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Expression of Autophagy and Reactive Oxygen Species-Related Proteins in Lacrimal Gland Adenoid Cystic Carcinoma

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Purpose: To investigate the difference of expression of autophagy and reactive oxygen species (ROS) related proteins in adenoid cystic carcinoma (ACC) of lacrimal gland in comparison with ACC of salivary gland.

Materials and Methods: Formalin-fixed, paraffin-embedded tissue samples from patients pathologically diagnosed as lacrimal gland ACC (n=11) and salivary gland ACC (n=64) were used. Immunochemistry was used to measure expression of autophagy related proteins [beclin-1, light chain (LC) 3A, LC3B, p62, and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)] and ROS related proteins [catalase, thioredoxinreductase, glutathione S-transferasepi (GSTpi), thioredoxin interacting protein, and manganese superoxide dismutase (MnSOD)]. The prognostic factors related to disease-free and overall survival (OS) in lacrimal gland ACC by log-rank tests, were determined.

Results: GSTpi in stromal cells was more highly expressed in lacrimal gland ACC (p=0.006), however, MnSOD in epithelial cells was expressed more in salivary gland ACC (p=0.046). LC3B positivity and BNIP3 positivity in epithelial component were associated with shorter disease-free survival (both p=0.002), and LC3A positivity in stromal component was the factor related to shorter OS (p=0.005).

Conclusion: This is the first study to demonstrate the expression of autophagy and ROS related proteins in lacrimal gland ACC in comparison with the salivary gland ACC, which would provide a basis for further study of autophagy and ROS mechanism as novel therapeutic targets in lacrimal gland ACC.

Key Words: Adenoid cystic carcinoma, autophagy, lacrimal gland, reactive oxygen species, salivary gland

INTRODUCTION

Adenoid cystic carcinoma (ACC) is a malignancy of secretory glands, including the lacrimal and salivary gland, which are highly aggressive and prone to local recurrence, and spread to adjacent tissues.[1] ACC is characterized by a slow but persistent progression, with multiple local recurrence, and metastasis to the lung, bone, and brain, occurring in approximately 50% of patients.[2,3] There are only a few studies of lacrimal gland ACC, in comparison with salivary gland ACC, due to rarity of the tumor. Advanced stage, solid architecture, high histologic grade, perineural invasion, and positive surgical resection margin are known as factors related to poor prognosis of salivary gland ACC.[4-6] Lacrimal gland ACC showed clinical features of younger age and worse prognosis compared to salivary gland ACC.[7] The causes of worse prognosis of lacrimal gland ACC were the high rate of incomplete excision rate due to complex orbital anatomy, invasion to nearby structures, and subsequent metastases.[1,8]

Primary treatments of lacrimal gland ACC are en bloc surgical excision and postoperative radiation, as needed.[9] Recently,

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neoadjuvant intra-arterial cytoreductive chemotherapy has been introduced to improve overall survival (OS) and decrease recurrence in 19 lacrimal gland ACCs, although controversy exists.[10,11] Neutron radiation therapy achieved 80% of 5 year local control in 11 cases, although late recurrence and distant metastasis remained as challenges.[12] No effective treatment is available in cases of recurrence or metastasis of lacrimal gland ACC. New treatments targeting this rare and life-threatening cancer are needed.

