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ABSTRACT

CD44 is a cell adhesion molecule that functions in cell proliferation, differentiation, migration and angiogenesis, as well as in signaling for cell survival. CD44 acts also as the main hyaluronan receptor. CD44 has been implicated to have a role in carcinogenesis.

Aim of the study: In this study the expression of CD44 in mucoepidermoid carcinoma was detected and correlated with the histopathological grade.

Material and methods: peroxidase-antiperoxidase immunohistochemical technique for the detection of CD44 expression in mucoepidermoid carcinoma.

Results: There was no statistically significant difference between the expression of CD44 in high and low grade mucoepidermoid carcinoma. Yet, the pattern of expression varied in both.

Conclusion: CD44 pattern of expression correlates with mucoepidermoid carcinoma histopathological grade. Correlates with increased proliferative activity and presumably increased cellular motility.

Summary: From this study it was deducted that over-expression of CD44 correlates with increased proliferative activity and presumably increased cellular motility.

1. Introduction

Cell adhesion molecules (CAMs), were thought to function only in attaching cells to the extracellular matrix components. Now however, this concept has changed, they are now recognized as having broader functions through their role in signal transduction [1]. CD44 is a CAM that is involved in cell-cell and cell-matrix signaling and adhesion [2,3]. CD44 is a single-chain molecule that is made up of three main parts extracellular, intracellular and transmembrane domains [4].

CD44 is associated with lipid rafts (glycolipid enriched membrane microdomains, GEMs), through the transmembrane domain. This association is cell type and cell activation state dependant [5]. The intracellular part (cytoplasmic tail) is linked with the cytoskeleton via ankyrin and ERM proteins and acts in signal transduction through its association with the Src phosphokinases. The cytoplasmic tail binds Merlin when the cell is in its' "quicent" state [5,6].

CD44 is expressed in normal basal epithelium of the skin and mucous membrane and in normal glandular epithelium, as well as, in tumors of these epithelial structures. CD44 is found in myoepithelium of normal salivary glands which may argue in favor of the role of these molecules in the regulation of growth and renewal under physiological conditions [7,8]. Researchers, also suggest that the binding of CD44 to HA is involved in cell guidance, as it was observed experimentally, that cells establish lamellipodia directed towards HA application [9]. Observations also point to a role for CD44 in inflammation, through phagocytosis and clearance of apoptotic cells and microbial pathogens by macrophages [10].

Added to its role in inflammation, CD44 was also found to have an important role in carcinogenesis under selective conditions [1,11]. Carcinogenesis involves abnormalities of cell adhesion, proliferation and angiogenesis [12].

CD44 may take part in carcinogenesis through its role in signal transduction which may be direct through binding to ligands or indirect by acting as a co-receptor [13]. CD44 acts as a co-receptor by binding to

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HA and then forming complexes with HER2 and c-Src kinase which triggers "cross-talk" between signaling pathways, during tumor development [14,15]. HER2 oncogene expression correlates with poor prognosis in many cancers [16].

At the same time, Cytoplasmic domains of CD44 have been shown to be involved in the recruitment of Src kinases `oncogenic proteins' family (Hck, Lyn, Fyn and Lck) in some tumors [17–19]. CD44 is also associated with the Rho members of the Ras superfamily. The overexpression of Rho GTPases is associated with poor patient outcome in several tumors. Their activity allows the formation of lamellopodia through the cleavage of CD44 and hence cellular motility [20,21]. CD44 cytoplasmic tail does not have an intrinsic receptor kinase or phosphatase activity, of its own. Nevertheless, it still acts as a docking site for growth factors [13].

Binding of HA to CD44 receptors, initiates extracellular clustering of CD44, in migrating. The clustering of the CD44 molecule may result in the activation of Ras which leads to tumor growth [13,19]. The cytoplasmic tail of CD44 binds to Ankyrin, which mediates HA dependant cell adhesion and motility [1,22]. Between the transmembrane region and the Ankyrin binding site on the cytoplasmic tail of CD44 lies a site for binding the ERM proteins which is the same binding site for the tumor suppressor protein Merlin (an ERM like protein) [23].

CD44-ERM association cross-links the CD44 molecule to the actin cytoskeleton, which is vital for the maintaining cell shape and regulation of cell movement [5]. In that sense, researchers suggest that CD44 can act as a metastases promoting factor on binding of the cytoplasmic tail to ERM and as a tumor suppressor on binding to Merlin [24]by negatively regulating CD44 function [25] (Table 1).

