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THE ROLE OF AUTOPHAGY IN SALIVARY GLAND TUMORIGENESIS

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ABSTRACT

Salivary gland tumors are a diverse group of tumors that exhibit a wide range of biologic characteristics, from entirely benign tumors to high-grade cancers. Also, because of the substantial morphologic overlap that is present in salivary gland tumors, it is frequently not possible to make a firm diagnosis without examining the entire tumor. In a number of human disorders, including cancer, autophagy is crucial in the control of signaling pathways that lead to survival and death. In the development of cancer, autophagy has two faces. Activation of autophagy works as a tumor suppressor by destroying damaged organelles and other cellular components. While cancer cells may use this system as a pro-survival strategy to produce nutrients and energy during periods of starvation, hypoxia, and stress induced by chemotherapy. The objective of the current study was to assess Beclin 1's potential as a prognostic marker of salivary gland tumorigenesis.

Material and methods: Five cases of normal salivary gland tissues, fifteen cases of pleomorphic adenoma and fifteen cases of adenoid cystic carcinoma (five cases of cribriform, five cases of tubular form and five cases of solid form). Immunohistochemical staining with Beclin 1 antibody was done for all specimens.

Results: Normal salivary gland tissue showed the highest mean area percent of immunoexpression, whereas ACC showed the lowest value.

Conclusion: Beclin 1 is involved in the tumorigenesis of salivary gland; in addition decrease in autophagic capacity associated with poor prognosis.

KEYWORDS Salivary gland tumors, Beclin 1, Autophagy

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INTRODUCTION

Salivary gland tumors can be classified by their location (parotid, submandibular, sublingual or minor glands) and by their pathology (benign or malignant).They differ greatly in terms of biological, phenotypic, and clinical behavior, as well as in their reactions to systemic treatments. [1] There is a wide morphologic spectrum across various tumor types and occasionally even within a single tumor mass. Additionally, this category of tumors is among the most demanding and intriguing in the head and neck due to the prevalence of hybrid tumors, dedifferentiation, and the possibility for some benign salivary gland tumors to progress to malignancy.[2]

Salivary gland tumors are mostly categorized based on their morphology, and the histologic features of these tumors correlate with the structure of normal salivary glands. However, this histologic similarity does not prove that a specific tumor develops from the structure it mimics. [2]

Pleomorphic adenoma (PA) is a benign salivary gland tumor with a diverse range of architectural features and cytomorphology. The tumor is made up of the following 3 elements: epithelial cells, myoepithelial cells, and mesenchymal-like component, the proportions of which can vary from tumor to tumor. Because this tumor has both an epithelial and a myoepithelial origin, it is also known as a benign mixed tumor.[3] ACC is a continually expanding tumor characterized by multiple local recurrences and perineural invasion. Regional lymph node metastases are typically thought to be uncommon. Hematogenous metastasis, in contrast, is frequent, particularly to the lung, liver, and bone. According to reserve cell theory, ACC arise from intercalated duct reserve cells.[4, 5] A process of intracellular disintegration known as autophagy helps cells adapt to stress by maintaining the basic tissue and cellular homeostasis and acting as a survival mechanism. It plays apart in both physiological and pathological conditions. Additionally, in response to a number of stresses and food scarcity, autophagy can be increased, supplying the cell with nutrients and energy by destroying damaged and aggregated proteins. [6, 7]

The first described mammalian autophagy protein was Beclin 1. Beclin 1 gene, which is known as BECN1/ATG6, was found on chromosome 17q21. Beclin 1 is localized within the cytoplasmic structures and is essential for initiating autophagy as it is one of the key signal-initiating complexes made up of p150 protein, PI3K-III /Vps34, and Beclin 1. [8, 9]

The Beclin-1 protein complex, which consists of the proteins Beclin-1 (Atg6), PI3K class III, p150, and Atg14L, could control autophagy. Phosphatidylinositol-3-phosphate (PI3P), which is needed for the formation of the autophagosome, is produced by the intrinsic activation of the PI3KC3/Beclin-1 complex. [10, 11] However, it is also known that the genes connected to autophagy play opposing roles in the tumors' development. Studies have shown that there is an association between the higher Beclin 1 expression and the poor differentiation of endometrial adenocarcinomas and ovarian carcinoma supporting this dual role. [12, 13] While decreased Beclin 1 expression was related to advanced clinical TNM stage, poor differentiation, and lymph node metastasis of squamous cell carcinoma (TSCC) of the tongue, demonstrating that Beclin 1 may play a role in the tumors' development and progression and that Beclin 1 overexpression prevented TSCC cells from proliferating and forming clones. [14] So this study was focused to understand the role of Beclin 1 as prognostic indicator of salivary gland tumorigenesis.