Recent advances have highlighted that alterations in both reactive oxygen species (ROS) and autophagy regulation are associated with cancer initiation and progression. It is now clear that these processes are mutually linked and play a crucial role in cancer progression and in response to cancer therapeutics.[13-16] Autophagy, a self-digestion process that facilitates cellular survival by maintaining energy homeostasis and macromolecular synthesis during cellular stress and nutrient deprivation, which can also induce ROS generation.[13,15-20] Recently, it was demonstrated that ROS can induce autophagy through several distinct mechanisms involving Atg4, catalase, and the mitochondrial electron transport chain, and some of the ROS stimulator, such as 2-methoxyestrodial and arsenic trioxide which are under clinical investigation as cancer treatments.[19,21] Autophagy can lead to cell-survival as well as cell-death responses and could be selective toward cancer cells. Cancer cell is able to survive in such an environment of hypoxia and nutrient deprivation, through angiogenesis and/or aerobic glycolysis. In highly aggressive malignant tumor requiring high metabolic demand, alternative metabolic pathway such as autophagy can provide cellular energy by recycle of cytoplasmic component, acting as a cytoprotective mechanism that help cancer cells resist anti-cancer treatments.[7,22] In the case of salivary gland ACC, ongoing investigations are taking place to better understand autophagy related proteins such as beclin-1 and YM155 and to develop chemotherapeutic agents targeting these markers.[23-26] However, no study has yet been conducted to investigate autophagy and ROS status in lacrimal gland ACC. The aim of this study was to investigate the expression and its implications of autophagy and ROS-related proteins in lacrimal gland ACC, compared to salivary gland ACC.

MATERIALS AND METHODS

Patient selection and clinicopathologic evaluation

Formalin-fixed, paraffin-embedded tissue samples of lacrimal gland ACC, collected from January 1997 to December 2012, at Severance Hospital, Yonsei University College of Medicine, were used for analyses. The study was approved by the Institutional Review Board of Severance Hospital. Clinical informations such as age at surgery, gender, tumor side, symptoms, and visual acuity were obtained from medical chart recordings. Tumor stage classifications followed the 7th American Joint Committee on Cancer staging system, and histologic features of hematoxylin and eosin slides were reviewed by a specialized pathologist (JSK). Histologic grading of tumors followed the indications established by Szanto, et al.[27] as follows: grade I, no solid component; grade II, ACC with less than 30% solid component; and grade III, ACC with more than 30% solid component. Histologic type was determined by predominant morphological growth patterns and divided into cribriform, tubular, and solid patterns. Perineural invasion, tumor margin (expanding, infiltrative), and tumor involvement in the surgical resection margin were evaluated. As a control group, 64 cases of salivary gland ACC in the same time periods of tissue collection were included.

Immunohistochemistry

The antibodies used for immunohistochemistry (IHC) in this study are listed in Supplementary Table 1 (only online). IHC was performed on formalin-fixed, paraffin-embedded tissue sections. After sectioning the tissue at a thickness of 3 μm, the samples were deparaffinized and rehydrated using xylene and alcohol solutions. IHC was performed using the Ventana Discovery XT automated staining system (Ventana Medical System, Tucson, AZ, USA). CC1 buffer (Cell Conditioning 1; citrate buffer, pH 6.0; Ventana Medical System) was used to wash samples for antigen exposure. IHC included the appropriate positive and negative controls.

IHC was performed to measure expression of proteins related to autophagy including beclin-1, light chain (LC) 3A, LC3B, p62 and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), and ROS including catalase, thioredoxinreductase, glutathione S-transferasepi (GSTpi), thioredoxin interacting protein, and manganese superoxide dismutase (MnSOD) in 11 cases of lacrimal gland ACC and 64 cases of salivary gland ACC.

Interpretation of immunohistochemical results

Results of IHC were defined as the proportion of stained cells× immunostaining intensity. The proportion of stained cell was defined as follows: 0 as negative, 1 as less than 30% positivity, and 2 as 30% or more positivity. Immunostaining intensity was defined as follows: 0 as negative, 1 as weak, 2 as moderate, and 3 as strong. The proportion of stained cells×immunostaining intensity was defined as follows: 0–1 was negative, 2–6 was positive.[28]

Statistical analysis

Data were statistically processed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). The Student's t-test and Fisher's exact test were used for continuous and categorical variables, respectively. Statistical significance was defined as $p<0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate survival time and time to tumor metastasis, respectively. Multivariate regression analysis was performed using the Cox proportional hazards model.

