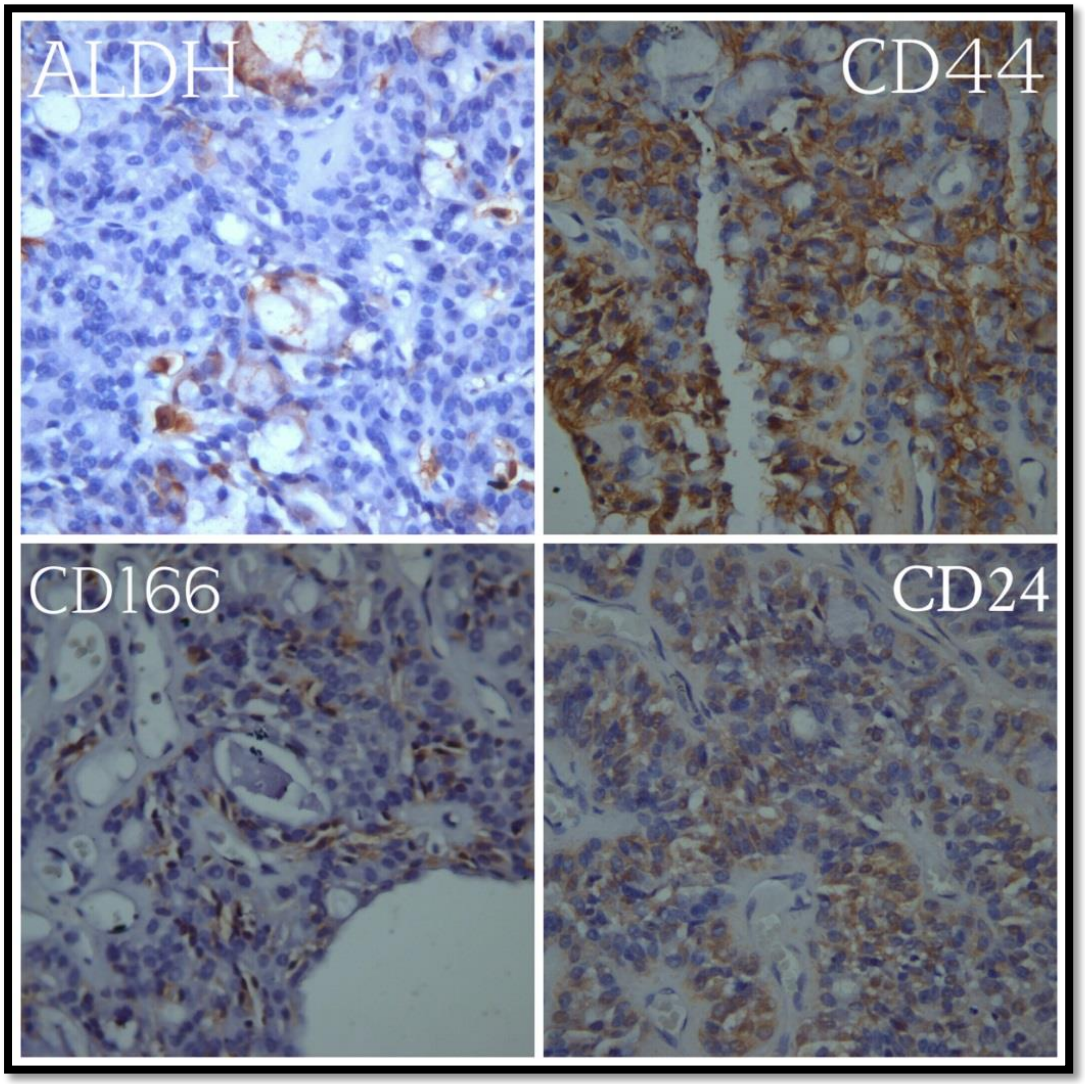


Figure 4.3. The expression of CSC markers in PLGA (DAB X400).

Ad CC:

Tumors that has cribriform pattern showed higher CSC expression than solid tumors. There was not any ALDH expression in Ad CC. Tumorcells showed similar expression for CD44, CD24 and CD166.

Table 4.5. The expression of CSC markers in Ad CC

MARKER	ALDH	CD44	CD24	CD166
Number and ratio of positive tumors (n =8)	(n =0) 0 %	(n =4) 50 %	(n =4) 50 %	(n =6) 75 %
Target Cells	-	Ductal + Myoepithelial + Basal +	Ductal + Myoepithelial + Basal +	Ductal + Myoepithelial + Basal +

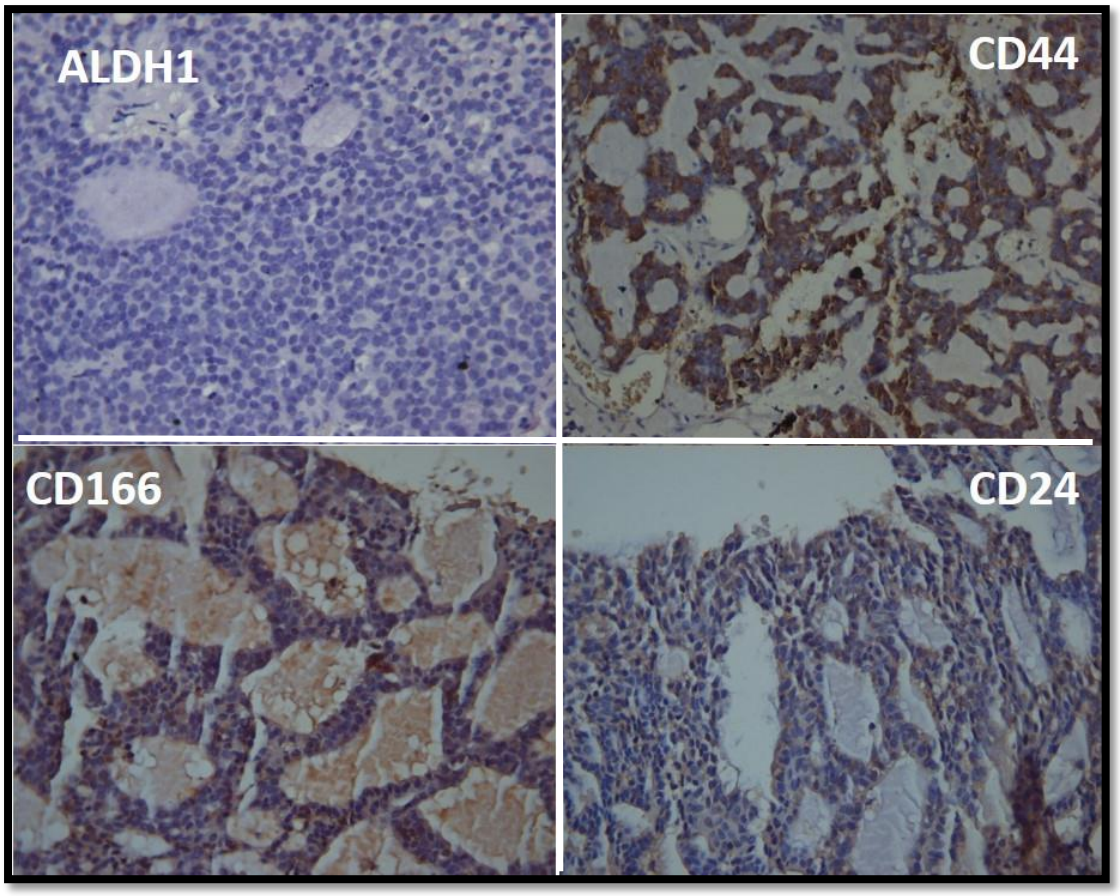


Figure 4.4. The expression of CSC markers in Ad CC (DAB X 400).