MATERIALS AND METHODS

Selection of cases

The specimens used in the present study were taken from the archives of Oral and Dental Pathology Department, Faculty of Dental Medicine for Girls, Al-Azhar University and Oral Pathology Department, Faculty of Dentistry, Alexandria University, in the form of paraffin blocks. Three groups were categorized from the cases: Group I, the control group, contains five normal cases (5 specimens of normal salivary gland tissue excised from patients with sialolithiasis). Group II, benign tumors of salivary gland, containing 15 cases of pleomorphic adenoma (PA). Group III, malignant tumors of salivary gland, in the form of adenoid cystic carcinoma (ACC) consisting of 15 cases exhibiting the different patterns. For this study, the Ethical code and approval was gained from the Ethical Committee of Faculty of Dentistry, Sinai University (Code. No. O.PATH 1-5-023).

Histological examination

To confirm the diagnoses and establish the histopathologic grading in accordance with WHO, the abovementioned cases were reevaluated using H&E examination [15].

Immunohistochemical analysis

In order to use streptavidin biotin technique, 4 μm thickness tissue sections on positively charged glass slides were obtained. The Paraffin embedded sections were deparaffinized in 4 changes of xylene and rehydrated using graded ethanol to distilled water. The endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide in methanol for 10 minutes. Antigen recovery was reached by addition of citrate buffer solution with pH about 6 and then was inserting in a microwave for three intervals, five minutes each at 95°C, then was washed with phosphate buffered saline (PBS). Then, one or two drops of the primary antibody Beclin 1, a mouse monoclonal antibody (G-11): sc-48381. Santa Cruz Biotechenology) in a dilution 1:50, in Tris buffer solution, were applied to the tissue sections, then were incubated overnight. Using the universal kit, detection was done by washing slides for 5 minutes in PBS, followed by 30-min incubation with a secondary antibody made of biotinylated goat serum conjugated mouse serum. Then sections were washed in PBS for 5 min, followed by visualization of antigen–antibody using diaminobenzidine in PBS with 40% H_2O_2. Sections were cleaned for 10 minutes using running water, counterstained with Mayer's hematoxylin, and mounted after that. Light microscopic examination of these immunostained sections was then carried out to assess the positive cases and to detect the immmunostaining within the prepared sections. In addition, the area percentage of the positively immunostained cells was determined using Leica image analyzer computer system image analysis controlled by Leica Qwin 500 software (Germany). The image analyzer was calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units.

Statistical analysis

The mean and standard deviation (SD) values of the data were displayed. One-way analysis of variance (ANOVA) test was applied to compare among more than two groups followed by Tukey's post hoc test if significant difference between groups was found. The significance level was established at $p \leq 0.05$. Statistical analysis was carried out with SPSS 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