Table 1. Clinicopathologic Characteristics of Lacrimal Gland Adenoid Cystic Carcinoma

Case number	Age/sex	Tumor size (cm)/ tumor side	Main histologic type	Histologic grade	Lymphovascular invasion	Ocular symptom	Local recurrence/ metastasis	Survival	Chemotherapy/ radiation therapy
1	41/F	3.0/right	Cribriform	2	No	Proptosis	Yes/yes	Dead	No/yes
2	21/M	2.6/left	Cribriform	1	No	Proptosis	No/yes	Alive	No/yes
3	63/F	4.0/left	Solid	3	No	Lid swelling	Yes/yes	Dead	No/no
4	54/M	3.5/right	Solid	3	No	Proptosis	No/yes	Dead	No/yes
5	28/M	3.5/right	Tubular	1	No	Proptosis	No/no	Alive	Yes/yes
6	72/M	3.0/right	Solid	3	No	Proptosis	Yes/no	Alive	Yes/yes
7	57/M	2.5/left	Cribriform	1	Yes	Palpable mass	No/no	Alive	No/yes
8	51/M	3.6/right	Tubular	2	No	Proptosis	No/yes	Alive	Yes/yes
9	35/M	3.0/right	Cribriform	1	Yes	Proptosis	No/no	Alive	Yes/yes
10	61/F	3.5/left	Tubular	2	No	Proptosis	Yes/yes	Alive	Yes/yes
11	43/M	2.5/right	Cribriform	1	No	Lid swelling	Yes/yes	Alive	Yes/yes

Table 2. Comparison to the Expression of Autophagy and Redox-Related Proteins between Lacrimal Gland ACC and Salivary Gland ACC

Parameters	Total, n=75 (%)	Lacrimal gland ACC, n=11 (%)	Salivary gland ACC, n=64 (%)	*p* value
Beclin-1 (T)				0.742
Negative	50 (66.7)	8 (72.7)	42 (65.6)	
Positive	25 (33.3)	3 (27.3)	22 (34.4)	
LC3A (T)				0.333
Negative	67 (89.3)	9 (81.8)	58 (90.6)	
Positive	8 (10.7)	2 (18.2)	6 (9.4)	
LC3A (S)				0.719
Negative	56 (74.7)	9 (81.8)	47 (73.4)	
Positive	19 (25.3)	2 (18.2)	17 (26.6)	
LC3B (T)				0.269
Negative	55 (73.3)	10 (90.9)	45 (70.3)	
Positive	20 (26.7)	1 (9.1)	19 (29.7)	
p62 (T)				0.272
Negative	68 (90.7)	9 (81.8)	59 (92.2)	
Positive	7 (9.3)	2 (18.2)	5 (7.8)	
BNIP3 (T)				0.477
Negative	71 (94.7)	10 (90.9)	61 (95.3)	
Positive	4 (5.3)	1 (9.1)	3 (4.7)	
Catalase (T)				0.742
Negative	50 (66.7)	8 (72.7)	42 (65.6)	
Positive	25 (33.3)	3 (27.3)	22 (34.4)	
Catalase (S)				1.000
Negative	70 (93.3)	11 (100.0)	59 (92.2)	
Positive	5 (6.7)	0 (0.0)	5 (7.8)	
TxNR				0.341
Negative	65 (86.7)	11 (100.0)	54 (84.4)	
Positive	10 (13.3)	0 (0.0)	10 (15.6)	
GSTpi (T)				1.000
Negative	16 (21.3)	2 (18.2)	14 (21.9)	
Positive	59 (78.7)	9 (81.8)	50 (78.1)	
GSTpi (S)				0.006*
Negative	60 (80.0)	5 (45.5)	55 (85.9)	
Positive	15 (20.0)	6 (54.5)	9 (14.1)	
TxNIP				1.000
Negative	72 (96.0)	11 (100.0)	61 (95.3)	
Positive	3 (4.0)	0 (0.0)	3 (4.7)	
MnSOD (T)				0.046*
Negative	12 (16.0)	4 (36.4)	8 (12.5)	
Positive	63 (84.0)	7 (63.6)	56 (87.5)	
MnSOD (S)				0.681
Negative	63 (84.0)	10 (90.9)	53 (82.8)	
Positive	12 (16.0)	1 (9.1)	11 (17.2)	

S, stromal; T, tumor; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; TxNR, thioredoxinreductase; GSTpi, glutathione S-transferasepi; TxNIP, thioredoxin interacting protein; MnSOD, manganese superoxide dismutase.