EX-MIX Tumor:

Tumor cells showed similar expression for CSC markers.

Table 4.6. The expression of CSC markers in EX-MIX TUMOR

Markers	**ALDH**	**CD44**	**CD24**	**CD166**
Number and ratio of positive tumors (n =2)	(n =2) 100 %	(n =2) 100 %	(n =2) 100 %	(n =2) 100 %
Target Cells	Ductal + Myoepithelial +	Ductal + Myoepithelial +	Ductal+ Myoepithelial+	Ductal+ Myoepithelial+

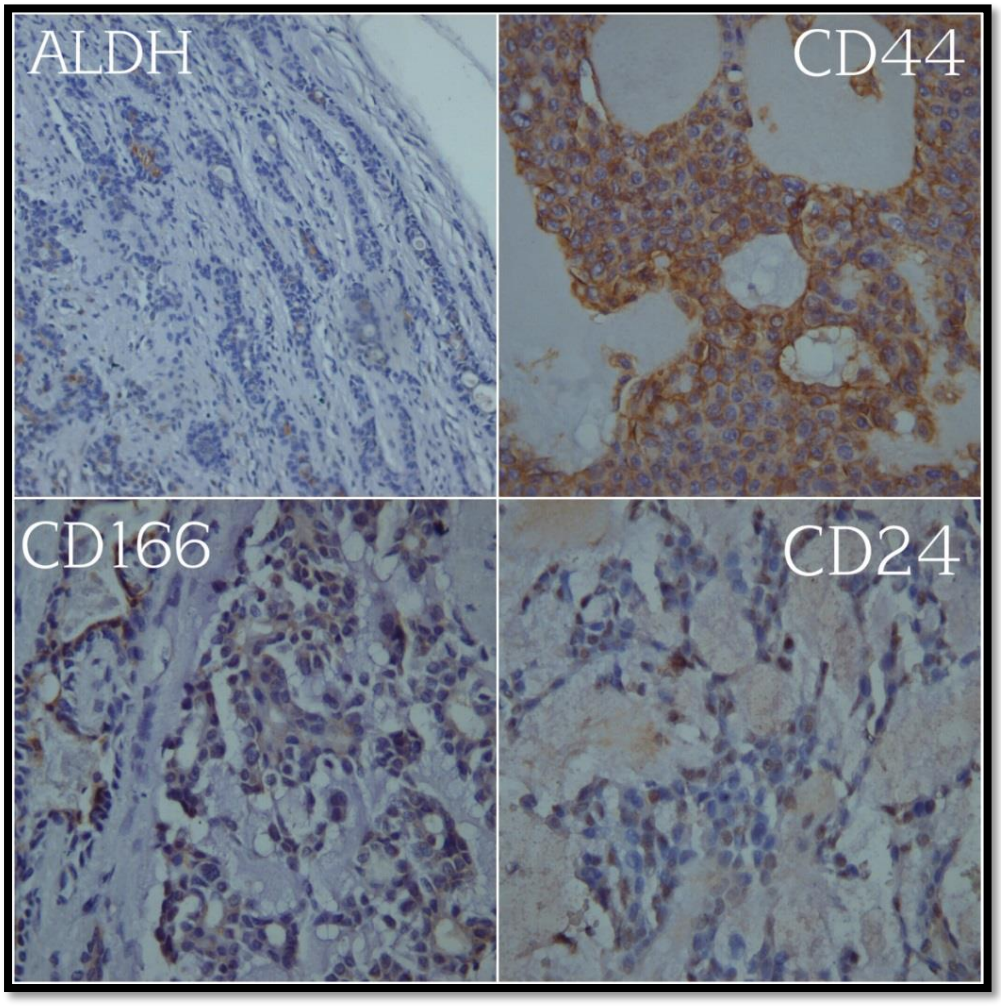


Figure 4.5. The expression of CSC markers in EX-MIX TUMOR (DAB 400).

Ac CC:

Tumor cells showed similar expression for ALDH, CD24 and CD166. There was not any CD44 expression in Ac CC.

Table 4.7. The expression of CSC markers in Ac CC

MARKERS	**ALDH**	**CD44**	**CD24**	**CD166**
Number and ratio of positive tumors (n =2)	(n =2) 100 %	(n =0) 0 %	(n =1) 50 %	(n =1) 50 %
Target Cells	Acinar cells +	-	Acinar cells +	Acinar cells +

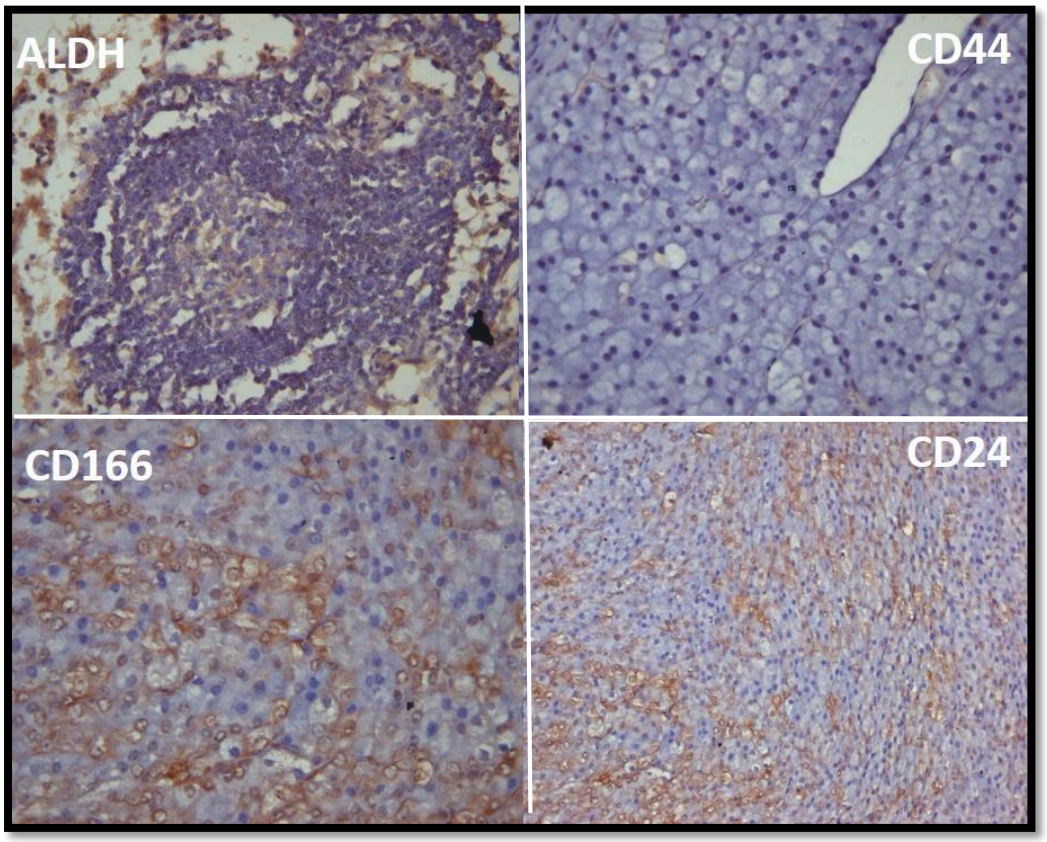


Figure 4.6. The expression of CSC markers in Ac CC (DAB X400).