Merlin is activated in the hypophosphorylated state, it acts as a tumor suppressor protein. Several theories were proposed as to the cause of Merlin inactivation [6]. The first theory, anticipated that at low cellular densities Merlin allows for cell proliferation while at high cell densities it depresses proliferation due to the effect of contact inhibition between cells. This contact inhibition of growth is lost in transformed cells (may be due to the presence of a HA coat enclosing the cells or the messed up signals from the tumor cells) [23].

The second theory presented that CD44 and Merlin cause cell cycle arrest, when the concentration of high molecular weight HA is elevated. While, upon degradation of this HA, the tumor benefits in two ways. The reduced concentrations of high-molecular-weight HA allows tumor cells to escape from CD44-Merlin mediated cell-cycle arrest. Simultaneously, the degradation products of HA, alter the tumor microenvironment, like for example the upregulation of MMPs. MMPs in turn cause the cleavage of the CD44, allowing for increased cell proliferation [26]. CD44 and HA are interconnected, one allowing the cleavage of the other [27,28]. The third theory for Merlin inactivation is through the sequestration of growth factors via CD44, which inhibit Merlin dephosphorylation and its tumor suppressive, anti-proliferative activity [6].

Ahrens, T., et al., 2001 [29] stated that cleavage of CD44 from the cell surface is mediated by proteases and metalloproteinases. This divides the CD44 molecule into fragments, one fragment remains in the cell membrane and the other is soluble and is released from the cell membrane (sol CD44). Further proteolysis of the retained part liberates a CD44 intracellular domain fragment (ICD) [30]. The mobilization of the CD44-ICD to the nucleus upregulate its own expression and other

molecules like integrin. Integrins in turn are involved in the regulation of cell adhesion, proliferation and metastases [31].

The CD44 molecule has binding sites for growth factors and matrix metalloproteinases (MMP). It has been observed that MMP binding to CD44 resulted in degradation of collagen type IV thus mediating cellular migration and activation of TGF-β, which caused neovasculization. Activation of MMP was also dependant upon binding of HA to CD44 [1].

Thus, through the interaction of CD44 with several molecules it imparts features to the tumor cell that may allow it to proceed through all steps of the metastasis [5]. Nevertheless, it should be noted that, binding of HA to the CD44 does not always activate a signaling cascade in the same manner, but this activation differs from cell type to cell type [28].

Native high molecular weight HA is anti-angiogenic while, Low molecular weight fragments of HA stimulate CD44-mediated angiogeneis in-vitro and in-vivo [28,32]. Reports have led to the assumption that, not only does HA and/or its degradation products induce angiogenesis [33,34] but it may also be involved in CD44 cleavage that enhances tumor motility [13,33]. HA is a ubiquitous ECM component The salivary glands like other tissues are made up of cells and extracellular matrix [32,34].

Mucoepidermoid carcinoma (MEC) is a common malignant salivary gland tumor [35,36]. MEC is classified either into low- and high-grade types by some authors or into high, low and intermediate grades by others. Prognosis varies according to the clinical stage, histological grade and sufficiency of the performed surgery [35–37].

Histopathologically, low grade MEC (MEC-LG) exhibit cystic areas containing mucinous material, as well as, numerous mucous cells. High grade MEC (MEC-HG) appear to be less cystic and with a more infiltrative pattern of growth. In MEC-HG the cells are mainly of the intermediate or epidermoid types. These cells show pleomorphism and increased mitotic activity. In the intermediate grade the intermediate cells predominate [38]. Cytokines have been implicated where there is stimulation of tumor growth, For example, TGF-β, is found consistently with MEC-HG [39,40]. In this study the expression of CD44 in MEC was detected and correlated with the histopathological grade.

2. Material and methods

2.1. Case selection

The material of this study consisted of 20 formalin fixed, paraffin embedded specimens of MEC salivary gland tumors. All cases included in the study were collected from the archives of the Pathology Department, National Cancer Institute, Cairo University, Egypt. MEC cases were further categorized into high grade and low grade according to their histological features. The study also included two cases of normal salivary gland tissue.

2.2. Histopathology

The sections were histologically reviewed using hematoxylin and eosin (H&E) stain, used for routine pathological diagnosis, in order to confirm the diagnosis, and to detect the grade of mucoepidermoid carcinomas(Table 2).