Data are presented as the number of cases with positive expression (%).

*Characters refer to statistical significance (p<0.05).

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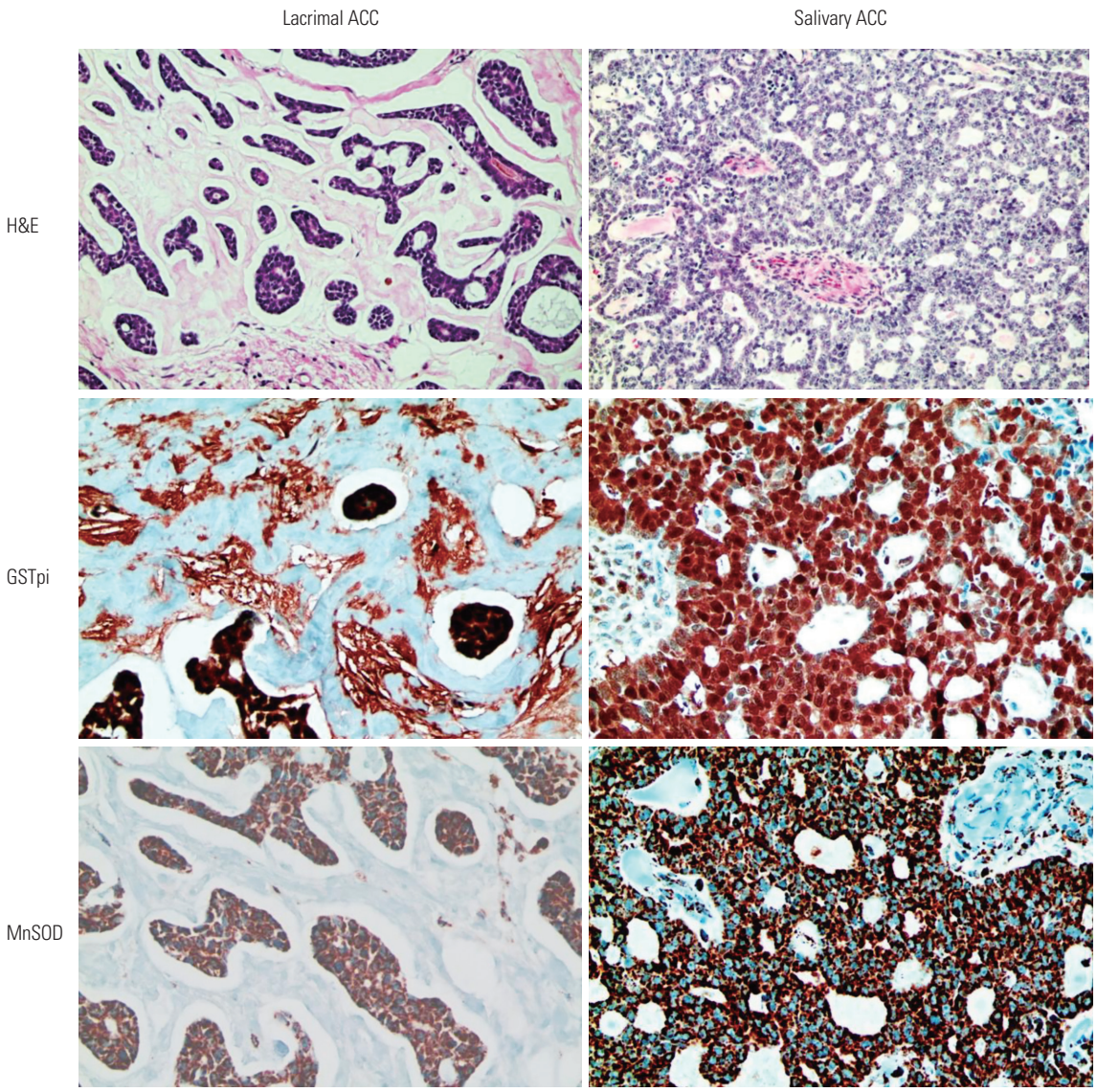

Fig. 1. Immunohistochemical expression of GSTpi and MnSOD in lacrimal and salivary gland adenoid cystic carcinoma (ACC). Expression of GST in stromal component was higher and MnSOD in cell component was lower in lacrimal gland ACC than in salivary gland ACC (×100). GSTpi, glutathione S-transferasepi; MnSOD, manganese superoxide dismutase; H&E, hematoxylin and eosin.

