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RESEARCH ARTICLE

IMMUNOHISTOCHEMICAL EXPRESSION OF CD44 AND CD56 IN MALIGNANT SALIVARY GLAND TUMORS

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Abstract

Introduction: Salivary gland cancers impose a huge part in oral and maxillofacial pathologies. Mucoepidermoid (MEC) and adenoid cystic carcinomas (ACC) are salivary malignancies, where (MEC) being the most common cancer. CD44, is a family of transmembrane non-kinase cell surface glycoproteins, that was broadly used as a marker for cancer stem cells (CSCs) in various malignancies. CD44 was found to be overexpressed in various types of cells including cancer stem cells (CSCs) and it has been postulated that CD44 has a part in carcinogenesis. Natural killer (NK) cells are one of the components of the innate immune system, which express the phenotypic marker CD56 in the absence of CD3, and has a vital role in tumor-cell surveillance. This study aimed to assess the expression of CD44 and CD56 in different grades of mucoepidermoid carcinomas and different patterns of adenoid cystic carcinoma, and correlate between the expressions of the markers (CD44 and CD56) in different grades and patterns of the tumors.

Material &Methods: Thirty-three paraffin embedded blocks of salivary gland tumors (18 mucoepidermoid carcinoma and 15 adenoid cystic carcinoma) were selected. Those tumorswere classified into four groups: intermediate grade (IG) MEC, high grade (HG) MEC, ACC (tub-crib) and ACC (solid) patterns.Immunohistochemical staining for CD44 and CD56 was performed. Data were analyzed using SPSS (24), the independent-T test and Anova to analyze the statistical significance.

Results: for CD44**,** inMEC group, we found no significant difference between both grades for CD44 expression, however, the difference was found to be significant between both patterns of ACC. While for CD56, the difference was statistically significant between both grades of MEC, and both patterns of ACC.

Conclusion:CD44 could be promising goal for cancer therapy, mostly for CD44 expressing cancers. NK cells have a crucial role in the anti-tumor immune mechanism. Moreover, more studies are significantly demanded to assess the promising role and function of CD56 in diagnosis and prognosis of salivary gland cancers, and the tumor-cell surveillance capacity of the NK cells.

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Introduction:-

Salivary gland tumors are one of the most important oral and maxillofacial pathologies, they are uncommon but they are not rare.The salivary gland tumors annual incidence ranges from 1 to 6.5 per 10,000 people worldwide.They account for 2% to 4% of all head and neck tumors around the world[36]. They appear among adults from the fourth to seventh decade of life, where benign tumors occur more often than malignant ones[23].

The most frequent malignant salivary gland tumors are the mucoepidermoid carcinoma (MEC), adenoid cystic carcinoma (ACC) and acinic cell carcinoma[36]. Of these tumors, MEC is the commonest salivary gland tumor affecting middle aged patients from 35 to 65 years of age, however, it appears to be the most common salivary cancer of childhood.Relying on the number of different cells (epidermoid cells, intermediate cells and mucus cells), grading of malignancy can be done and has shown clinical rapport[3]. As the tumor can setback unheeding to histological appearance, the tumor has 5-year survival rate about 70%. There is geographic variation in the tumor frequency as the difference in frequency found between the United States and Great Britain[36].

ACC is recognized as a mixture of epithelial and myoepithelial cells, which is arranged in different patterns: cribriform, tubular, and solid.Generally, an integration of these patterns is found, and the tumors are categorized according to the predominant structure. The cribriform pattern represents the most well-known pattern[36]. This pattern is characterized by slow-growing behavior and infiltrative pattern and has the tendency to invade into nerves and this is why it is accompanied by pain in some patients[18]. According to the literature, lungs, bones, brain, and liver metastatis may occur[42], while regional lymph nodes metastasis is uncommon[36].

CD44 is one of the members of the non-kinase, single span transmembrane glycoproteins family, it is detected in bone marrow, embryonic stem cells and connective tissue by various degrees [11,19]. It is also detected in a population of cancer stem cells (CSCs) and so it is considered as marker of CSCs [56]. The hyaluronic acid (HA) is an abundant component of the extracellular matrix (ECM) expressed by cancer and stromal cells and it is the main ligand for CD44 [4]. It binds the CD44 ligand binding domain which permits fixing of proteins to intracellular domains which stimulate many signaling pathways causing cell adhesion, invasion, proliferation and migration[39,58].