2.3. Immunohistochemistry (IHC)

2.3.1. IHC staining

For the detection of CD44s antigen, a monoclonal mouse IG_1, prediluted antiCD44s antibody was used. The antibody was purchased from Visionbiosystems Novocastra™ Laboratories, Ltd, United Kingdom. These antibodies are designed for the specific localization of antigen in formalin fixed paraffin embedded tissue sections. Peroxidase-anti-peroxidase technique was performed for the immunohistochemical

Table 1
Quick review on Merlin and the ERM.

Merlin and ERM
Merlin and ERM are structurally related proteins. ERM proteins binds to actin and is involved in cytoskeletal and membrane remodeling. These proteins localize to the membrane ruffles and microvilli. Merlin acts as a tumor suppressor protein [1].

Table 2
Summary of the MEC grades included in the study.

Lesion	No.	%
MEC-LG	8	40%
MEC-HG	12	60%
Total	20	100%

staining using the ready-to-use universal kit, Visionbiosystems Novocastra ™ Detection System, Purchased from Visionbiosystems Novocastra ™ laboratories,Ltd., United Kingdom.

2.3.2. IHC evaluation

For each MEC case, four fields at a $40\times$ magnification, showing highest immunopositivity were captured using a digital video camera (C5060, Olympus, Japan), mounted on a light microscope (BX60, Olympus, Japan). The captured images were digitally analyzed by the computer, using image analysis software (Image J, 1.41a, NIH, USA).

Phase analysis was calculated automatically to give the area fraction of the positive cells. The area fraction was given by the percentage of immunopositive area per total microscopic field area. Finally, the area fraction for each microscopic field was measured.

2.3.3. Assessment of the results

The collected data was tabulated using Microsoft Excel (Microsoft Office, 2003). For all cases, the area fraction of immunopositivity for at least 4 different microscopic fields was measured. The mean area fraction for each case was then calculated and used for statistical analysis. Also, the hitopathological pattern of expression was carefully evaluated and noted.

2.4. Statistical assessment

All MEC cases of this study were incorporated in the statistical analysis. The data was tabulated using the Statistical Package for Social Science (SPSS 15.0) Software. The statistical tests performed included the independent sample T-test for comparison of means. This test was done for the mean area fraction of immunopositivity to CD44s in MEC-LG and MEC-HG. The results were considered significant when the P value was ≤ 0.05.

Comparison between Mean area fractions of immunopositivity to CD44s in MEC-LG and MEC-HG was performed using analysis of variants test (ANOVA -for more than two groups-). Graphs were performed using Microsoft power point Software (Microsoft Office, 2003).

3. Results

3.1. Light microscopic results

Immunopositivity appeared as brown staining in the cytoplasm or on the plasma membrane or both. The lesions were considered positive when at least one positive tumor cell was detected.

3.1.1. Normal salivary gland tissue

The two stained sections of normal salivary gland tissue showed immunopositivity to CD44s in the cytoplasm and on the plasma membrane of the acinar epithelial cells, the staining was most intense on the basal and the lateral cell membranes in serous (Fig. 1) and mucous acini (Fig. 2). Myoepithelial cells were immunopositive (Fig. 1). The cells lining the ducts were immunonegative.

3.1.2. MEC

All lesions revealed immunopositivity to CD44s. In MEC-LG, some of the cells lining the duct like structures were immunonegative, others were immunopositive (Figs. 3 and 4). The neoplastic cells forming masses in MEC-LG were mostly immunopositive. The positive reaction was membranous in most of the cells with only few cells showing cytoplasmic reaction (Fig. 4). For MEC-HG, all neoplastic cells forming masses exhibited cytoplasmic and membranous immunopositive reaction (Figs. 5–7).

3.2. Image analysis and statistical results

The study showed that the mean area fraction of MEC-LG was 10.5375% and 14.9167% for MEC-HG. The mean area fraction of immunopositive cells in MEC-LG were fewer than the MEC-HG. (Table 3) (Fig. 8).