RESULTS

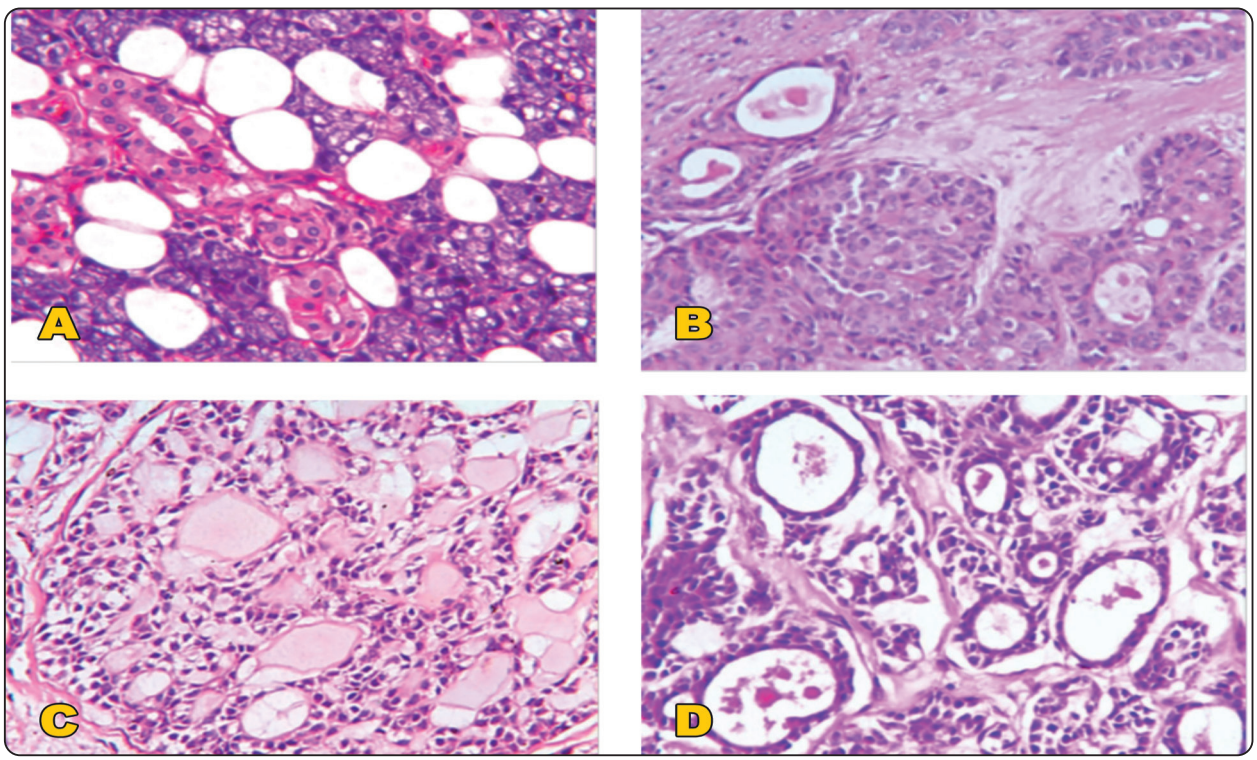
Histopathological results:

Histopathological examination of the specimens of normal group showed normal salivary gland tissues consisted of normal serous acini and normal

duct system arranged in glandular lobes and lobules separated by connective tissue septa. The serous acini lined by pyramidal cells with moderately basophilic cytoplasm and rounded basal nuclei (Fig., 1 A). The cases of pleomorphic adenoma displayed a mesenchymal-like background in which a mix of glandular epithelium and myoepithelial cells found. The epithelial element revealed strands and solid sheets of epithelial cells in addition to duct-like structures. In certain cases, chondroid tissue was also visible. In other regions, there were varying numbers of myoepithelial cells visible as spindle or stellate-shaped cells in mucoid or myxoid tissue (Fig., 1 B). Moreover, the cribriform pattern of ACC showed small basaloid cells, with sparse cytoplasm and darkly stained hyperchromatic nuclei, surrounding cystic spaces. Slightly basophilic mucinous areas were also found (Fig., 1 C). While the tubular pattern showed rows or small duct-like structures consisted of ductal and myoepithelial cells, which found in hyaline connective tissue (Fig., 1 D). Furthermore, the solid pattern displayed little eosinophilic hyalinized stroma in which collections of small uniform and undifferentiated tumor cells in the form of islands or sheets were embedded. There were very few spaces or glandular structure present (Fig., 1 E).

Immunohistochemical results:

Group I: Normal salivary gland, Beclin1 immunostaining was revealed in the cytoplasm of ductal and serous acini cells (Fig., 1 F). While in Group II: (PA), the immunostaining was visible in the cytoplasm of epithelial cells, duct like structures and myoepithelial cells lining the ducts and myxoid as well as chondroid tissue cells (Fig., 1 G). Group III: The immunostaining was seen in the cytoplasm of some cells in cribriform, tubular and solid tumor patterns (Fig., 1 H, I, J).