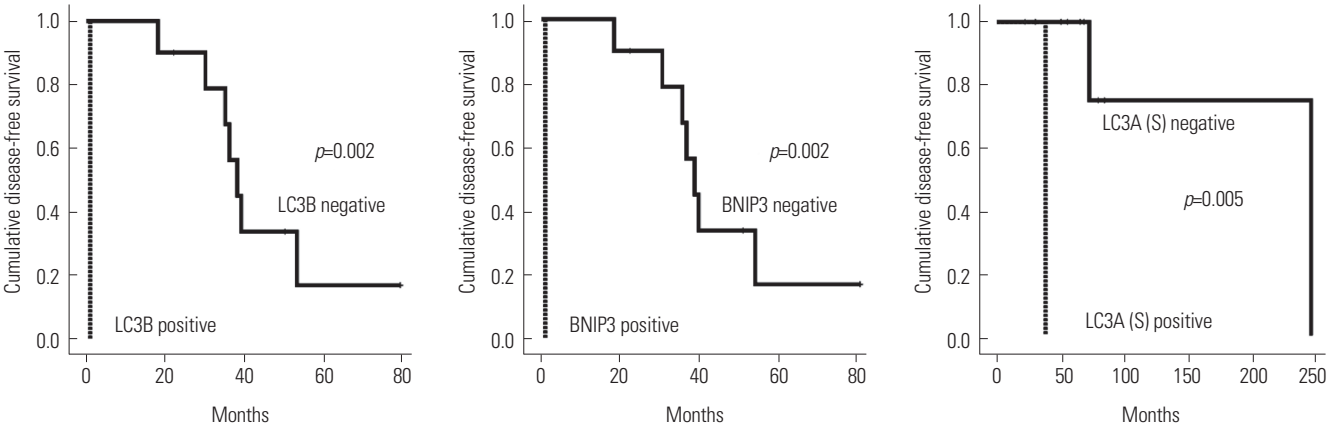

Fig. 2. Kaplan-Meier curves for disease-free survival and overall survival of lacrimal gland ACC, correlated with expression of the autophagy related proteins such as LC3A, LC3B, and BNIP3. BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; ACC, adenoid cystic carcinoma; LC, light chain.

RESULTS

Basal characteristics of lacrimal gland ACC

This study included 11 cases of lacrimal gland ACC (Table 1). Clinical characteristics were 21–72 years of age, and 3 were female and 8 were male. The size of the tumor was 2.5 to 4.0 cm, and the main histologic type was cribriform type (n=5). The most common histologic grade was grade 1 (n=5). Lymphovascular invasion was observed in 2 cases, local recurrence in 5 cases, and distant metastases in 7 cases [brain (n=5) and lung (n=2)]. Three patients died of disease.

Expression of autophagy and ROS-related proteins in lacrimal gland ACC: comparison with salivary gland ACC

Autophagy and ROS-related proteins expression was compared between lacrimal gland ACC and salivary gland ACC (Table 2). Compared to salivary gland ACC, lacrimal gland ACC showed higher expression of GSTpi in stromal component (p=0.006), and lower expression of MnSOD in epithelial component (p=0.046) (Fig. 1).

Impact of expression of autophagy and ROS-related proteins on patient prognosis in lacrimal gland ACC

In lacrimal gland ACC, the effect of expression of autophagy and ROS-related proteins on the prognosis was evaluated using univariate analysis (Fig. 2, Table 3). Factors associated with a shorter disease-free survival (DFS) were LC3B and BNIP3 positivity in epithelial component (both p=0.002). The factor associated with shorter OS was LC3A positivity in stromal component (p=0.005), however, no independent influencing factors on prognosis were found by multivariate Cox analyses (Table 4).