When the Cancer cells begin the epithelial to mesenchymal transition (EMT) they acquire the properties of stem cells and increase in CD44 expression [31]. This cancer cells that begin the EMT become more invasive and become irresponsive to chemotherapy [57]. The impact of CD44 in promoting tumorigenesis promise for its molecular targeting in cancer therapy [27]. Also, CD44 is an important prognostic marker due to its ability to maintain the stemness of the cells, and due to the role of CSCs in tumor regression after therapy.

The hypothesis of CSCs is that the developing neoplasm is caused by stem cells and another type of cancer cells having properties of stem cells, these cells cause tumor initiation, progression,metastasis, and recurrence[7]. CD44 is one of the stem cell markers, and it is documented that it is overexpressed in many neoplasms[9,26].

Natural killer (NK) cells are innate immune effectors, their role is the clearance of virally infected cells or tumorigenic cells and also it modulates the immune responses.NK cells are about 10% of the circulating lymphocytes and is termed among lymphocytes as CD56+ CD3⁻ cells (T-cell marker). There are two subsets of CD56 cells which are $CD56^{dim}$and $CD56^{bright}$, the $CD56^{dim}$ cells are the most abundant subgroup in peripheral circulation while $CD56^{bright}$is the minority of the circulating NK cell populace. Both subsets have special expression of cell surface receptors that correlate to their specific phenotypic and functional capabilities [6,15].

CD56 is also called neural cell adhesion molecule (NCAM). It is sufficiently detected in cells coming from neural source, and similarly expressed in additional tissues comprising the heart, kidney, muscles, liver and on hematopoietic-derived cells as human natural killer (NK), dendritic cells and NKT cells [17]. NCAM is a member of the cell adhesion molecule family, it has multiple isoforms due to RNA splicing [48].

Owing to its discovery site, CD56 is considered a marker of neural lineage commitment[30]. But in hematopoietic system, CD56 expression is not limited to, natural killer (NK) cells[8]. On other lymphoid cells, it is detected as activated CD8+ T cells and gamma delta (γδ) T cells, and on dendritic cells (DCs) [25,43,53]. Abnormal CD56 expression is detected in some hematological cancers [37,54] as well as solid tumors [20,40,51].

Our aim was to study the expression of CD44 and CD56 in different grades of mucoepidermoid carcinomas and different patterns of adenoid cystic carcinoma, and correlate between the expressions of the markers (CD44 and CD56) in different grades and patterns of the tumors.

Material &Methods:-

Tissue Samples

The studied cases were selected from the archives of the General Pathology Department, Faculty of Medicine, Cairo University. Tumors diagnosed as mucoepidermoid carcinoma, and adenoid cystic carcinoma were selected for inclusion in the study. This work was approved by the Medical Research Ethics committee, NRC, registration number 19317.

Histopathology

All available hematoxylin and eosin (H and E) slides were re-examined to confirm the diagnosis and to select representative tissue blocks. Thirty-three tumors were selected based on the availability and adequacy of tissues and are as follows: mucoepidermoidcarcinomasranging from intermediate (IG) to high grade (HG), and adenoid cystic carcinomas with different patterns, solid and tubular-cribriform patterns.In addition, 2 cases of normal salivary gland tissues were included in the study as control.

Immunohistochemical Analysis

Consecutive slides from paraffin-embedded tissue blocks were cut by microtome into sections of 4 µm thickness, and were mounted on positively charged glass slides (Opti-Plus, BioGenex Laboratory, USA) to give better adhesion between the tissue sections and the slides.

Then the sections were dewaxed and labeled for the following commercially available markers, mouse anti CD44 monoclonal antihuman antibody (clone BH0185, SunLong Biotech Co., LTD, China) and mouse anti CD56 monoclonal antihuman antibody (clone 123C3, Dako, Agilent Tech. Inc, USA), all of which were ready-to-use using automated stainer (Thermo Scientific, Lab Vision Corporation^{TM}, USA).The immunoreactions were visualized using the ultraViewUniversal DAB (diaminobenzidine) QuantoDetectionKit in an automated autostainer (Thermo Scientific, Lab Vision Corporation^{TM}, USA).

Assessment of the results

The immunostained sections were then examined in the Basic Dental Science Department, Oral and Dental Research Institute, National Research Center using Ordinary Light Microscope to assess the prevalence of immunopositivity of CD44 and CD56 staining in the studied cases.Image Analysis Computer System was used to assess the area percentage of the positive cells. The image analysis was performed using a computer system consisting of color video digital camera (Olympus Soft Imaging Solutions GMBH, Model # LC20, Germany), mounted on a light microscope (Olympus CX 41, Germany)which in turn connected to the computer where the images are viewed using LC micro Imaging Software (Olympus, Germany). The images were then analyzed using Image J, (1.50i, USA) image analysis software.Using the image analysis software, the area fractions of CD44 and CD56 immunoreactivity were evaluated in 4 high power fields (x200) in each slide, and then the mean values (MAF) were calculated.