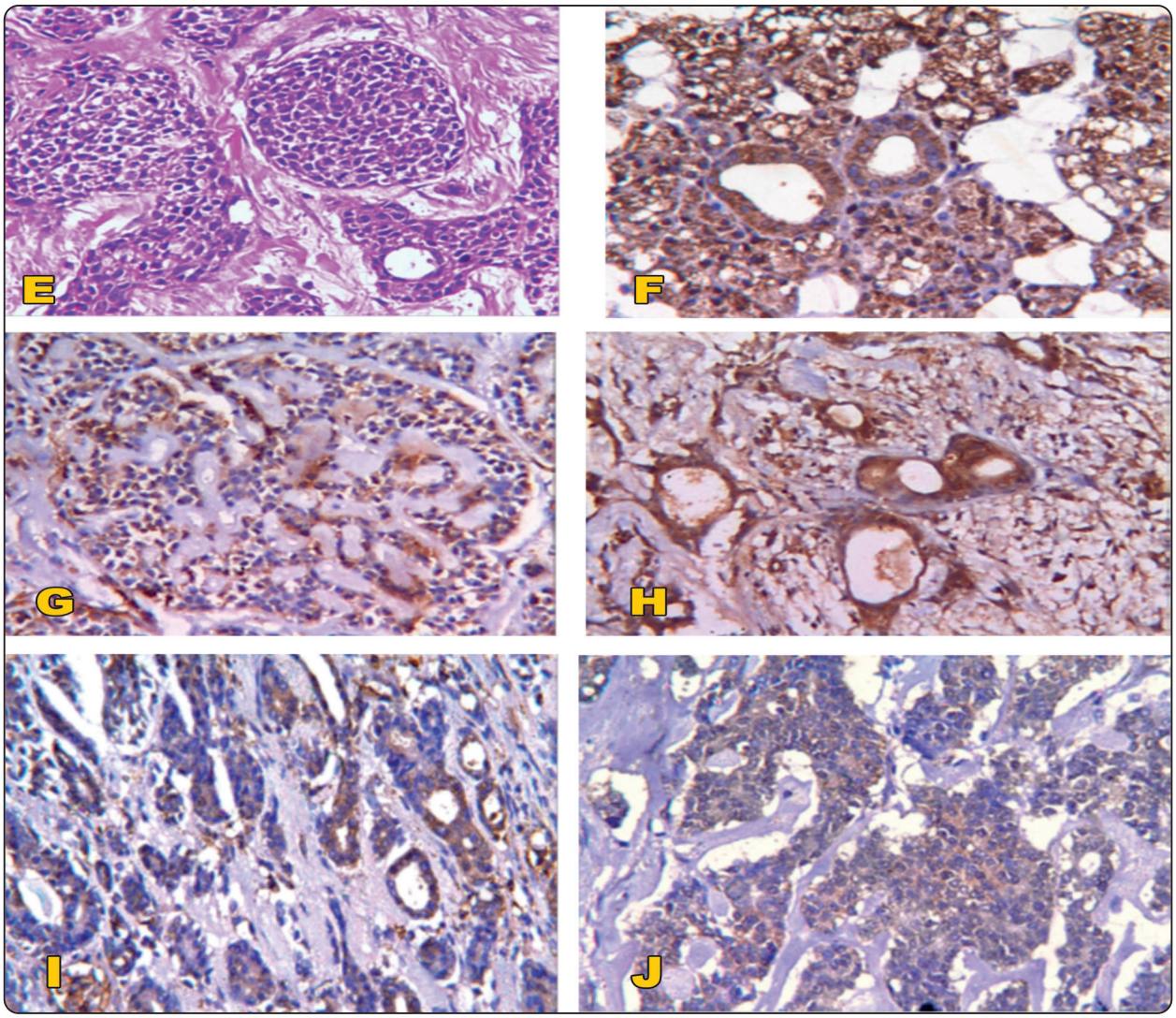


Fig. (1) Normal salivary gland tissues showing serous acinar and duct structure surrounded by myoepithelial cells(A), PA showing nests of uniform glandular epithelial cells and duct like structure containing eosinophilic mucin secretion and surrounded by myoepithelial cells(B), Adenoid cystic carcinoma showing hyperchromatic cells forming cribriform structure, tubular structure and solid pattern (C, D, E), (H&E X 200). Positive Beclin 1 immunostaining was detected in the cytoplasm and few nuclei of normal, benign and malignant salivary gland tissues (F, G, H, I, J) (Beclin 1, X 200).