Using the independent *t*-test for the comparison, it was found that there was no significant difference between the mean area fraction of immunopositivity to CD44s in the MEC- LG and MEC-HG, (p-
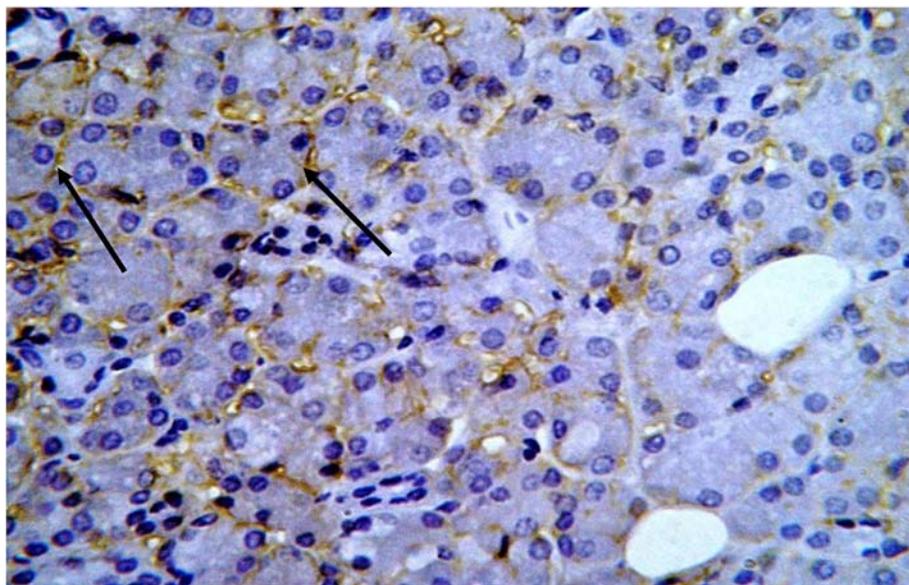
Fig. 1. Photomicrograph of a normal salivary gland showing immunopositivity on the cell membrane of serous acini, also note the immunopositivity of myoepithelial cells (arrows) (CD44s, orig. magx40).
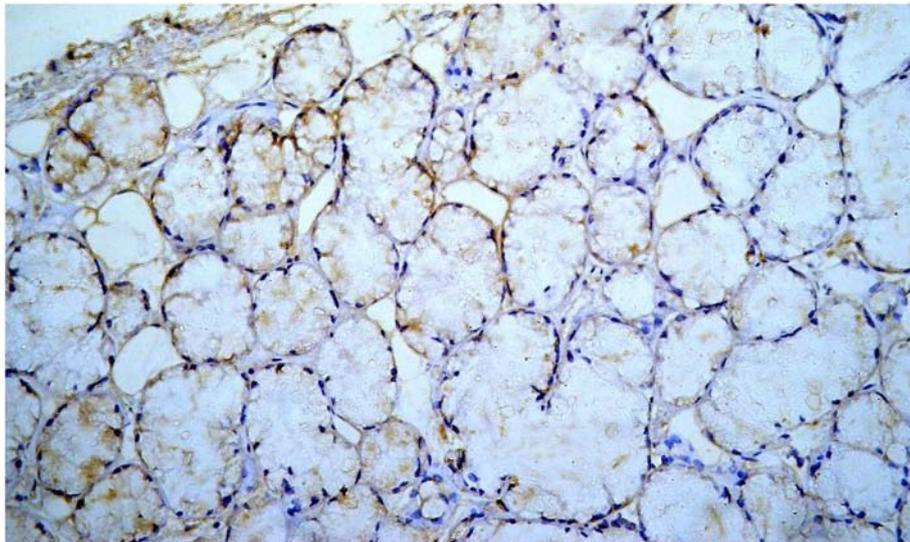
Fig. 2. Photomicrograph of a normal salivary gland showing immunopositivity on the cell membrane of mucous acini (CD44s, orig. magx20).
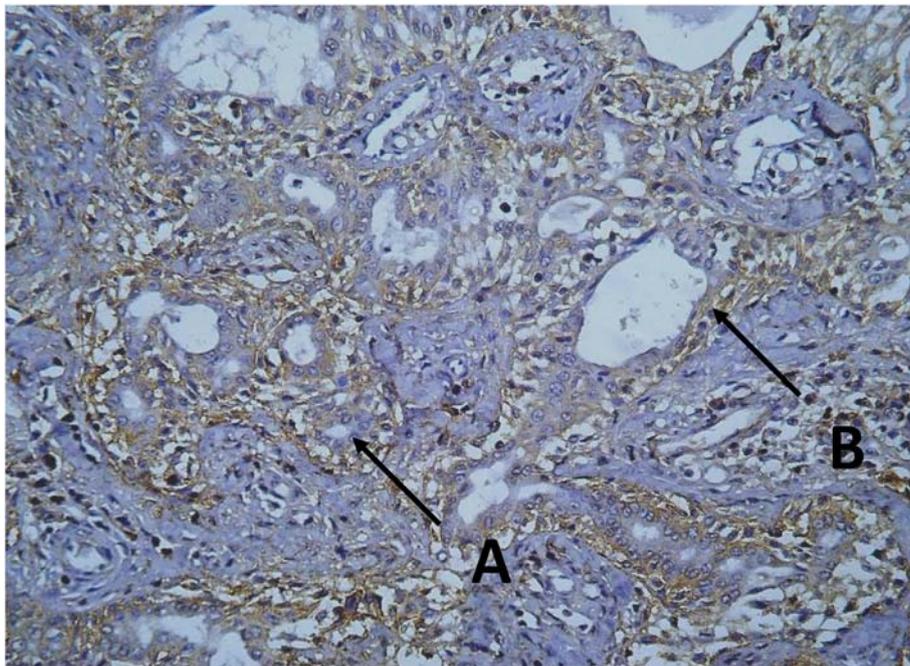

