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A POSSIBLE CORRELATION OF EZRIN AND FASCIN-1 IN ADENOID CYSTIC CARCINOMA OF SALIVARY GLAND: AN IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Adenoid cystic carcinoma (ACC) of salivary gland is one of the most common epithelial salivary gland carcinomas and is characterized by extensive local tissue infiltration and poor long-term survival. All forms of ACC are characterized by prolonged but persistent growth, extensive perineural invasion and hematogenous metastases. Distant sites of metastasis are primarily lung, although bone and liver are also common. The incremental motility of malignant cells is a critical step in their migration, invasion and metastasis, which is regulated by reorganization of actin cytoskeleton and regulation of focal adhesion. Fascin-1 and ezrin are essential components of these cellular structures. The aim of this study is to evaluate the expression of these cytoskeleton-associated proteins in ACC as a prognostic indicator of invasiveness. Five cases of normal salivary gland tissue and 43 cases of adenoid cystic carcinoma (10 cases cribriform form, 15 cases tubular form and 18 cases solid form). Immunohistochmical staining for ezrin and fascin-1 antibodies were done for all specimens. Statistical analysis was performed using a commercially available software program (SPSS 19; SPSS, Chicago, IL, USA). The level of significance was set at $P < 0.05$. For both markers, statistically, the greatest mean area percent was recorded in solid pattern ACC, whereas the lowest values were recorded in normal tissue. These results indicating a synergistic effect between ezrin and fascin-1 during ACC invasion.

KEY WORDS: Adenoid cystic carcinoma, ezrin, fascin, invasion

INTRODUCTION

Adenoid cystic carcinoma (ACC) of salivary gland is one of the most common epithelial salivary gland carcinomas and is characterized by extensive local tissue infiltration and poor long-term survival.[1,2] Adenoid cystic carcinoma (ACC) most commonly arising in the submandibular and minor salivary glands from latent epithelial cells of intercalated ducts that differentiate into either epithelial or myoepithelial subtypes. ACC are histologically categorized into three distinct growth patterns; tubular, cribriform and solid types that can all exist within the same tumor,

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with the predominant form allowing for some degree of outcome prediction. All forms of ACC are characterized by protracted but persistent growth, extensive perineural invasion and hematogenous metastases. Distant sites of metastasis are primarily lung, although bone and liver are also common.[3]

The molecular mechanisms for the aggressive invasiveness and distant metastasis remain unclear. Therefore, identification of the key regulatory molecules and signal transduction is crucial for understanding tumor dissemination and for the development of novel interventions. The incremental motility of malignant cells is a critical step in their migration, invasion and metastasis, which is regulated by reorganization of actin cytoskeleton and regulation of focal adhesion. Fascin-1 and ezrin are essential components of these cellular structures. Ezrin (EZR) is a predominant member of the Ezrin-Radixin-Moesin protein (ERM family) encoded by EZR gene located at chromosome 6q25.2-q26, which was described as a cross-linker between membrane proteins and cytoskeletal actin filaments, through its N-terminal and C-terminal domains, respectively. It is implicated in several important cellular complexes and processes, e.g., in cell adhesion to the extracellular matrix and in cell-cell interactions, and as a conduit for signals between metastasis-associated cell surface molecules (such as CD44) and signal transduction components.[2, 8-11] Ezrin expression has been found to be positively related to the degree of malignancy in many tumors, and its expression has also been linked to poor survival in several cancers, including carcinomas of the breast, endometrium and in melanomas and soft tissue sarcomas.[8, 12-13]

Fascin-1 has emerged as an interesting potential biomarker due to its low or absent expression in the majority of normal adult epithelia, colonic, breast, ovarian, stomach, pancreas, oral cavity, oropharynx, nasopharynx and larynx. Fascin-1 was originally described in the extracts of unfertilized sea urchin eggs as an actin-binding protein of 55 kDa. It is encoded by a gene located on chromosome 7p22 in humans.[7,14-18] . Fascin-1 appears to provide cancer cells with an efficient mechanism to assemble stable longliving invasive protrusions, which allow tumor invasion into the extracellular matrix and disrupt epithelial junctions. Fascin-1 overexpression was found associated with unfavorable prognosis in many malignant tumors such as colon cancer, gastric cancer, oral squamous cell carcinoma, esophageal squamous cell carcinoma, non-small cell lung cancer and breast cancer.[7,14, 17-21] Till now there are no studies involving the evaluation of expression of both ezrin and fascin-1 in normal salivary gland tissues and adenoid cystic carcinoma and no comparative analysis of the expression of these markers in these tissues. Consequently, the present study has been performed aiming to evaluate the expression of these cytoskeleton-associated proteins in ACC as a prognostic indicator of invasiveness.