Statistical assessment

Data were represented by mean and standard deviation (SD) values. The statistical tests performed included independent T-test for comparison of means, ANOVA for analysis of variants and to analyze the statistical significance of the datawhen applicable. $P \leq 0.05$ was considered statistically significant.

Results:-

Light microscopic results

The selected cases comprised 18 cases of MEC and 15 cases of ACC. In MEC, 8 were of intermediate grade and 10 were of high grade. In ACC, based on their histopathologic patterns, 7 cases were of intermediate grade showing cribriform and tubular patterns, and 8 cases were of high grade where most of which showed solid pattern.

Expression of CD44 and CD56 in normal salivary gland tissue

The two cases of the normal salivary tissue showed immunopositivity for CD44 and CD56 mostly on the plasma membrane of the acinarcells with few cytoplasmic staining, the expression was mostly evident on the basal and

lateral cell membranes in the serous and mucous acini. Immunopositivity was also detected in themyoepithelial cells.

Expression of CD44 in MEC and ACC

CD44 was expressed in neoplastic cells of all tumor types. In MEC (IG), the expression was mostly membranous, with few cytoplasmic reactions which was evident in the intermediate, epidermoid and acinarcells. Moreover, the inner cells lining the ducts were immunonegative, while the outer ones showed positive membranous and few cytoplasmic reactions. In MEC (HG), all the cells were showing membranous and cytoplasmic immunopositive reactions. As for the ACC cases, the positive expressions were seen as prominent membranous staining in the acinar and myoepithelial compartments in the solid and tub-crib ACCs (fig.1).