PA:

Tumor cells showed similar expression for CSC markers.

Table 4.8. The expression of CSC markers in PA

MARKERS	**ALDH**	**CD44**	**CD24**	**CD166**
Number and ratio of positive tumors (n =21)	(n =19) 90 %	(n =19) 90 %	(n =17) 81 %	(n =10) 52 %
Target Cells	Ductal + Myoepithelial +	Ductal + Myoepithelial +	Ductal + Myoepithelial +	Ductal + Myoepithelial +

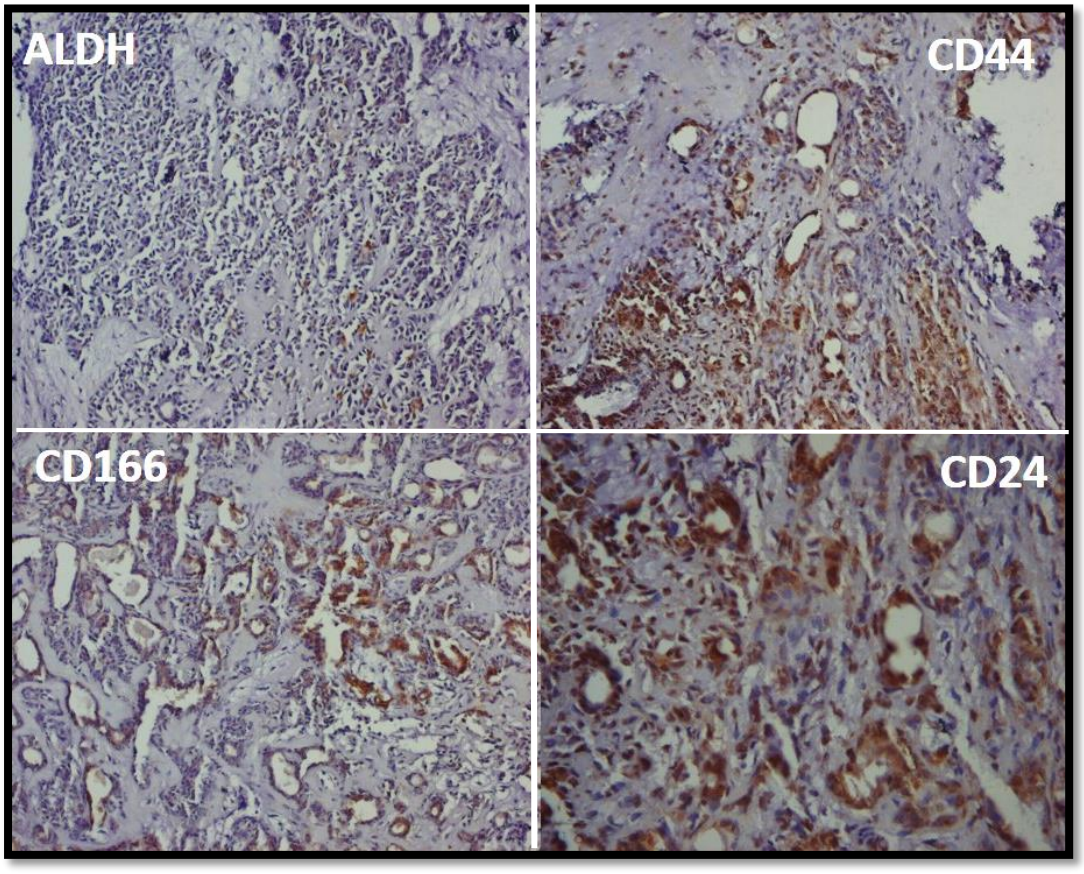


Figure 4.7. The expression of CSC markers in PA (DAB X200)

BCA:

There was not any ALDH or CD166 expression in BCA. Tumor cells showed similar expression of CD44 and CD24.

Table 4.9 The expression of CSC markers in BCA

Markers	**ALDH**	**CD44**	**CD24**	**CD166**
Number and ratio of positive tumors (n =3)	(n =0) 0 %	(n =1) 33 %	(n =3) 100 %	(n =0) 0 %
Target Cells	-	Basal cells +	Basal cells +	-

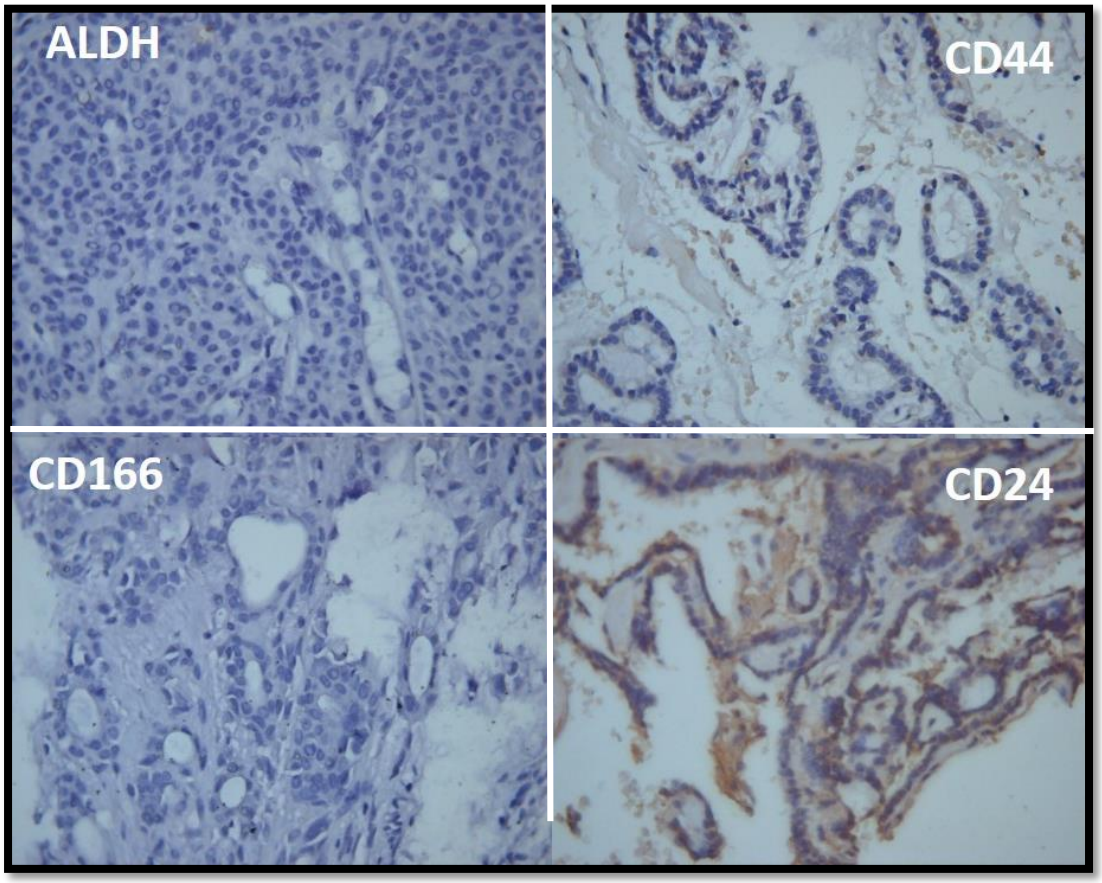


Figure 4.8. The expression of CSC markers in BCA (DAB X400).