MATERIALS AND METHODS

Case Selection

The specimens of this study were collected as formalin-fixed paraffin-embedded blocks from archive of Oral and Dental Pathology Department, Faculty of Dental Medicine for Girls, Al-Azhar University The specimens were divided into two groups, normal oral salivary gland tissues (5 cases of normal salivary gland tissue were taken as normal tissue adjacent to tumor of parotid gland) and 43 cases of adenoid cystic carcinoma (10 cases cribriform form, 15 cases tubular form and 18 cases solid form).

Histopathological Analysis

Using H&E for reevaluation of the aforementioned samples was carried out to confirm their diagnosis, and establish the histopathologic grading.

Immunohistochemical Analysis

Sections of 4 μm thickness were mounted on electrically positive charged glass slides and deparafenized by overnight incubation with xylene then,rehydratedingradualdescendingconcentrations of ethanol followed by Phosphate buffered saline (PBS) wash. Blocking the endogenous peroxidase activity was performed by 3% hydrogeneperoxide (H_2O_2) for 5 minutes at room temperature. For antigen retrieval, tissue section were put in glass jar containing 0.01M sodium citrate buffer (pH 6.0) and boiled in a microwave oven twice for 5 minutes each to enhance immunoreactivity (reserve the loss of antigenicity that has been occurred with some epitopes of formalin fixed paraffin embedded tissues).

The slides were allowed to cool and rinsed with PBS, pH 7.2. The immunohistochmical staining for ezrin and fascin-1 antibodies were done according to the manufacturer's instructions using "mouse monoclonal antibodies Clone 3c12 Cat. No. S-661, 1ml concentrated, Thermo Scientific, USA", for ezrin and "mouse monoclonal antibodies clone D-10: SC 46675 Dako, Denmark" for fascin-1 concentrated. The dilution used for both markers was 1: 50 in phosphate buffered saline.

Detection was carried out using the universal kit (DAKO, Denamark) by washing slides in PBS for 5 minutes and incubated with secondary antibody that was biotinylated goat serum conjugated rabbit and mouse sera for 30 minutes. Sections were then washed for 5 minutes in PBS followed by development of antigen antibody visualization by di aminobenzidine [DAB] in PBS containing 40% H2O2. Sections were washed under running tap water for 10 minutes, then counterstained with Mayer's haematoxylin and mounted.

Histmorphometric Analysis

Immunoreactivity for both ezrin and fascin-1 were evaluated by estimating the percentage of positive immunostained cells in relation to the area examined in each field, using Leica image analyzer computer system image analysis (Germany). The image analyzer consisted of color video camera, colored monitor, hard disc of hp personal computer connected to light microscope and controlled by Leica Qwin 500 software. The image analyzer was calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area and area percentage of both ezrin and fascin reactive areas were measured with reference to a standard measuring frame of area 11434.9 micrometer^2 using magnification (x200). Using the color detection, reactive areas of positive immunostaining were masked by a blue binary color. Ten fields per each slide section of each patient were successively taken to be histomorphometrically evaluated. Mean values were then obtained for each specimen.

Statistical analysis

Data were presented as mean and standard deviation (SD) values. ANOVA test used to compare means of more than two groups. Tukey Kramer multiple comparisons were used in procedure of pair wise comparisons between the groups when ANOVA test is significant. The P value is significant if less than or equal 0.05 ($P \leq 0.05$). Statistical analysis was performed by using instate graph pad version 3.10 and Microsoft® excel 2007.

RESULTS

Histopathological Findings:

The normal salivary gland tissue specimens showed acini of serous cells with duct system (fig1, A). The cribriform pattern of adenoid cystic carcinoma revealed cystic spaces surrounded by small basaloid cells and showed slightly basophilic mucinous areas. The cells revealed scanty cytoplasm and darkly stained hyperchromatic nuclei (fig1, B). While the tubular pattern showed ductal

and myoepithelial cells arranged in rows or small duct like structures, these duct cells were present in hyaline connective tissue (fig1, C). The solid pattern showed island or sheets of small uniform and undifferentiated tumor cells (The tumor cells were embedded in little easinophilic hyalinized stroma, very few spaces or glandular structure were present (fig1, D).