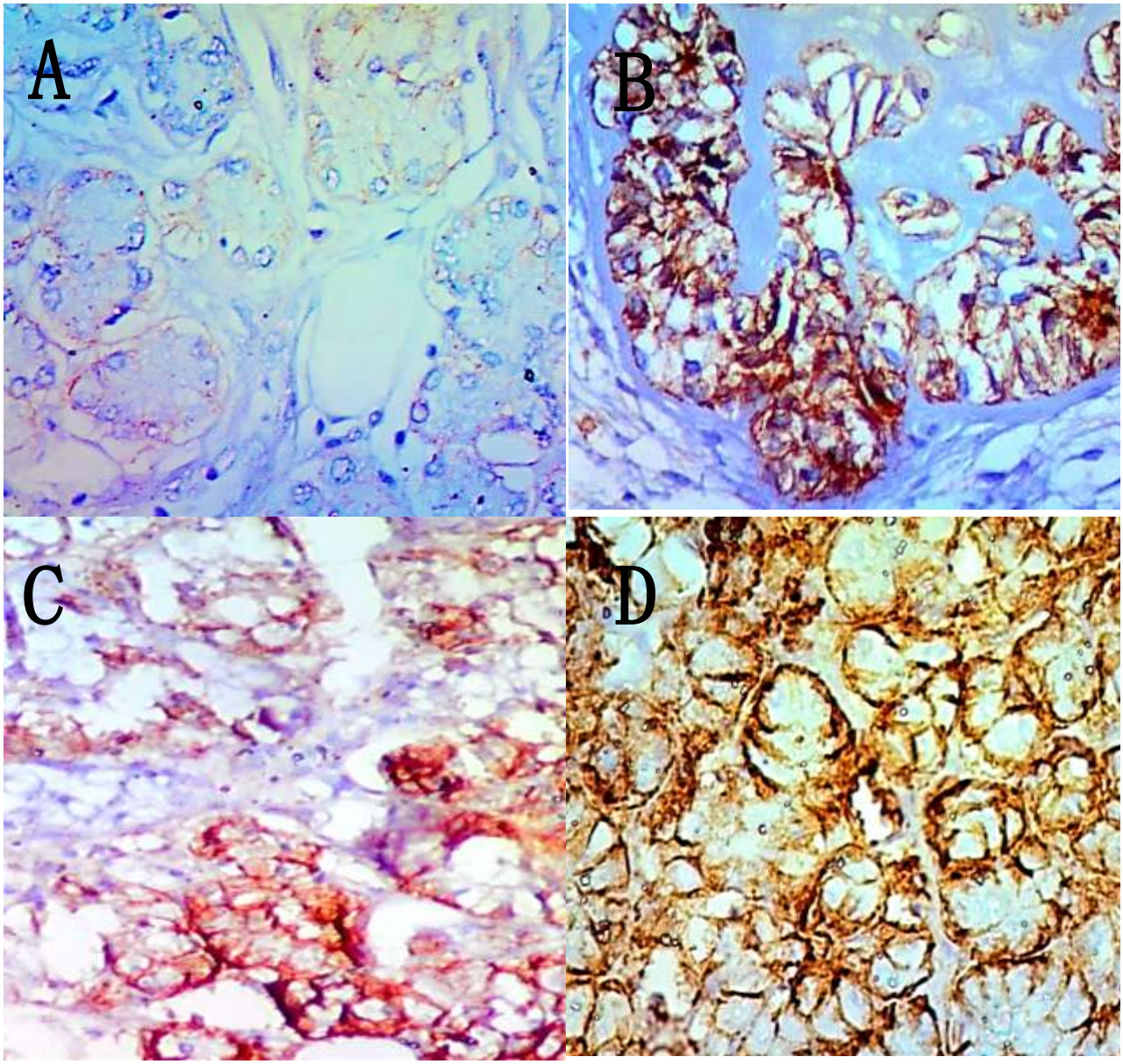

Fig. (1)Immmunohistochemicalstaining of CD44, (A) MEC (IG) showing mostly membranous immunopositivereactionsin most of the acinar cells(CD44, orig.mag.x20), (B) MEC (HG) showing epidermoid masses with positive membranous and cytoplasmic immunoreaction in most of the epidermoid cells(CD44, orig.mag.x20), (C) ACC (tub-crib) showing positive membranous acinarstaining,note the membranous immunopositivity in the myoepithelial cells (orig. mag. X10), (D) ACC (solid) showing positive membranous staining (CD44, orig.mag.x20).

Expression of CD56 in MEC and ACC

CD56 was expressed exclusively cytoplasmic in intermediate and epidermoid cells of intermediate and higher-grade cases of mucoepidermoid carcinoma, moreover, CD56 cytoplasmic staining was detected amongthe histological patterns of ACC (fig.2).