Table number 4.10 summarizes the details of CSC expression on the basis of tumor types.

Table 4.10. The expression of CSC in different tumor types

	n	ALDH				CD166				CD44				CD24			
		(+)		(-)		(+)		(-)		(+)		(-)		(+)		(-)	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	N	%
MEC	6	6	100%	0	0 %	6	100%	0	0 %	3	50%	3	50%	3	50%	3	50%
PLGA	6	4	67%	2	33%	5	83%	1	17%	5	83%	1	17%	3	50 %	3	50 %
AD CC	8	0	0 %	8	100%	6	75%	2	25%	4	50%	4	50%	4	50%	4	50%
AC CC	2	2	100%	0	0%	1	50%	1	50%	0	0%	2	100%	1	50%	1	50%
EX-MIX	2	2	100%		0 %	2	100%	0	0%	2	100%	0	0%	2	100%	0	0 %
PA	21	19	90%	2	10%	11	52%	10	48%	19	90%	2	10%	17	81%	4	19%
BCA	3	0	0%	3	100 %	0	0%	3	100%	1	33 %	2	67%	3	100%	0	0 %

The lack of ALDH1 expression in adenoid cystic carcinomas (P 0,000) and basal call adenomas (P 0,026) in comparison to other tumors were statisticallysignificant.

The lack of CD166 expression in basal call adenomas in comparision to other tumors was statistically significant.(P 0,039)

Although it wasn't statistically significant, there was a lack of CD44 expression in acinic cell carcinomas.

4.3. Immunohistochemical Correlations with the Clincopathological Features

The analysis of CSC markers expressions on the basis of tumor biology and age is given in table 4.11.

ALDH1 expression was highest in normal salivary glands followed in descending order by benign and malignant tumors. Malignant SG tumors had statistically significant down regulated ALDH1expressionwhen compared with normal SG tissues (P 0,034) (Graphic 4.2 and table 4.11).

For CD166 the highest expression was by malignant tumors followed in descending order by benign tumors and normal salivary glands. Malignant SG tumors had statistically significant up regulated CD166 expression when compared with normal SG tissues (P 0,002) (Graphic 4.2 and table 4.11).

There was a prominent decrease of CD44 expression in malignant tumors in comparison to benign and normal SG tissues. (Graphic 4.2 and table 4.11)

Malignant SGT showed lower CD24 expression in comparison to benign SGT. (Graphic 4.2 and table 4.11)

There wasn't any statistical significance in comparison of metastasizing/recurrent with non-metastasizing/ non recurrent tumors on the basis of CSCs expressions, however prominent decrease in CD166 and slight increase in CD44, CD24 and ALDH in metastasizing/recurrent tumors were detected (Graphic 4.3 and table 4.11).

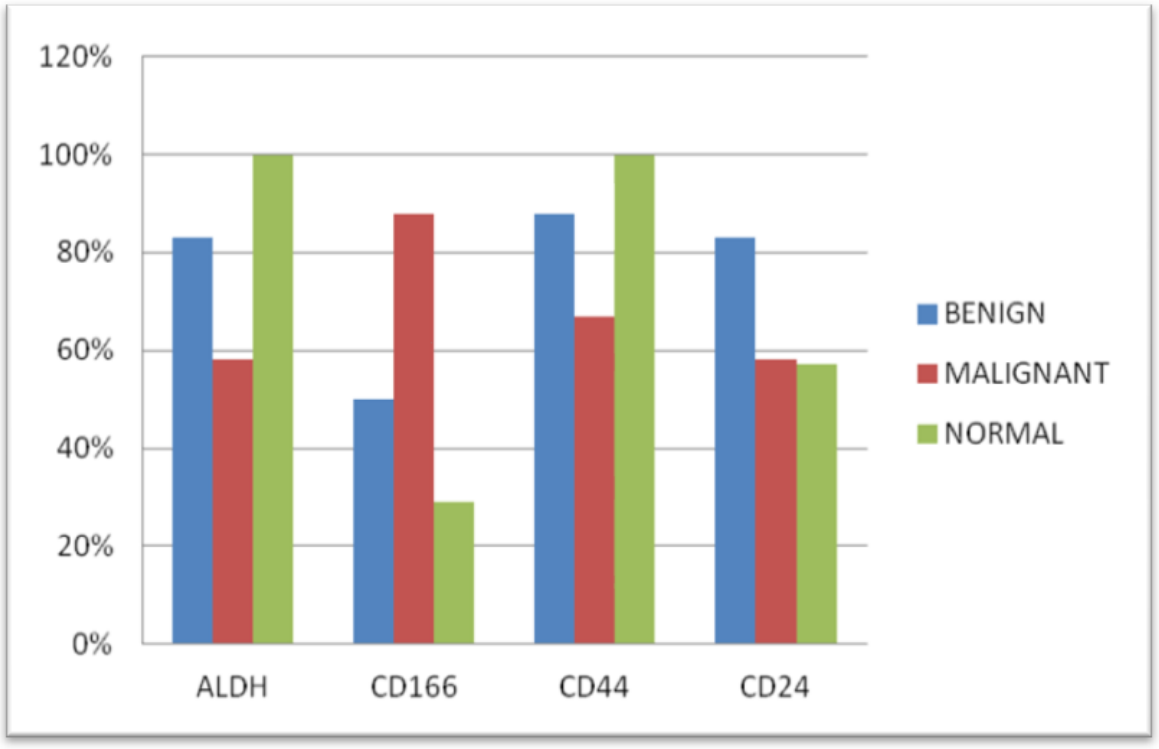
There was not any statistically significant difference in CSC expression between high grade and low grade tumors. However we noticed a prominent decrease in CD166 and slight decrease in CD44 and ALDH in high grade tumors.(Graphic4.4 and table 4.11).

When CSC expression analysed on the basis of patient's age with reference age of 60 years, it was found that patients over 60 years old had lower ALDH expression (P 0,007). (Table 4.11).

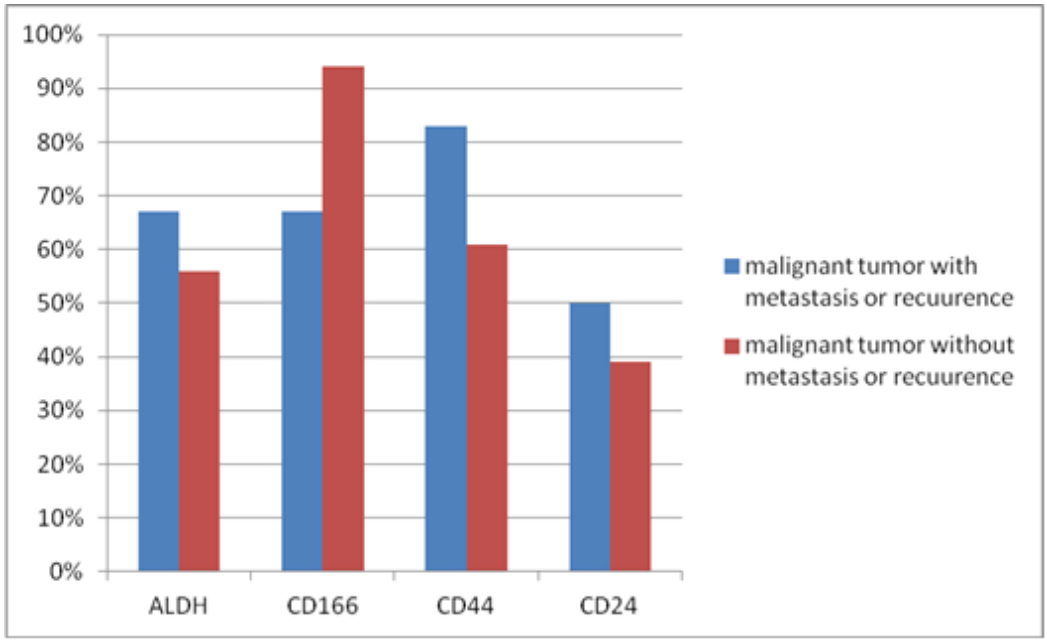
No statistically significant difference of CD44/CD24 or CD44/CD24/ALDH1 immunophenotype was detected between malignant tumors, benign tumors and normal SG tissues. These immunophenotypes dind't show any statistical difference between metastasizing/recurrent and non-metastasizing/nonrecurrent tumors, or between high and low grade tumors.