Immunohistochemical findings:

The ezrine immunostaining was detected in all normal cases in the cytoplasm of ductal cells only (fig1, E). While, ezrin immunostainingardg was seen in cytoplasm of neoplastic cells in all pattern of adenoid cystic carcinoma. Regarding the cribriform pattern 8 cases showing positive ezrin immunostaing (fig1, F), while, tubular pattern 12 cases showing positive ezrin immunostaing (fig1, G) and all cases of solid pattern showing positive ezrin immunostaining (fig, H). Regarding ezrin area percent of immunoexpression, the highest mean value was recorded in solid pattern (37.36), while the lowest value was recorded in normal salivary tissue (12.6), with a statistically significant difference ($p<0.0001$). Tukey's post hoc test revealed no significant difference between normal salivary tissue, cribriform and tubular patterns (Table1)

The fascin-1 immunostaining was detected in the cytoplasm of both ductal and some of acinar cells only in 3 normal cases (fig1, I). While, fascin-1 immunostaining was seen in cytoplasm

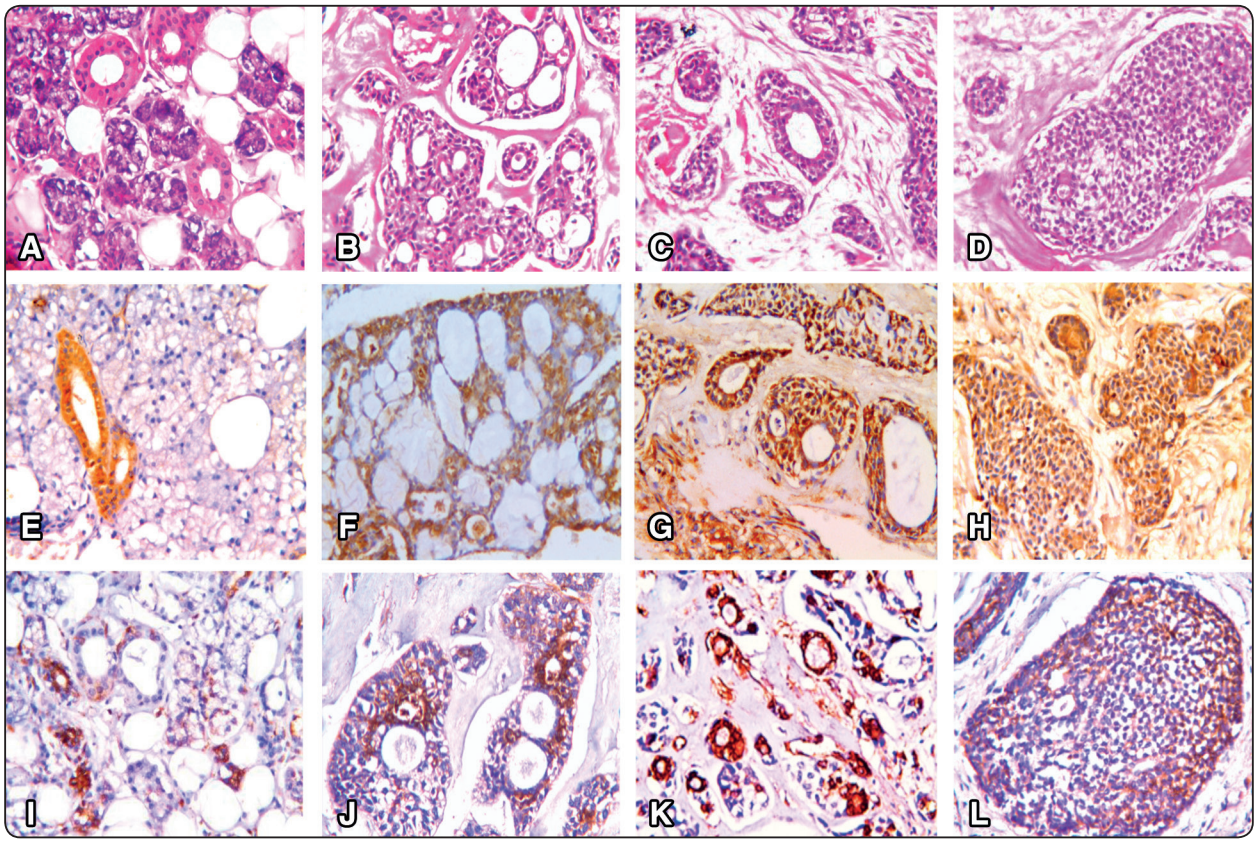

Fig. (1) Normal salivary gland tissue composed of acini of serous cells with duct system A, Adenoid cystic carcinoma showing hyperchromatic cells forming cribriform structure B tubular structure C and solid structure D, (H&E, X200). Ezrin immunostaining of cytoplasm of ductal cells only in normal salivary gland tissue E and cytoplasm of neoplastic cells of cribriform structure F, tubular structure G and solid structure H, (ezrin, X200). Fascin-1 immunostaining of cytoplasm of ductal cells some acinar cells in normal salivary gland tissue I, and cytoplasm of neoplastic cells of cribriform structure J, tubular structure K and solid structure L, (fascin-1 X200).

of neoplastic cells in all pattern of adenoid cystic carcinoma. Regarding the cribriform pattern 6 cases showing positive fascin-1 immunostaing (fig1, J), while tubular pattern 13 cases showing positive fascin-1 immunostaing (fig1, K) and all cases of solid pattern showing positive fascin-1 immunostaining (fig1, L). Regarding fascin area percent of immunoexpression, the highest