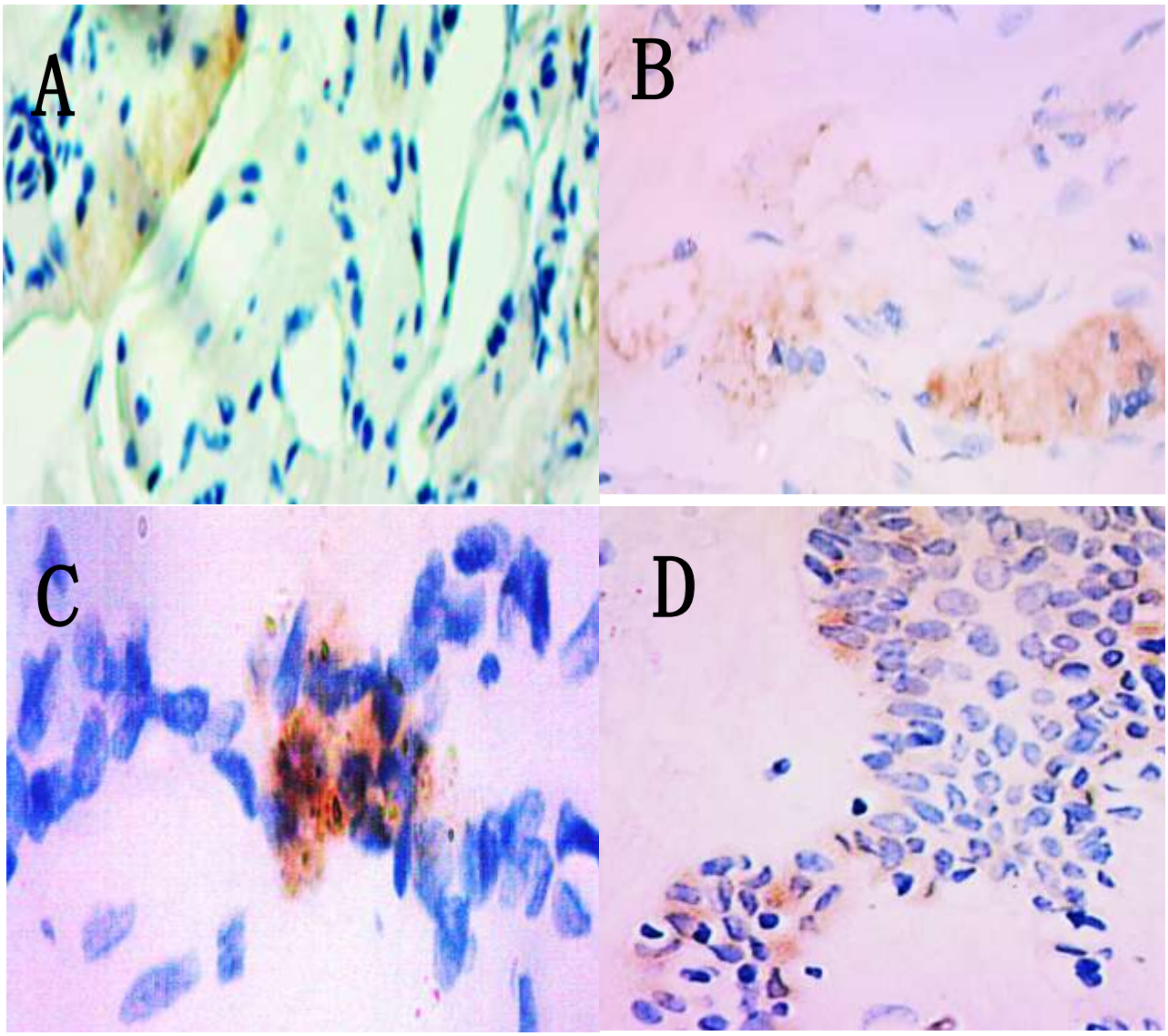

Fig.(2) Immunohistochemical staining of CD56, (A) MEC (IG) showing positive cytoplasmic reaction in the tumor cells (orig.mag.x20), (B) MEC (HG) showing positive cytoplasmic reaction in the tumor cells with high magnification (x40), (C) ACC (tub-crib) showing positive cytoplasmic reactions in the tumor cells (orig.mag.x40), (D) ACC (solid) showing positive cytoplasmic reactions in the tumor cells.(CD56, orig.mag.x20).

Image analysis and statistical results

Expression of CD44 and CD56 were represented as mean (m) and standard deviation (SD) for all groups, MEC and ACC, graphically drawn in figures. The collected records were statistically analyzed by Microsoft Excel ® 2016, Statistical Package for Social Science (SPSS)® Ver. 24. and Minitab ® statistical software Ver. 16.

Regarding MEC group, mean ± standard deviation of CD44 and CD56 expression were (207.25±29.9) and (235.92±7.15) respectively. While for normal group, mean ± standard deviation of CD44 and CD56 expression were (146.7±33.4) and (163.4±29.4) respectively. Using independent t-test for significance assessment of both groups

regarding MEC, there was significant difference between both groups for CD44 and CD56 expression as P-value < 0.05, fig. (3).

Regarding ACC group, mean ± standard deviation of CD44 and CD56 expression were (242.5±17.2) and (259.35±23.8) respectively. While for normal group, mean ± standard deviation of CD44 and CD56 expression were (146.7±27.1) and (163.4±17.2) respectively. Using independent t-test for significance assessment of both groups regarding ACC, there was significant difference between both groups for CD44 and CD56 expression as P-value < 0.05 fig. (3).

Regarding MEC group, it was subdivided into two subgroups as IG and HG for immunohistochemical analysis of CD44 and CD56 expression. For CD44 expression, mean ± standard deviation of IG and HG of MEC were (200.75±51.5) and (213.75±15.2) respectively. Using independent t-test for significance assessment of both grades of MEC group, there was insignificant difference between both grades for CD44 expression as P-value > 0.05, fig. (4).

While for CD56 expression, mean ± standard deviation of intermediate and high grade of MEC were (212.5±6.12) and (259.35±10.9) respectively. Using independent t-test for significance assessment of both grades of MEC group, there was significant difference between both grades for CD56 expression as P-value < 0.05, fig. (4).

Regarding ACC group, it was subdivided into two subgroups as tub-crib and solid pattern for immunohistochemical analysis of CD44 and CD56 expression. For CD44 expression, mean ± standard deviation of tub-crib and solid pattern of ACC were (230 ±14.63) and (255±21.4) respectively. Using independent t-test for significance assessment of both grades of MEC group, there was significant difference between both patterns for CD44 expression as P-value < 0.05, fig. (4).