Fig. 3. Photomicrograph of MEC-LG Showing, duct like structures. Most of the inner cells lining the ducts were immunonegative (A), while the outer ones were immunopositive (B). Also, note the positive reaction was mostly membranous (CD44s, orig. mag. x20).

value = 0.49). (Table 4).

4. Discussion

Several studies were performed to detect the expression of CD44 in malignancy. Some researchers found that the increased expression does correlate with increased grade of malignancy [1,3]. While, others found that there was no change in CD44 expression between normal, benign or malignant lesions [41]. In this study, we evaluated the expression of CD44 in MEC as an example of a malignant salivary gland tumor and correlated the expression of CD44 in its low and high grades.

Salivary gland tumors are rare, comprising less than 3% of all neoplasms of the head and neck region. Reports from several parts of the world have shown differences in the incidence of salivary gland tumors [42]. Data collected from the pathology department, National Cancer Institute, Cairo University, showed that MEC was the most common malignant salivary gland tumor during the period from 2004 to 2016. This is why these tumors were used as material for this study.

The results of this study revealed that, in normal salivary glands, CD44 expression was located in the acinar cells and the myoepithelial cells of the ducts. The expression was found to be on the basal and lateral cell membranes of the acinar cells. These results were consistent with the results of Franchi et al. (2001) [43].

It was observed in this study that, the immunopositivity was relatively higher-even though the difference was not significant-in MEC-HG than MEC-LG. A finding that could be explained by the belief that, CD44 may be up-regulated in high grade tumor cells, due to the expression of abnormal or amplified growth factor receptors, such as EGF, TGF-beta and HER2 oncogene, which result in the up-regulation of CD44, especially in the MEC-HG [39].