mean value was recorded in solid pattern (38.53), while the lowest value was recorded in normal salivary tissue (16.72), with a statistically significant difference (p=0.001). Tukey's post hoc test revealed no statistically significant difference between solid pattern and cribriform pattern. Moreover, there was no significant difference between tubular and cribriform patterns. Tubular pattern and normal salivary tissue were not significantly different (Table 2)

Correlation Between Expression of Ezrin and Fascin

Pearson's correlation test revealed a strong positive correlation between ezrin and fascin area percent of immunoexpression (Table 3, Fig., 2).

TABLE (1) Comparison between normal salivary tissues and adenoid cystic carcinoma regarding Ezrin immunoexpression (ANOVA test)

Ezrin	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Normal	16.72 [c]	1.65	.74	14.67	18.77	14.25	17.90
Cribriform pattern	25.49 [b,c]	4.73	2.11	19.62	31.35	17.51	29.43
Tubular pattern	29.74 [a,b]	7.49	3.35	20.44	39.05	22.65	40.79
Solid pattern	38.53[a]	10.45	4.67	25.56	51.50	27.21	51.55
F	8.65						
P	0.001*						

Significance level p<0.05, *significant. Tukey's post hoc test: means sharing the same superscript letter are not significantly different

TABLE (2) Comparison between normal salivary tissues and adenoid cystic carcinoma regarding Fascin-1 immunoexpression (ANOVA test)

Fascin-1	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Normal	12.06[b]	6.87	3.07	3.53	20.59	5.99	21.73
Cribriform pattern	16.11[b]	6.80	3.04	7.66	24.55	9.79	27.00
Tubular pattern	20.68[b]	1.54	0.69	18.77	22.59	18.06	22.09
Solid pattern	37.36[a]	6.75	3.02	28.98	45.74	27.79	45.73
F	17.49						
P	0.000*						

Significance level p<0.05, *significant
Tukey's post hoc test: means sharing the same superscript letter are not significantly different

TABLE (3) Correlation between Ezrin and Fascin area percent of immunoexpression

R	R^2	Interpretation	P value
0.645	0.4163	strong positive	0.002* (significant)

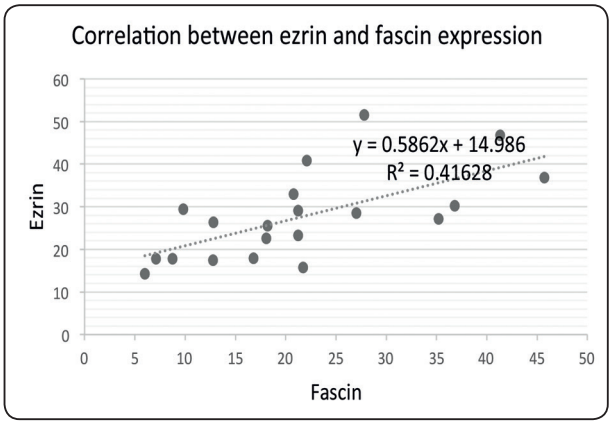

Fig. (2) Scatter plot showing correlation between Ezrin and Fascin area percent of immunoexpression