While for CD56 expression, mean ± standard deviation of tub-crib and solid pattern of ACC were (242.5±37.6) and (276.2±14.1) respectively. Using independent t-test for significance assessment of both patterns of ACC group, there was significant difference between both patterns for CD56 expression as P-value < 0.05, fig. (4).

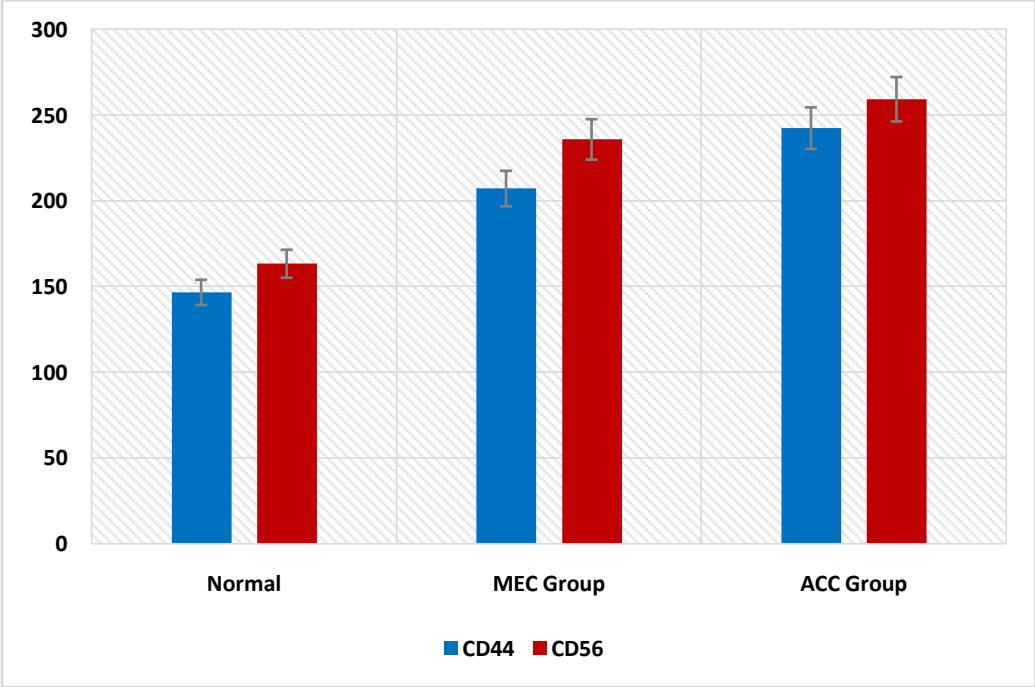

Figure (3):- Expressionof CD44 and CD56 in MEC and ACCimmunohistochemically.

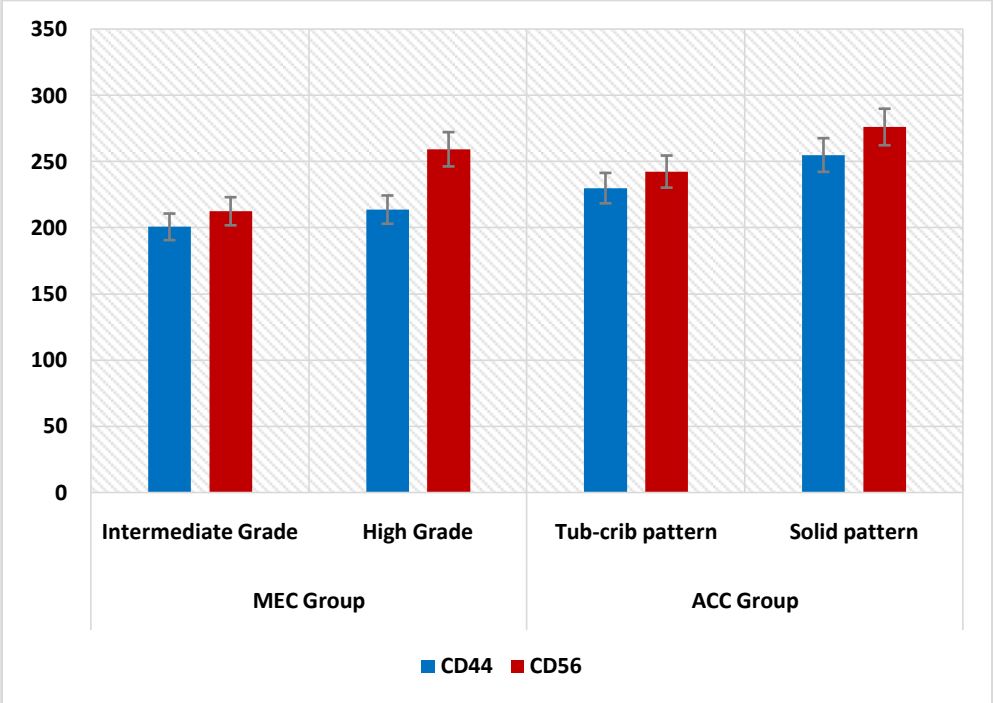

Figure (4):- Expressionof CD44 and CD56 in different subgroups of MEC and ACC.