Table 4.11.The analysis of CSC marker expression on the basis of tumor biology and age.

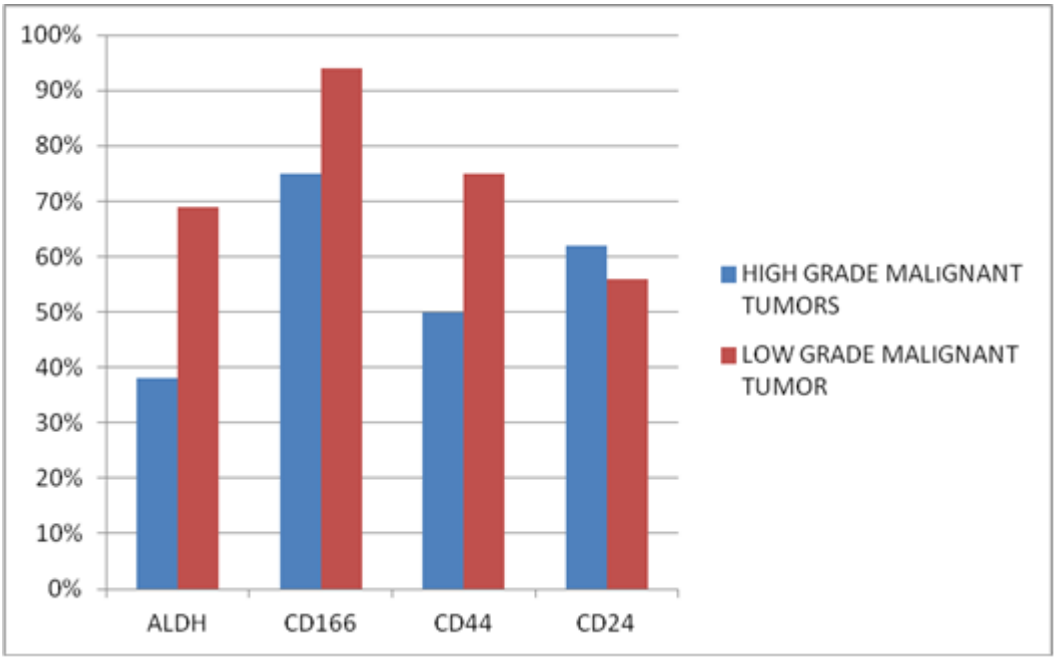
	Variable	(n)	ALDH				CD166				CD44				CD24				P
			(+)		(-)		(+)		(-)		(+)		(-)		(+)		(-)		
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
	Normal	7	7	**100%**	0	**0%**	2	**29%**	5	**71%**	7	**100%**	0	**0 %**	4	**57%**	3	**43 %**	
Tumor	Benign	24	20	**83%**	4	**17%**	12	**50%**	12	**50%**	21	**88%**	3	**12%**	20	**83%**	4	**17 %**	**ALDH(p) =0,034** CD44(p) =0,073 **CD166(p)=0,002** CD24(p) =0,137
	Malignant	24	14	**58%**	10	**42%**	21	**88%**	3	**12%**	16	**67%**	8	**33%**	14	**58%**	10	**42 %**	
metastasis + recurrence	(yes)	6	4	**67%**	2	**33%**	4	**67%**	2	**33%**	5	**83%**	1	**17%**	3	**50%**	3	**50 %**	ALDH(p) =0,650 CD44(p) =0,339 CD166(p)=0,081 CD24(p) =0,650
	(no)	18	10	**56%**	8	**44%**	17	**94%**	1	**6 %**	11	**61%**	7	**39%**	7	**39%**	11	**61%**	
grading	High	8	3	**38%**	5	**62%**	6	**75%**	2	**25%**	4	**50%**	4	**50%**	5	**62%**	3	**38 %**	ALDH(p) =0,156 CD44(p) =0,239 CD166(p)=0,081 CD24(p) =0,650
	Low	16	11	**69%**	5	**31%**	15	**94%**	1	**6 %**	12	**75%**	4	**25%**	9	**56%**	7	**44 %**	
Age	<60	40	22	**55%**	18	**45%**	15	**38%**	25	**63%**	30	**75%**	10	**25%**	23	**58%**	17	**43%**	**ALDH(p) =0,007** CD44(p) =0,121 CD166(p)=0,716 CD24(p)=0,854
	≥60	14	3	**21%**	11	**79%**	5	**36%**	9	**64%**	7	**50%**	7	**50%**	8	**57%**	6	**43%**	



Graphic 4.2. Expressions of CSC markers in benign SGT, malignant SGT and normal salivary glands



Graphic 4.3. Expression of CSC markers in metastasis/recurrence (+) and metastasis/ recurrence (-) malignant tumors



Graphic 4.4. Expression of CSC markers in high and low grade malignant tumors

5. DISCUSSION

The CSCs has several implications in terms of future cancer treatment and therapies. These include disease identification, selective drug targets, prevention of metastasis, and development of new intervention strategies. The concept of CSC has been demonstrated in several human cancers including leukaemia, brain tumor, breast cancer, prostate cancer, lung cancer, pancreas cancer and colon cancer [7-9]. However there are limited studies on salivary gland tumors.

Salivary neoplasms show a complex pattern of tumor cell differentiation and organization. The diversity of epithelial SGTs as well as their rarity and varied morphological aspects often makes diagnosis difficult [13-14]. Due to the limited understanding of pathogenesis and lack of proper chemotherapy regimens, surgery is still the main treatment option in salivary gland tumors. The most challenging issue in treatment of malignant salivary gland tumors is the resistance to chemotherapy [14]. Considering the role of CSC in resisting the therapy in other organs, we could speculate that this unique sub-population of cells may also be involved in resistance to treatment and aggressiveness of salivary gland tumors. Further more these cells may be used for diagnostic and prognostic evaluation of tumors. Understanding the role of CSC in SG can lead to better understanding of the pathobiology of salivary gland malignancies as well as to development of more effective therapies. The present study was designed to evaluate the consequence of cancer stem cells in salivary gland neoplasm. We analysed cancer stem cells expression and its correlation with the clincopathological features of SG tumours.

Diverse cell surface markers have been used for the identification of CSCs in human tumors [75, 97, 98]. ALDH, CD44, CD24, and CD166 have been used extensively for detection of CSC in adenoid tissues like SG and breast [75, 78, 80, 85, 92, 93]. These markers were extensively involved in researches in other organ tumors like melanoma, prostate and intestinal cancerss as well [62-64].