The histopathological pattern of CD44 expression was different between both tumor grades in this study. In MEC-LG, the expression
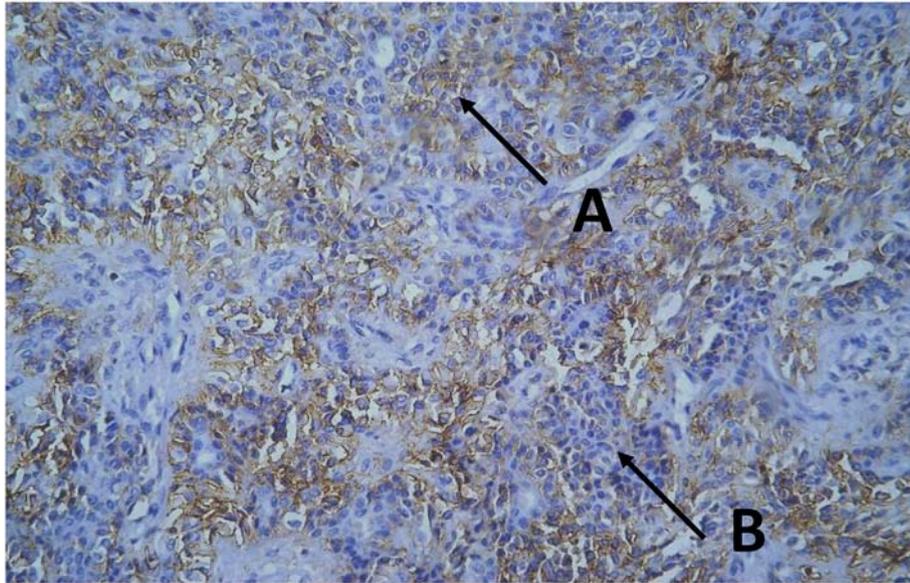
Fig. 4. Photomicrograph of MEC-LG Showing, duct like structures and epidermoid masses. In the masses some epidermoid cells were immunopositive (A) and others were immunonegative (B). The immunopositive reaction was membranous in most of the epidermoid cells. (CD44s, orig. mag. x20).
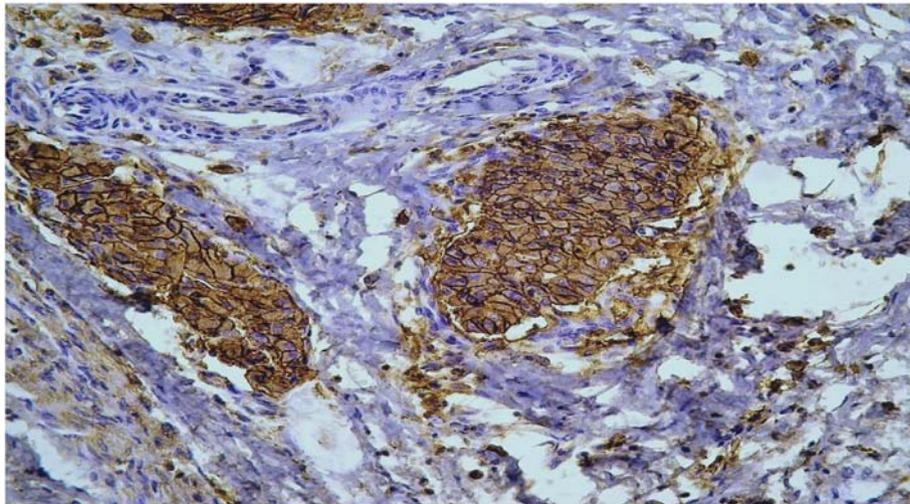
Fig. 5. Photomicrograph of MEC-HG Showing, epidermoid cells forming masses. Note that, all epidermoid cells were immunopositive (CD44s,orig. mag. x20).

was mostly membranous while the MEC-HG exhibited cytoplasmic expression as well. In the MEC-LG the luminal surface of the cells forming the duct like structures were mostly immunonegative and the outer layer showed immunopositivity. The absence of expression of CD44 on the luminal surface of the cells is probably due to the absence of the ligands of the CD44 molecule as CD44 functions in cell to cell and cell-matrix adhesion.

The cytoplasmic expression of CD44 noted in MEC-HG could result from the Proteolytic cleavage of CD44 resulting in the release of a CD44 intracellular domain fragment (ICD). The CD44-ICD translocates to the nucleus and thus giving a positive reaction through out the cytoplasm. Several studies proved that the presence of this ICD fragment correlates with increased proliferation and metastases and hence the cytoplasmic expression increases with increased grade of malignancy in salivary gland tumors [25,31,32].

The tumors studied showed masses of epithelial cells in MEC-HG. CD44 increases the chances of clustering of cells, this is in agreement with Ponta et al., 2003 [1]. Sugahara et al., 2003, also stated that, the over expression of CD44 by tumor cells enhances the homotypic aggregates of tumor cells which in turn results in stabilizing receptors and

not allowing ligands that negatively regulate kinase activity to attach to the receptors [33]. CD44-HA interactions result in cell matrix cross bridging and hence mediating cell aggregation. Also, up-regulation of CD44 expression on the cells forming masses may be due to the role of CD44 in signal transduction participating in increased proliferation and mass formation. This is through it's association with c-erbB2, c-Src and Ras and by acting as a harboring site for growth factors [17,19,21].

Another explanation for the increased mass formation with increased CD44 expression is through the inactivation of Merlin (a tumor suppressor protein that is associated with CD44) causing loss of its growth inhibitory function [25,26]. The inactivation of Merlin may be through; loss of contact inhibition between cells, presence of low molecular weight HA and/or sequestration of growth factors via the CD44 molecule [27,29]. Besides its role in tumor growth and proliferation CD44 may be involved in the enhancement of cell survival. The increased cell survival may be due to the presence of a HA coat which protects the cells from immune surveillance and thus allowing them to survive longer [28,30].