Discussion:-

Cancer stem cells (CSCs) comprise an entity of cells that perform a huge job in recurrence, progression and poor prognosis of tumors.Of which belongs the CD44[44].
CD44 has been detected in epithelial tumor cells of benign and malignant salivary tumors with minor variances. The immune-expression of CD44 wasstudied in many salivary gland tumors, such as pleomorphic adenoma (PA), ACC, and MEC [5].

Numerous searches were done to assess the immunoexpression of CD44 in cancer. Scientists concluded the fact that higher expression is associated with higher cancer grades[39,47]. Though, others concluded not any alteration in expression of CD44 among benign or cancerous tissues[52].

Our results showed that CD44 expression in normal salivary glands was evident in ductal myoepithelialandacinar cells. The immunoexpression was clear in the acinar cells on the lateral and basal plasma membranes. Those findings are similar to those of Franchi et al. 2001[14] and Ayoub et al. 2018 [2].

In our research, we studied the immune-expression of CD44 in MEC and ACC as examples of salivary carcinomas and detected CD44 expression in intermediate, high grades of MEC and histological patterns of ACC. It was detected in our research that, the immunopositivity of CD44 in MEC was rather higher -although the difference was insignificant- in MEC-HG than MEC-IG. This finding might be elucidated that,CD44 might be over expressed in higher grades, owing to the presence of amplified or abnormal growth factor receptors, such as TGF-beta, HER2 oncogene and EGF, that in turn upregulates CD44, particularly the MEC-HG. These results are consistent with those found by Ayoub et al. 2018 [2].

Immunohistochemicalexpression of CD44 in MEC showed that higher expression of CD44 was associated with higher grade tumors. Likewise, a positive correlation was found amid CD44 and the grade of salivary gland cancers, which is consistent with studies by Fok et al., 2013; Binmadi et al., 2016 and Mesrati et al., 2021 [5,13,32].

In MEC, expression of CD44 was evident in all types of cells. Similar results have been obtained by other researchers [5,13,50].The pattern of expression of CD44 was histopathologicallydivergent among the two grades in our research. In MEC-IG, the expression was mainly membranous whereas the MEC-HG cases showed both cytoplasmic as well as membranous expression. The luminal surface of the duct like structures,in the MEC-IG cases,

were mainly immunonegative,while the outer layer exhibited immunopositivity.These findings are consistent with those found by Ayoub et al. 2018[2]. The immunonegativity of CD44 in the luminal surface of cells is possibly owing to the lack of CD44 ligands for CD44 has roles in cell-cell and cell-matrix adhesion.

Detection of CD44 expression in the cytoplasm shown in MEC-HG cases might be due to the proteolytic breakdown of CD44 causing the liberation of CD44 intracellular domain fragment (ICD). The CD44-ICD moves towards the nucleus therefore showing a positive immunoreaction in the cytoplasm. Various studies verified that the existence of this ICD fragment relates with increased metastases and proliferation, henceforth the immune reaction in the cytoplasm upsurges with higher grades of salivary cancers.

However, Irani and Jafari 2013 [21] revealed that the correlation between the tumor grade and the expression levels of CD44 had a statistically significant difference, which is inconsistent with our current study.

Bearing in mind the part of CSCs in cancer recurrence and therapy resistance toin further glandular malignancies (e.g. breast, pancreas),it is possible to presume that those cells probably have an additional part in the persistent growth and resistance to therapy typically shown by MECs. This indicates that patients with MEC may get use of the targeted elimination of these distinctively tumorigenic CSCs.

Strong data indicates that isoforms of CD44 are meticulously associated with clinicopathological features of many malignancies. CD44 is thought to undertake structural and functional modifications into malignant transformation, that in turn, assist in the cancer cells detachment from their original site, then invading the neighboring tissues[32].