For detection of cancer stem cells several methods have been used including Colony Forming Cell Assay, Side Population Assay, ALDH Activity Assay, PKH staining Assay , and Staining for Surface Antigens. Colony forming assay is unable to detect quiescent CSCs and the reliability is controversial [99]. Side population assay and ALDH activity assay are

easy and simple but specificity is low. Immunohistochemical staining for detection of surface antigen is inexpensive, suitable for paraffin based tissues and highly specific technique for detection of cancer stem cells [99,100]. Aside from its advantages since paraffin embedded tissues were enrolled in the study, immunohistochemistry method was preferred.

In the present study besides benign and malignant salivary gland tumours normal salivary gland tissue was evaluated for CSC markers. We found that serous acinous cells of normal salivary gland tissue mainly express CD44, while mucinous acinous cells mainly express CD166 and ALDH1, there was no difference in the expression of CD24 in any cell types.

Maria and colleagues [101] also reported that serous acinar cells express CD44 , while mucous acinar cells express CD166. They concluded that these two cell surface markers will be useful in the identification of specific populations of salivary acinar cells, which is concordant with our results.

Fonseca et al also characterized the distribution of CD44 isoforms (CD44 v3–v6) by immunohistochemistry and immunoelectron microscopy in human salivary tissue. They reported that CD44v3 and CD44v6 were positive in serous acinar and myoepithelial cells, while CD44v3 was additionally positive in basal ductal cells [84]. In our study ductal or myoepithelial cells didn’t show any CD44 positivity. This discrepancy might be due to the antibody used which doesn't include different isotypes.

CD44 is an important receptor for hyaluronic acid (HA). The functions of this transmembrane receptor include coordination of cell motility, cell-cell adhesion, lymphocyte activation, cell migration and cellular-extracellular matrix interaction. 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. Besides the interaction with HA, CD44 protein has also been shown to interact with other proteins in the extracellular matrix including fibronectin, collagen types I and IV, serglycin and osteopontin. The CD44 receptor is one of the most frequently studied CSC markers, associated with epithelial tumours, although the results show different correlations in each tissue analysed [78-79].

The effect of CD44 expression on malignant neoplasms still demonstrates conflicting results, some papers demonstrated that CD44-expression could be important for tumor suppression, while others suggested the role of CD44 for tumor promotion [79-81].

It has been demonstrated that CD44 is expressed on cancer cell surface and assists haematogenous spread while interacting with P- or L-selectins. It is also involved in numerous complex signalling cascades enhancing tumour initiations by interacting with neighbouring receptors like tyrosine kinase. There are contradictions in validating CD44 expression level and correlation with disease prognosis. For several years, the usefulness of CD44 as CSC marker has been uncertain. But some studies have shown that, CD44 plays a major role in initiation, metastasis, and promoting tumorigenesis [79,80]; while other studies opposed this relationship in other human cancers like breast and prostate cancers, where its high expression was not related to carcinogenesis [102]. CD44 is expressed in almost all normal and cancer cells leading to discrepancy and reflecting the ambiguity regarding functional aspects of CD44 in CSC maintenance and mechanisms involved in cross-talk with expression of stemness genes [103].

Certain studies reported that CD44 could promote apoptosis through activation of caspase-3, and similarly, it can inhibit PI3K activation and AKT thus inhibit tumor initiation [79,102,103].

Furthermore CD44 has been implicated in the inhibition of angiogenesis, particularly by high molecular wight (HMW) hyaluronan engagement. HMW hyaluronan can inhibit induction of the immediate early genes c-fos and c-jun, and it can inhibit migration of endothelial cell [102,103].

All these suggested properties of CD44 are crucial in preventing carcinogenesis. The loss of CD44 may facilitate tumor initiation and progression. The prominent decrease in CD44 expression in malignant salivary gland tumours in comparison to benign tumours and normal SG tissue, detected in our study, was in accordance to that knowladge. CD44 expression was 100 % in normal SG, 88% in benign SGT and in 67% in malignant SGT.

In the current study all types of malignant tumors displayed variable CD44 expression except Ac CC. Both two acinic cell carcinoma cases were totally negative for CD44. However as we had only two Ac CC cases, it was statistically insignificant. Nevertheless this data gave

rise to thought that as the tumour cells mostly demonstrate serous acinar differentiation which show CD44 expression. Loss of CD44 adhesion molecule may play role in carcinogenesis of Ac CC and hence it might be a candidate for diagnostic marker. Advanced researches on large series should be carried out to elucidate this suggestion.

In our study metastasizing tumor displayed higher CD44 expression in comparison to non-metastasizing tumors.

In certain studies it has been shown that CD44 play an important role in metastatic process by means of its adhesive, locomotion and growth transduction functions. As hyaluranic acid (HA) creates a microenviroment with low resistance to cell traffic, CD44 as HA receptor may also play an important role in cell motility, a crucial factor in formation of metastasis [106].

CD44 and other similar adhesion molecules, initiates a cascade of events that can be started by adherence to the extracellular matrix. This leads to activation of the molecule itself, binding to additional ligands, such as growth factors and matrix degrading enzymes, complex formation with additional transmembrane molecules and association with cytoskeletal elements and signal transducing molecules. Thus, through the interplay of CD44 with its ligands and associating molecules CD44 modulates adhesiveness, motility, matrix degradation, proliferation and cell survival, features that together may well allow a tumor cell to proceed through all steps of the metastatic cascade[107].

Based on our data we suggested that loss of CD44 may be important step toward early SG malignancy, whereas increase of this molecule in the course of tumor progression may facilitate metastasis.

CD24 is a mucin-like adhesion molecule expressed by neutrophils, pre-lymphocytes and a large variety of solid tumors. Functionally, it is identified as an alternate ligand for P-selectin, an adhesion receptor on platelets and endothelial cells through which their interaction facilitates the passage of tumour cells in blood stream during metastasis. It increases proliferation and adhesion of tumour cells to fibronectin, collagen, and laminin . The metastatic associations of CD24 increased its importance as a prognostic factor and a new CSC marker [103].

It has been shown that CD24 has distinct expression and function in different cancers like breast, colon, stomach, gallbladder, pancreas and ovary [89]. High expression of CD24 was associated with tumour progression and metastasis [103].

Soave et al.has shown positive CD24 expression in 9 malignant salivary gland tumors. The analysis of the CD24-positive cells in SGMN showed an association between CD24 expression, and clinical stage [92]. On the other hand Ma and colleagues reported that metastatic adenoid cystic carcinoma cell lines lack CD24 expression [109].

In our study malign SGT displayed lower CD24 expression in comparison to benign tumors whereas metastasising/recurrent tumors showed higher CD24 expression. This data supported the suggestion that CD24 expression might be associated with tumor progression and metastasis.

The levels of CD24 expression show great variation between cell lines even in cells of the same cancer subtype. They are related with distinct functionalities at different time periods during tumour progrssion and metastasis [103].

With advancement of studies in CSCs, the correlation between marker expression, tumour initiation, invasion, and metastatic properties has been questioned [8,92,110]. Furthermore, coexpression of surface markers in CSCs is sometimes debatable in several cancer types. Every marker shows independent expression level but seems to have coordination with each other in developing tumours at different stages [92,110].