TABLE (1) Comparison of Beclin1 area percent in normal salivary gland tissues, benign and malignant tumors

Group	Mean	S.D	Minimum	Maximum	*P- value*
Normal	53.96[a]	8.71	43.65	66.72	
Benign	41.03[b]	10.34	29.45	63.09	
Cribriform ACC	19.92 [c]	5.03	12.74	26.17	<0.0001*
Tubular ACC	18.76 [c]	3.94	13.10	23.46	
Solid ACC	16.69 [c]	7.77	8.46	29.95	

Significance at P ≤ 0.05. Tukey's post hoc test: means with different superscript letter are statistically significantly different.* *(Table 1, Fig. 2).***

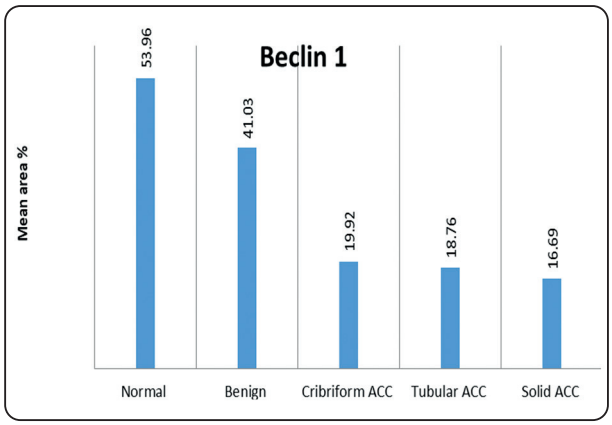


Fig. 2: Column chart showing mean Beclin 1 area percent in different groups.

Statistical results

ANOVA test revealed that there was extremely statistically significant difference between mean area percent in the three groups ($p<0.0001$). The normal salivary gland tissue group exhibited the statistically significant highest mean area percent, followed by benign salivary gland tumor group, while the statistically significant lowest mean area percent was seen in the group of malignant salivary gland tumors. **(Table 1, Fig.2).**

DISCUSSION

The propensity of salivary glands to produce a significant number of tumors with diverse histopathological features, the very variable immunohistochemical profile of tumoral cells, and their usually unpredictable behavior present significant diagnostic and therapeutic challenges. Thus, it becomes clear that there is a need to identify novel diagnostic and prognostic difficulties to help the pathologist make the best diagnoses and treatments for each patient. [16, 17] So, the main purpose of this study was to assess Beclin1 immunohistochemical expression in benign, malignant as well as normal salivary gland tissues and correlate the results with histopathologic findings. Considering Beclin 1 results among different groups in this study, the highest mean area percent of Beclin 1 immunoexpression was recorded in normal salivary gland tissues, followed by benign tumors then malignant one. This result is coincident with previous results in which Beclin 1 expression was down-regulated in parotid PA and Carcinoma ex PA than in normal parotid gland tissue. It was speculated that there is a correlation between the reduction of autophagic capacity and the development of salivary gland tumors. [18]

As for cellular expression of Beclin 1 in the current study, it was observed as cytoplasmic reaction of normal salivary gland tissues and benign neoplastic cells of PA while in malignant tumors it appeared nuclear as well as cytoplasmic. Beclin 1 is expected to have cytoplasmic patterns of immunohistochemistry expression since it normally localizes primarily in the intracytoplasmic organelles including: mitochondria, endoplasmic reticulum and perinuclear membrane. Beclin 1 nuclear expression was rarely mentioned and it was shown that its high nuclear expression was frequently related to vigorous pathologic characteristics in colorectal cancer [19, 20]. Beclin 1 expression was shown to be minimally expressed in the majority of cancer of oral tissues. The cytoplasmic expression of the Beclin 1 protein frequently accompanied the nuclear expression. Additionally, Beclin 1 nuclear expression had no correlation with the markers for autophagosomes, suggesting that Beclin 1 has functions other than autophagy in the nucleus. [20]