DISCUSSION

The incremental motility of malignant cells is a critical step in their migration, invasion and metastasis, which is regulated by reorganization of actin cytoskeleton and regulation of focal adhesion. [4-7] In the current study, ezrin immunostaining was detected in the cytoplasm of ductal cells of normal saliary gland tissue. These findings were in accordance with that reported by **Wang et al., 2011** [2] who reported weak ezrin immunostaining in ductal cells of normal salivary gland. Regarding adenoid cystic carcinoma, ezrin expression was significantly higher in solid pattern than in tubular and cribriform patterns. These results were in accordance with that described by **Wang etal., 2011** [2] who confirmed that ezrin immunostaining was significantly higher in solid pattern than in tubular and cribriform patterns, also the overexpression of ezrin was related to distant metastasis and poor outcome of ACC patients. Other studies found that significant correlations between ezrin expression and invasiveness as well as metastasis in several common cancers.[22,23]

The exact mechanism of ezrin's contribution to tumor invasion and metastasis remains to be clarified. As a cross-linker between membrane proteins and the cytoskeleton, and participating in signal-transduction pathways, functional activation of ezrin upon threonine and tyrosine phosphorylation can mediate many changes in the metastasis-associated cell-surface signals and intracellular signaling cascade and, ultimately, in the nucleus that confer the metastatic capability in tumor cells. It has been shown that ezrin is necessary for several signaling pathways important to tumor dissemination, including mitogen-activated protein kinase, protein kinase B, protein kinase C, PI3K, Rho, transforming growth factor β, CD44, and others. In addition, ezrin has been reported to associate with micro-RNA and c-Myc in invasion and metastasis of cancer cells.[22,24,25]

As regard fascin-1 immunostaining, it was detected in the cytoplasm of ductal cells and some acinar cells of normal salivary gland tissue with the lowest mean area percentage. These findings were in accordance with that reported by **Konstantinos et al., 2014**[14] who reported that fascin-1 expression has been found to be low or absent in the majority of normal adult epithelia of varying origin. However, increased fascin expression in the basal layer of nasopharyngeal epithelial tissues has previously been reported, which supports the observation of frequent and increased fascin expression in healthy, although tumoradjacent, epithelial tissue. The observed upregulated fascin expression may reflect a tissuespecific expression pattern or an association between fascin-1 and the proliferating capacity of cells. Alternative explanations may be that the epithelial tissue that was examined was directly adjacent to the tumor and the tumor may condition its microenvironment, or that the macroscopicallynormal epithelial tissue was not normal at a molecular level [16].

Regarding adenoid cystic carcinoma, fascin-1 expression was significantly higher in solid pattern than in tubular and cribriform patterns. These results were in accordance with that described by **Gao et al., 2012**[7] who reported that a positive correlation between high expression of fascin-1 and advanced tumor stage (T3+T4), poor cancer differentiation, N+ cancer, and advanced clinical stage (III+IV). This observation was in line with the work of **Durmaz et al.** [31] and **Zou et al**.[21] for LSCC and other researchers for a number of other malignant neoplasms. [17,18,26]

Cytoskeleton-associated proteins regulate polarity, differentiation, proliferation, migration and invasion of neoplastic cells by their intimate association with the actin cytoskeletal network, a complex mechanism. Fascin-1 and ezrin are essential components of these cellular structures. Their interactions could contribute to the signal transduction, which is involved in the transforming growth factor β, β-catenin-TCF, MARK, and twist pathways between cellular surface projections (microvilli and membrane ruffle) and cytoplasmic microfilaments, which might be the structural elements responsible for the migratory invasive properties of LSCC. Fascin-1, ezrin are regulated by microRNAs and take part in the modulation of malignant progression in carcinoma. [7,27-30]

CONCLUSIONS

Our study demonstrated a positive correlation among the proteins level of ezrin and fascin-1, so they may have a synergistic effect during ACC invasion. The expression of ezrin and fascin are significantly associated with histopathologic pattern and distant metastasis. Further in vitro and in vivo experiments are encouraged to address the exact mechanism. However, these results expand the understanding of local invasiveness and distant metastasis in SACC, which suggests that Ezrin and fascin-1 might represent potential targets for future anticancer therapeutic strategies in SACC.

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