Recent investigations found that CD24 is coexisting with CD44, CD29, and CD31 in various cancers and gained new interest as a CSC marker. Al-Hajj et al. found that only the population of CD44+/CD24-/low lineage–cells could initiate the process of breast carcinogenesis in immunodeficient mice. Those cells possessed capacities of self-revival, differentiation, unlimited proliferation, and tumorigenesis, whereas CD44+CD24+ cells did not have the capacities[61].

In another study in breast tumors CD44+/CD24-/low population has shown tumorigenic ability. The prognostic value of CD44+/CD24-/low population’s prevalence has been analysed in 136 patients with breast cancer, with and without local recurrence. In normal mammary tissue the presence of CD44+/CD24-/low population was between 0% and 40%, and it increased until 80% in cancer tissue. CD44+/ CD24-/low was expressed in less than

10% of the total cancer cells in 122 cases, while the remaining tumor expressed it with more percentage [90].

Sheridan et al. studied the invasive and proliferative abilities of CD44+/CD24- cell populations coming from metastatic sites. Results demonstrated a high percentage of CD44+/CD24-, more than 30% in the primitive tumor, but a reduction of their proliferative and expansive capabilities in metastatic sites, suggesting that CD44 is necessary but insufficient to identify metastatic cells with stem properties [110].

Another study by Saove et have presented some evidence that the analysis of isolated CD44 and CD24 immunoexpression or the CD44/CD24 immunophenotypes could give prognostic information associated to clincopathological features of malignant SGT. They showed that SGMN with CD44+/CD24+ profile may represents the tumours with most aggressive behaviour and worst prognosis. The immunophenotype CD44+/CD24- was the most prevalent, appearing in 52.2% of salivary glands tumours. They found no correlation between CD24 expression (total, cytoplasmic or membranous) and overall survival. They have also found no correlations between CD24 expression (total, cytoplasmic or membranous) and disease free survival [92].

Recent studies [92,110] demonstrated no correlation between marker expression and tumourigenic potentiality in CSCs of the same cancer type obtained from different patients which was concordant with our data, where none of the correlations in CSC markers could be associated with the aggressiveness of the tumours or metastasis. Also no significant difference could be detected between normal and tumoral SG.

ALDHs are a family of enzymes involved in the maintenance of cellular homeostasis by metabolizing both endogenous and exogenous reactive compounds. ALDH play an important role in protection against a variety of environmental stressors such as dehydration and ultraviolet radiation. The ability to act as an "aldehyde scavenger" during lipid peroxidation is another ostensibly universal ALDH function found across species. Mutations in various ALDHs are associated with a variety of pathological conditions in humans, highlighting the fundamental importance of these enzymes in physiological and pathological processes [66,67]

Mutations in ALDH genes lead to a defective aldehyde metabolism which is the molecular basis of several diseases, including cancer. Interestingly, several ALDH enzymes appear to be markers for both normal and cancer stem cells [113].

ALDH1 belongs to the aldehyde dehydrogenase superfamily which is responsible for the oxidation of reactive form aldehydes to their corresponding non-reactive form carboxylic acids. Moreover, it exhibits high activity for oxidation of aldophosphamide and has a role in the detoxification of some commonly used anticancer drugs, such as oxazaphosphorines. It has been demonstrated that cancer cell-acquired drug resistance is associated with the transcriptional activation of ALDH1 expression [66, 67, 113, 114, 115].

In the literature ALDH1 studies revealed conflicting results. Jelski et al [116] had found that the total activity of ALDH is lower in colorectal, gastric and oesophageal cancer cells than in healthy mucosa. The decreased ALDH activity in cancer tissue suggested that the cells have a greater capability for ethanol oxidation and less ability to remove acetaldehyde than healthy mucosa [115]. On the contrary, some scholars have found that high ALDH1 activity can be used to identify and isolate CSCs. Ginestier et al. [69] suggested that normal and cancerous mammary epithelial cells with increased aldehyde dehydrogenase activity (ALDH) have stem/progenitor properties. In breast cancer ALDH1 positive cells possess self renewal, and differentiation potential, which accounted for only 5% of the total number of breast cancer cells.

In breast cancer many studies showed that ALDH1 is expressed in breast cancers and they are overexpressed in invasive cancer compared to carcinoma in situ [118]. Furthermore many data suggested the role of ALDH1 in resisting anticancer drugs [98]. On the contrary, Kurlandsky et al had found that there is no difference in ALDH between breast cancer and healthy mucosa [119]. Subsequently, several studies have shown that ALDH1 can also serve as stem cell markers of head and neck squamous carcinoma [70], lung cancer [71, 72], cervical cancer [74], prostate cancer [73] and colorectal cancer [75].

Extremely limited data about ALDH are available in salivary gland tumors. L. Sreerama documented elevated level of class 3 ALDH in Warthin tumor and mucoepidermoid carcinoma of human parotid gland. In the same study they reported lower level of ALDH in pleomorphic adenoma, undifferentiated carcinoma and an adenoid cystic carcinoma of the parotid in relation to normal salivary gland tissue [76].

In the present study the highest expression of ALDH1 was by normal salivary gland tissues followed by benign then malignant salivary tumours. Eventually malignant SG tumours showed statistically significant lower ALDH1expression in comparision to normal salivary gland tissue (P 0.034).

The decrease of ALDH1 in malignant SG tumours might be explained by the biological role of that protective and detoxifying enzyme in cancer process. This enzyme has a role in protection against a variety of environmental stressors such as dehydration and ultraviolet radiation. Furthermore it's responsible for the oxidation of reactive form aldehydes which is carcinogenic to their corresponding non-reactive form carboxylic acids[66-70].

In our study there was a loss of ALDH1 with age, older patients had lower ALDH1 expression which may be correlated with its biological role as a protective enzyme. The loss of ALDH1 with normal process of aging may decrease the protection against carcinogenic agents also the decreased capacity of DNA repair genes may facilitate carcinogenesis.

As a result, the loss of ALDH1 maybe an important step toward SG malignancy and it might be a CSC marker for malignant salivary gland tumors. This evidence, which may lead to better insight of biologic background of SG cancer, might bring new treatment modalities.

In our study we noticed a higher expression of ALDH1 in low grade tumors, which may be explained by the protective role of ALDH1. In other words loss of ALDH1 may couse more agresive and higher grade tumors.

In our study we noticed a slight increase in ALDH expression in metastasizing/ recurrent tumors. ALDH may play a role in tumor progression and metastatic process, however further studies are needed with larger samples to confirm the role of ALDH in metastasis.

SUN and colleagues indicated that $ALDH^{high}$ cells infected with luciferase vectors showed increased ability to metastasize when compared to $ALDH^{low}$ cells [77]. Zhou and colleagues who investigated ALDH in adenoid cystic carcinomas suggested that beside CSC, other unkwon factors may play an important role in tumorigenesis and metastasis [117].

When we analysed ALDH1 expression on the basis of tumour types we noticed an important difference in the expression of ALDH1 in Ad CC which was statistically significant (P 0.000). Same results was also noted for BCA (P 0.026). Both tumors showed lack of ALDH1.

This data was interesting as both tumors have basal cell component. Further studies might be planned to elucidate the effects of ALDH1 on basal cells of SG.

The lack of ALDH1 in Ad CC and BCA could be important in differential diagnosis of these tumors.Thus further studies should be carried to elucidate the value of ALDH1 as a diagnostic marker.