Table 3. Univariate Analysis by Log-Rank Test of the Impact of Autophagy and Redox-Related Proteins Expression in Lacrimal Gland ACC on Disease-Free Survival and Overall Survival Times

Parameters	Disease-free survival		Overall-survival	
	95% CI	*p* value	95% CI	*p* value
Beclin-1 (T)		0.201		N/A
Negative	44 (30–58)		N/A	
Positive	24 (0–50)		N/A	
LC3A (T)		0.761		0.431
Negative	44 (30–59)		74 (61–86)	
Positive	27 (0–77)		245 (245–245)	
LC3A (S)		0.634		0.005*
Negative	40 (24–55)		201 (97–305)	
Positive	35 (35–35)		38 (38–38)	
LC3B (T)		0.002*		N/A
Negative	44 (31–56)		N/A	
Positive	1 (1–1)		N/A	
p62 (T)		0.100		N/A
Negative	45 (31–58)		N/A	
Positive	18 (0–52)		N/A	
BNIP3 (T)		0.002*		N/A
Negative	44 (31–56)		N/A	
Positive	1 (1–1)		N/A	
Catalase (T)		0.101		0.061
Negative	44 (31–58)		201 (97–305)	
Positive	23 (0–49)		53 (32–73)	
Catalase (S)		N/A		N/A
Negative	N/A		N/A	
Positive	N/A		N/A	
TxNR		N/A		N/A
Negative	N/A		N/A	
Positive	N/A		N/A	
GSTpi (T)		0.534		N/A
Negative	43 (33–52)		N/A	
Positive	37 (23–52)		N/A	
GSTpi (S)		0.714		N/A
Negative	39 (32–46)		N/A	
Positive	37 (17–56)		N/A	
TxNIP		N/A		N/A
Negative	N/A		N/A	
Positive	N/A		N/A	
MnSOD (T)		0.397		0.808
Negative	51 (24–78)		75 (70–80)	
Positive	34 (22–46)		210 (123–297)	
MnSOD (S)		N/A		N/A
Negative	N/A		N/A	
Positive	N/A		N/A	

ACC, adenoid cystic carcinoma; CI, confidence interval; LC, light chain; T, tumor; S, stromal; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; TxNR, thioredoxinreductase; GSTpi, glutathione S-transferasepi; TxNIP, thioredoxin interacting protein; MnSOD, manganese superoxide dismutase.
*Characters refer to statistical significance (p<0.05).

DISCUSSION

In this study, expression of autophagy and ROS-related proteins was examined in lacrimal gland ACC, in comparison to salivary gland ACC, and effects of prognostic variables effects on DFS and OS in lacrimal gland ACC were explored using the log-rank test. First, GSTpi isoenzyme protein expression level was higher in lacrimal gland ACC than salivary gland ACC. Until now, there has been no study on the expression of GSTpi in ACC. GSTpi isoenzyme is known to suppress toxin-induced DNA damage by catalyzing the conjugation of electrophilic molecules with glutathione.[29,30] High GSTpi expression is consistently found in tumor cells, and seems to be directly related to the development of chemotherapeutic resistance in several types of cancer, especially in breast cancer by detoxifying chemotherapeutic drugs inside neoplastic cells.[11,12,31,32] GSTpi expression in stromal cells in breast tumor microenvironment, namely cancer-associated fibroblast, is also recognized to have major roles in cancer progression.[32] Likewise, higher level of GSTpi in stro-

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Table 4. Multivariate Analysis of Disease-Free Survival and Overall-Survival in Lacrimal Gland ACC