Moreover, it was found that the expressions of Beclin 1 in PA samples were significantly higher than that Carcinoma ex PA. Oppositely expressions of p53 in PA samples were significantly lower than that in Carcinoma ex PA. So it was concluded that the reduced autophagy and enhanced anti apoptosis coexist in the process of malignant transformation [21]. Also, it has been found that Beclin 1 expression was lower in salivary gland ACC samples than in normal salivary gland tissue [22]. In addition, the expression of Beclin 1 was reduced in tongue squamous cell carcinoma (TSCC) tissues when

compared to surrounding non-cancerous tissues, which indicated that loss of Beclin 1 may have a role in the development of cancer and the overexpression of Beclin 1 significantly reduces the clonogenicity and growth of TSCC cells. [14]

Autophagy, as is well known, inhibits tumorigenesis by reducing inflammation and necrosis in the tumor microenvironment. Metabolic stress specifically produces necrosis when autophagy and apoptosis in tumors are suppressed, followed by chronic inflammatory response that aids in the development and progression of tumors. Additionally, autophagy generates minimum amount of ATP needed for DNA repair. Moreover, the damaged cytoplasmic organelles (including mitochondria) in addition to, unfolded proteins and reactive oxygen species (ROS) can be eliminated from the cells by autophagy. This indirectly reduces the genomic instability. [23].

Opposite to our results, Beclin 1 expression levels in early stage human gastric cancer tissues were higher compared to those of the nearby normal tissue, which suggests that as cancer progresses, malignant cells eventually develop the ability to evade cell death. In tumors, autophagy is a significant form of cell death, and Beclin 1 may help to induce it. Although Beclin 1 was highly expressed in gastric tissues in early stages of gastric cancer, such changes were not found in the specimens of late-stage gastric cancer. It appeared that as the gastric cancer progress, the gastric tissues tended to lose their potential for autophagy.[19] Therefore, it had been found that both beclin 1 increased or decreased expression were detected in human malignancies, as it may had a cancer suppressor role, by interacting with bcl-2 protein members**.** Otherwise the tumor stimulatory role of beclin 1 overexpression in which it was associated with cancers aggressive behavior was done by anti-apoptotic machinery potentiation and another mechanism. Beclin 1 overexpression during adverse cancer environmental conditions like hypoxia and increased acidity, as a tumor grows, nutrients will become limited and oxygen levels will decrease in its inner region. As a mechanism of stress tolerance, autophagy may enable cancer cells to survive in this harsh microenvironment.[19,24,25] Beclin-1 overexpression, however, may be able to slow the development of cancer by reducing chromosomal instability and the incidence of additional mutations. That could result from Beclin 1-dependent autophagy that triggered an immunological response. [26, 27]

Regarding Beclin 1 expression in normal salivary gland of the current study, it was found in duct cells and acini. This was in agreement with speculation that in salivary acinar cells, autophagy may have a significant role in the homeostatic regulation of cellular proteins and organelles.[7] In normal cells, autophagy helps control cellular homeostasis and the turnover of organelles and long-lived proteins. Additionally, Beclin 1 was shown moderate level of expression in normal colon tissue, suggesting that it is a typical component of the colonic mucosa and may play a role in triggering the basal level of autophagy required for cellular homeostasis.[28]

Considering Beclin 1 immunoexpression ACC in this study, the highest mean area percent was recorded in cribriform pattern, then tubular pattern whereas the lowest mean value was measured in solid one. While the difference between these three patterns was not statistically significant. These results are in agreement with previous study reported that there was an association between Beclin 1 expression and the histological type and prognosis of ACC, where Beclin 1 expression was higher in cribriform pattern, the least aggressive pattern of ACC. High Beclin 1 mRNA and protein expression were considered as favorable indicators regarding ACC prognosis and the results of survival analysis indicated that high expression of Beclin 1 in ACC cells is tumor suppressive[22]. Moreover, it was demonstrated that salivary ACC progression was associated with the reduced Beclin 1 expression [21]. Furthermore, Beclin 1 expression was shown to have a significant statistical correlation with the histological grade and growth pattern of ACC. In addition, the clinical

features were favourable in patients with high Beclin 1 expression and unfavourable in those with low Beclin 1 expression[29]. Autophagic capacity is reduced as many tumors progress. Numerous antitumor agents and suppressors can stimulate autophagy; on the other hand, abundant oncoproteins can also suppressed autophagy[30-32].

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