CD166 is a transmembrane glycoprotein (called activated leukocyte cell adhesion molecule, ALCAM) of immunoglobulin superfamily of adhesion molecules and also transduce signals to intracellular signalling pathway. It mediates heterophilic and hemophilic cell–cell interactions. It also regulates n-cadherin. ALCAM plays role in the development of different tissues during embryogenesis and in adults [88,111]. The CD166 expression has been detected in subgroup of cells which are involved in migration and dynamic growth; it has been discovered in cancer stem cells as well [120].

Since its discovery CD166 expression has been regarded as a cause of tumour progression and metastasis in a subset of tumors, such as cutaneous melanoma [121] prostate carcinoma [120,122], breast cancer [123], bladder cancer [124]. However previous studies addressing ALCAM's role in cancer have yielded conflicting results. Existing reports are paradoxical, with ALCAM gene expression being highly up regulated in some cancers and greatly down regulated in others [120]. Furthermore depending on the tumor cell type, ALCAM expression has been reported to be both positively and negatively correlated with cancer progression and metastasis in the literature [85, 86].

Van Kempen et al. stated that there is an increased expression of CD166 in vertical phase growth in melanoma [121]. Another study by Kristiansen G et al demonstrated that there is up regulated CD166 expression in prostate cancer compared with benign prostatic hyperplasia and normal epithelia [122].

In breast cancer King et al. stated that reduced expression of CD166 is associated with poor prognosis (nodal involvement, higher grade, higher TNM stage) and clinical outcome (local recurrence and death) [123]. On the other hand Burkhardt [125] suggested that strong cytoplasmic ALCAM expression in primary breast cancer might be a new marker for a more aggressive breast cancer. Weichert reported that ALCAM was frequently up regulated in colorectal cancer compared to normal colonic mucosa. Up regulation was an early event in

malignant transformation and membranous ALCAM/CD166 expression correlated with shortened patient survival. So he concluded that it might be a new independent prognostic marker for colorectal cancer [111].

Verma et al stated that overexpression of ALCAM/CD166 is associated with poor prognosis in esophageal squamous cell carcinoma: (late clinical stage, enhanced tumour invasiveness, and nodal metastasis [126]. In another study on oral squamous cell carcinoma it was found that membranous ALCAM expression at the invasive front serves as a molecular marker for lymphatic metastasis [127].

Another study by Tachezy et al suggested the importance of CD166 as a prognostic biomarker for pancreatic neuroendocrine tumour patients. ALCAM was abundantly expressed in these tumors and decreased expression was significantly associated with poor prognosis [128].

However there is only one published literature concerning CD166 expression in salivary gland tumours [88]. In that study they found that CD166 expression in malignant salivary gland tumours (MEC and AD CC) was significantly higher than that of benign salivary gland tumors (PA), and higher in PA than normal salivary gland. They also found that CD166 expression was significantly higher in high grade tumors compared to low grade tumors.

In our study the highest CD166 expression was by malignant tumors followed in descending order by benign tumors and normal salivary gland tissue (P0.002). Additionally, although it wasn't statistically significant there was a prominent decrease in the expression of CD166 in the metastasizing/rec and high grade tumors in contrast to non-metastasizing/ non-rec and low grade tumors.

Higher CD166 expression in malignant tumors could be explained by the fact that CD166 has signalling role in proteolysis and can enhance MMP 2 activity and the breakdown of the extracellular matrix resulting in increased tumor invasiveness and progression [124-128]. There are conflicting results regarding ALCAM expression and tumors biological behaviour. Yet studies pointed out that CD166 mostly up regulates in the early malignancy, and down regulates in advanced malignancy and metastasis [124-126]. This is correlated with our data. In SG malignancy, the increase of CD166 might be an important step in the early stages of tumors while for tumor progression and metastsis, the loss of CD166 adhesion molecule

could be a critical step as it facilitates detachment of the invading cells from their contacts and extracellular matrix.

Despite many researchs and gathering information, the ambiguity of cancer stem cells persists. Further studies are needed to determine specific markers for spesific tumor types as it appear to have high discrepancy even among the same tumor type. As it might be expected, SGT which originate from various cell types with diverse histopathological features may also have disrepancy in CSC markers expression.

To define CSCs and their functions is crucial as it will lead to better understanding of biology of tumors and to establish more effective treatment modalities. Intensive studies on large series may help to comprise a concensus on CSCs and to determine potential prognostic CSC markers.

6. CONCLUSION

Malignant salivary gland tumors showed statistically significant down regulated ALDH1 expression in comparison to normal salivary gland tissue (P 0.034). A decreased ALDH expression was noted in high grade tumors. This data suggested that loss of ALDH1 maybe an important step toward SG malignancy and it might be a CSC marker for malignant salivary gland tumors.

The lack of ALDH1 expression in adenoid cystic carcinomas (P 0.000) and basal call adenomas in comparision to other tumors (P 0.026) were statistically significant. ALDH1 might be a potential diagnostic marker in differential diagnosis of these tumors.

Malignant SG tumors displayed statistically significant up regulated CD166 expression (P 0.002) which gave rise to thought that its expression may have role in tumorogenesis and might be a CSC marker for malignant salivary gland tumors.

Loss of CD166 was determined both in metastasising/recurrent and high grade tumors in comparison to non-metastisizin/non-recurrent and low grade ones. This result is concordant with the literature knowledge that CD166 mostly up regulates in the early malignancy, and down regulates in advanced malignancy and metastasis.

Diminishing CD44 expression was noted in benign and malignant tumors in descending order while metastisizing/recurrent tumor had higher CD44 expression in comparison to non-metastasizing/ non-recurrent tumors. We suggested that loss of CD44 may be important step toward early SG malignancy, whereas increase of this molecule in the course of tumor progression may facilitate metastasis.

Acinic cell carcinoma cells, which mostly demonstrate serous acinar differentiation that show CD44 expression, were devoid of CD44. This data suggested that loss of CD44 molecule may play role in carcinogenesis of Ac CC and hence it might be a candidate for diagnostic marker. Advanced researches on large series should be carried out to elucidate this suggestion

Malign SGT displayed lower CD24 expression in comparison to benign tumors whereas metastasizing/recurrent tumors showed higher CD24 expression. This data supported the suggestion that CD24 expression could be associated with tumor progression and metastasis.

Down regulation of ALDH expression by age showed statistical significance (P 0.007). Loss of ALDH1 by aging may play an important biological role in malignancy.

The presented thesis pointed out that ALDH1, CD166, CD44 and CD24 are potential CSC markers for SGT. Besides they might have role in tumor initiations as well as progression and metastasis. Discrepancies in results particularly regarding metastasizing/recurrent tumors could be due to low sample number. Advanced studies on large number of cases may help to comprise a consensus on CSCs and to determine potential prognostic CSC markers.

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1) **Metastasis from Lung Cancer Presenting as Pyogenic Granuloma in the Lower Gingiva Case Report.** ALAA M. SHUİBAT, EMRE BARIŞ, BEGÜM KARAN, TARKAN YETİŞYİĞİT. **Acta Stomatol Croat 2012:46:(4):307-311** (International Journal Of Oral Science And Dental Medicine)
2) **Rare actinomycosis limited to the bone of the mandible in a child: Case report**. BENAY YILDRIM. ALAA M. SHUIBAT. İndian J Dent Adv 2013 5(1):1147-1149.
3) **Sol maksiller sinüste izole *Aspergillus* enfeksiyonu** Yıldırım B, Shuibat AM. Olgu bildirimi. *ActaOdontol Turc* 2014;31(2):99-101.

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GAZİ GELECEKTİR..