Ligand binding to CD44, is not a passive task, but on the contrary, it initiates a cascade of events through CD44 activation. When CD44 is
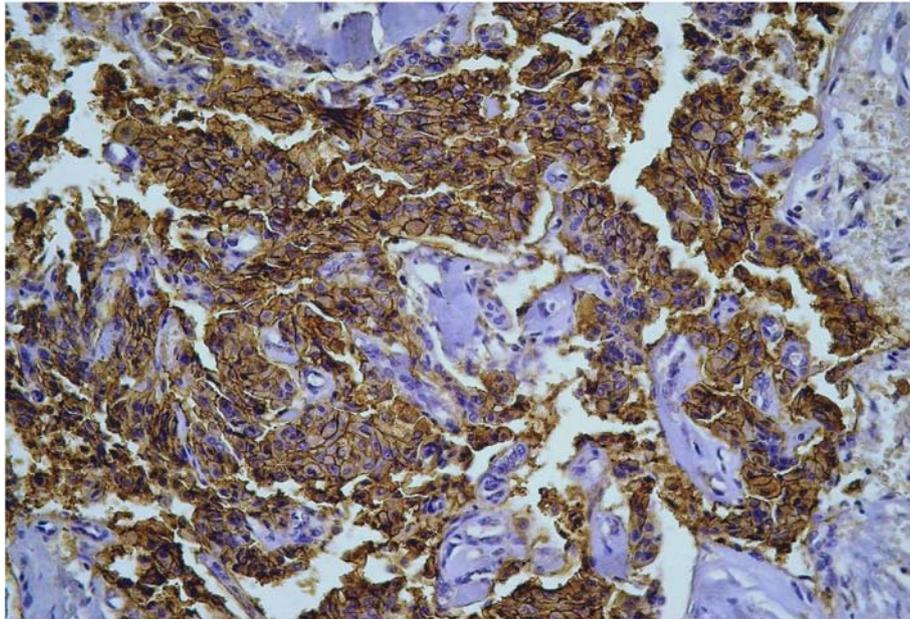
Fig. 6. Photomicrograph of MEC-HG, note immunopositivity in the cytoplasm and the plasma membrane. (CD44s,orig. mag. x20).
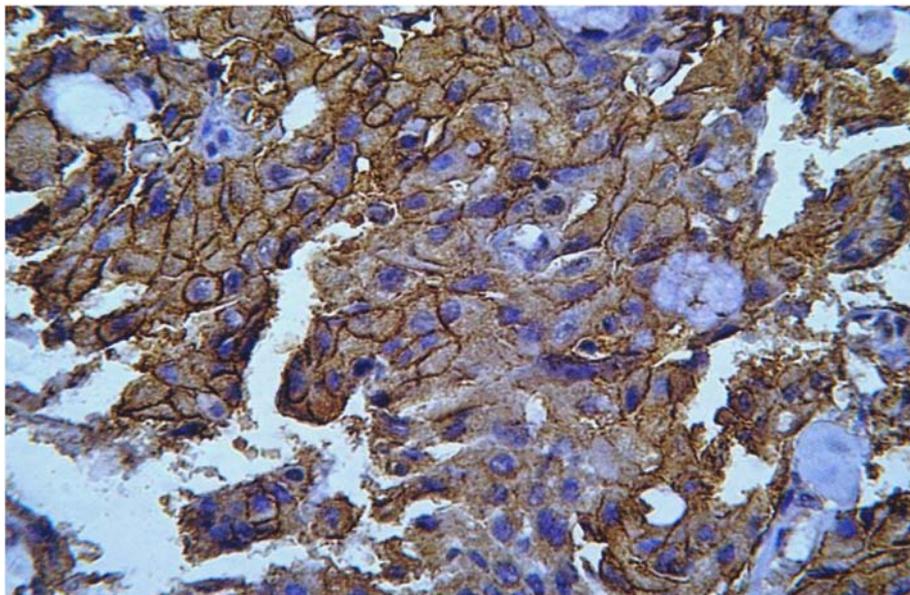

Fig. 7. Higher magnification of the previous photomicrograph of MEC-HG Showing, immunopositive epidermoid cells, the reaction was in the cytoplasm and the plasma membrane. (CD44s,orig.mag. x40).