In several malignancies, the increase of CD44 expression doesn't have to be permanently related to the worst prognosis. Quite the reverse, its upregulation in quite a few tumors is associated with a better prognosis. In many researches, studying the same tumor came to opposing results concerning CD44 expression and prognosis of the disease [34]. This might be attributed to variances in methodologies. Eventually, these conflicting results have to be resolved in order to apply anti-CD44 as a targeted therapy.
Adenoid cystic carcinoma is a cancer characterized by a lethargic and locally invasive growth. Distant metastasis and local recurrence are usual behaviors of this malignancy [18]. In our study, CD44 was expressed in the myoepithelial cells, though the ducts showed negative expression. These results are in accordance with previous researches [1,13,16,50]. It was postulated that CD44 immunopositive cells have significant roles in tumor morphogenesis by means of reciprocal action with the ECM [16]. Seifi et al. 2016 [46] postulated that the extracellular matrix, by means of CD44+ and CD133+ SCs, has an integral part in controlling cell morphogenesis and the several histological shapes of ACC.

This study revealed a significant difference between both patterns of ACC, with higher expression in the solid type of ACC, which is consistent with Fok et al. 2013 [13]. Furthermore, a study on breast cancer revealed that higher CD44 expression is related to a higher tumor histological grade[28,55]. Though, in another study, researching invasive breast cancer, found that positive expression of CD44 was not related to clinicopathological features involving histological grade [22]. Higher expression of CD44 may have a significant part in tumorigenesis,moreover, we spotted from our data that CD44 expression could be used as a predictor of tumor behavior but more researches with larger samples would be more supportive in this matter.

NK cells are members of the innate immune system and characterized by expression of CD56 in the absence of CD3. They are subdivided according to their surface level expression of CD56 into two subsets. Where most NK cells in peripheral blood are $CD56^{dim}$, and in tissues are the $CD56^{bright}$ NK cells [8].

Our results revealed that the expression of CD56, as a marker of NK cells, is expressively higher in MEC (HG) and ACC (solid pattern). According to our results, it seems that an elevated proportion of NK cells in salivary cancers signifies the body's immunityversus cell mutilation. This is in accordance with study by Seifi et al. 2016 [46] who found that CD56 was significantly higher in high grade salivary tumors. However, they found no statistically significant difference among different histological patterns of ACC regarding CD56 expression. Our results were contradictory to these shown by Mamessier et al. 2011[29], who found that the higher-grade malignant tumors had a reduced count of NK cell activating receptors as CD56.

They are the primary line of defense against harmful factors,though the number and role of NK cells differs according to the tumor type, its stroma and the tumor grade. A possible explanation, of NK cells being within the same long-term vicinity with tumor antigens, is that a sort of compatibility among the immune cells and stroma of the tumor, and sensitivity of NK cells to tumor cells is gone. Moreover, factors, for instance, TGF-β1, might result in a drop in the count of CD56+ NK cells and a rise in NK cell inert receptors. However, a drop in the count of CD56+ NK cells might arise along with a rise in further categories of NK cell. Though, with higher grades, dysfunction of the normal NK cells arises, thus the count of cytotoxic NK cells rises in salivary tumors. Therefore, the upsurge in cytotoxic NK cells is coupled with tumor progression, higher malignancies, and metastasis.

Former researches have shown contradictory results on the diagnostic and prognostic importance of CD56. To date, limited studies have searched CD56 expression in salivary gland tumors. Nakatsuka et al. 2011 [33]described a case of invasive adenocarcinoma in the accessory parotid gland which exhibited immunopositivity of CD56 in an irregular form,though they employed CD56 as a neuroendocrine marker. Dutsch et al. 2010 [12]assessed the expression of CD56 in adenocarcinomas in the parotid gland, and showed that 14% of which turned out to be CD56+.

Conclusions:-

This research has concluded that CD44 SC marker is expressed in epithelial tumor cells of MEC and ACC, and correlates with the histopathological grade. CD44 could be promising goal for cancer therapy, mostly for CD44 expressing cancers. However, additional studies with a larger samples and appropriate documentation are essential for the study of CD44 expression in this subgroup of tumors.NK cells, which are upregulated in higher grade salivary cancers, are significant gears of the antitumor immune mechanism. Consequently, impairment of those cells may bring about tumor progression. Our results may perhaps be beneficial in the development of molecular-targeted therapies in salivary gland cancers.It is clear that additional researches are crucial to evaluate CD56 expression in immune cells. Illustrating the upregulation and function of CD56 on cells of the immune system that ought to be thought of, in order that the present and future immune therapeutic modalities would likely get benefit from.

Conflicts of interest

There are no conflicts of interest.

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