Included parameters	Disease-free survival			Overall-survival		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Tumor size			0.456			0.632
≤3 cm vs. >3 cm	0.419	0.042–4.132		0.012	0.000–815171	
Lymphovascular invasion			0.287			0.635
Absent vs. present	0.003	0.000–116.7		0.012	0.000–977358	
Histologic grade			0.415			N/A
1/2 vs. 3	0.444	0.063–3.128		N/A	N/A	
LC3A (S)			0.289			N/A
Negative vs. positive	5.210	0.247–109.9		N/A	N/A	
LC3B (T)			N/A	Not included		
Negative vs. positive	N/A	N/A				
BNIP3 (T)			N/A	Not included		
Negative vs. positive	N/A	N/A				
Catalase (T)			N/A			N/A
Negative vs. positive	N/A	N/A		N/A	N/A	

ACC, adenoid cystic carcinoma; CI, confidence interval; LC, light chain; T, tumor; S, stromal; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3.

mal cells of lacrimal gland ACC could be related to chemoresistance, although the mechanism is unclear and requires a further investigation.

The major antioxidant enzyme that scavenges superoxide anion radical in mitochondria is MnSOD.[33] In our study, the expression level of MnSOD was lower in lacrimal gland ACC than salivary gland ACC. MnSOD was reported to be expressed lower in tumor tissue than in normal tissue, playing a role as a tumor suppressor.[10] MnSOD have been demonstrated to play a critical role in the development and progression of cancer.[33] Many human cancer cells such as neuroblastoma, lung cancer, hepatoma, esophageal cancer, and colorectal cancer harbor low levels of MnSOD proteins and enzymatic activity.[34-38] Enzymatic activity of MnSOD rapidly declined in stage IV colon cancer tissue, suggesting that a decrease of in MnSOD in cancer tissue could be related to aggressiveness of tumor.[39] However, some cancer cells possess high levels of MnSOD proteins and enzymatic activity,[40] suggesting that differential regulation of MnSOD exists in cancer cells, depending on the type and stage of cancer development. Lower expression of MnSOD proteins in lacrimal gland ACC could be associated with poorer prognosis of lacrimal gland ACC than salivary gland ACC.

Cancer is one of the first diseases found to genetically be linked to autophagy malfunction.[18,41] A study has reported that beclin-1, an autophagy related protein, was correlated with OS in salivary gland ACC.[42] In this study, there was no statistical difference in beclin-1 expression level between lacrimal gland and salivary gland. Also, LC3B and BNIP3 were closely associated with shorter DFS in lacrimal gland. Expression of LC3B in breast cancer and BNIP3 in lung cancer, larynx cancer, and breast cancer were related to poor prognosis, compatible to our results.[43-46] In addition, LC3A expression in stromal component was associated with shorter OS in our study, which was also compatible to the previous reports that the expression of LC3A was a poor prognostic factor in other cancers including stomach cancer, ovary cancer, and lung cancer.[47-49] Current cancer therapies, including chemotherapy and radiation, are known to induce autophagy within tumor cells.[50] Recently, autophagy related to ROS, pathway is thoroughly discussed as a target of anticancer treatment.[19,21] ROS produced endogenously, by deranged metabolism of cancer cells, or exogenously, by ROS-generating drugs, have been shown to promote macroautophagy, a lysosomal pathway of self-degradation with essential prosurvival functions.[16] Furthermore, there are safe, clinically available drugs known to both inhibit and stimulate autophagy, however, there are conflicting positive and negative effects of autophagy reported and no current consensus on how to manipulate autophagyto improve clinical outcomes.

In conclusion, lacrimal ACC showed different expression of ROS related protein from salivary gland ACC. Lacrimal gland ACC was shown to express higher level of GSTpi in stromal component and lower level of MnSOD in epithelial component than salivary gland ACC. Also, autophagy related proteins such as LC3A, LC3B, and BNIP3 were associated with poor prognosis in lacrimal gland ACC. We found that some of autophagy and ROS related proteins were expressed in both cell and stromal component of lacrimal gland ACC. Further studies are mandatory to understand the role of autophagy in the pathogenesis, and to confirm association between autophagy and ROS pathways, in order to find out whether inhibition or stimulation of autophagy and/or ROS is beneficial in the treatment of lacrimal gland ACC. Our data would provide a basis for further study of investigation of autophagy and ROS pathway as targets for possible anticancer treatment.

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