Table 3
Descriptive analysis of immunopositive mean area fraction with CD44 among the studied cases.

Tumor	Frequency	Mean area fraction	± S.D
MEC –LG	8	10.538	4.3025
MEC –HG	12	14.917	4.696

activated it binds to more ligands like growth factors, MMP and elements of the cytoskeleton. Thus, the interaction of CD44 with its ligands may well allow a tumor cell form metastases [13,16,19,21,32].

The presence of MMP in the tumor environment has a crucial role in modulating CD44 function as a cell adhesion molecule. One assumption is that, MMP cleaves CD44 molecule, so the increased expression detected in this study may be due to, the accumulation of cleavage products of the molecule between and with in cells as opposed to the presence of the whole molecule so, in spite of the increased expression there is loss rather than gain of adhesion [29–31]. Another assumption is that, MMP bound to CD44 is protected from its inhibitors and thus is allowed to cleave extra-cellular matrix components and consequently allowing migration of tumor cells [1,30,31]. On top of that, MMP activate angiogenic factors, e.g., the proform of TGF-B, leading to neo-angiogenesis [1,5]. Hence, the higher expression of CD44 in high grade MEC signifies increased cellular motility.

CD44 localization is an additional important factor to consider, not just the mere increase in expression. Tzircotis et al., 2005 [17], Cherukuri, et al., 2001 [18] and Bourguignon et al., 2001 [19]. Researchers [5,25], have demonstrated before that the association of CD44 with lipid rafts and/or the actin cytoskeleton, may affect lateral movement of CD44 proteins on plasma membrane that are important in altering cell to cell and cell matrix adhesion.
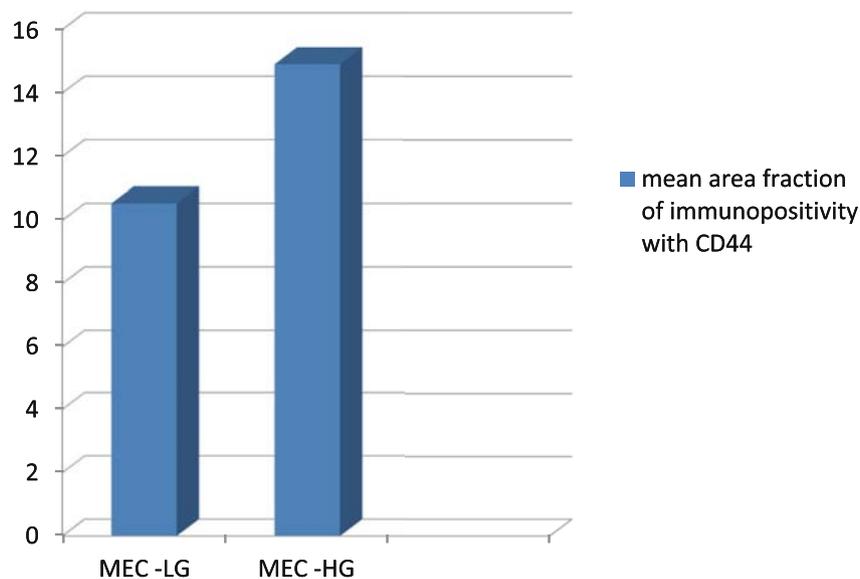
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Fig. 8. Histogram showing immunopositive mean area fraction with CD44 among the studied cases.

Table 4

Comparison between the studied salivary gland tumors regarding mean area fraction of immunopositivity with CD44 using independent student t-test.

Groups		t- test	p- value
MEC-LG	MEC-HG	2.11	0.49

Significane: P ≤ 0.05.

In many cancers an upregulation of CD44 expression is not always associated with bad prognosis. On the contrary, its upregulation in some tumors is associated with a better prognsis. Not just that, but in many cases different research groups analyzing the same type of tumor came to contradicting conclusions as regards to CD44 expression and disease prognosis [8]. These differences in results may possibly be due to differences in methodologies. Regardless of the reason behind these differences they must be resolved before applying anti-CD44 targeted therapy for human cancers safely.

5. Conclusions

CD44 pattern of expression correlates with MEC histopathological grade.

Conflicts of interest

We declare that there is no conflict of interest with any financial institute regarding the material discussed in the manuscript.

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