Expression of MIF, Beclin1, and LC3 in human salivary gland adenoid cystic carcinoma and its prognostic value

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Abstract

Adenoid cystic carcinoma (ACC) is an uncommon salivary gland malignancy with a poor long-term prognosis. Clinical reports show the high rates of local recurrences and distant metastases. This study aimed to investigate the expression of MIF, Beclin1, and light-chain 3 (LC3) in salivary adenoid cystic carcinoma (SACC).

Tissue specimens were obtained from 48 salivary glands adenoid cystic carcinoma (SACC) patients and 15 oral squamous cell carcinoma (OSCC) patients. Immunohistochemical staining was performed to estimate the level of LC3, Beclin1, and MIF. All SACC patients were followed up. The Kaplan–Meier method was used to compare the prognosis of patients after treatment.

The 3-year, 5-year-, and 10-year-survival rates of the SACC patients were 83.9\%, 69.9\%, and 46.6\%, respectively. MIF, LC3, and Beclin1 in SACC were all obviously over-expressed. MIF showed an increased tendency in cases with advanced TNM stages, and at the same time, there was an inversely proportional relationship between MIF and LC3, Beclin1.

The long-term survival of SACC patients is poor. MIF might be a risk factor for SACC patients, whereas, LC3 and Beclin1 might be an effective strategy for treatment of SACC.

Abbreviations: ACC = adenoid cystic carcinoma, ATG6 = autophage-related-gene-6, LC3 = light-chain 3, MIF = macrophage migration inhibitory factor, SACC = salivary adenoid cystic carcinoma.

Keywords: adenoid cystic carcinoma, Beclin1, LC3, MIF

1. Introduction

Adenoid cystic carcinoma (ACC) is a rare low-grade malignancy generally in the major salivary glands responsible for 1\% of head and neck tumors and approximately 10\% of the salivary gland epithelial tumors.\textsuperscript{[1]} It is generally characterized by slow growth, high frequency of local multiple recurrences and high incidence of perineural invasion, and has a predisposition at the primary site. Also, it can metastasize to the distant sites especially the lung.\textsuperscript{[2]} At present, the most ordinary treatment is operation along with surgical adjuvant radiotherapy.\textsuperscript{[3]} Despite it achieves optimistic 5-year survival, the long-term survival among the patients is still not very high.\textsuperscript{[4–6]} In a single-center study involving 105 ACC cases,\textsuperscript{[4]} the 5-, 10-, and 20-year survival rates were 68\%, 52\%, and 28\%, respectively. Therefore, it is urgent to understand the molecular pathogenesis in order to develop effective therapeutic options.

Macrophage migration inhibitory factor (MIF), generally recognized as pro-inflammatory soluble cytokine, is initially discovered by Bloom and Bennett, which was produced by activated T-lymphocytes that can inhibit the random migration of macrophages in guinea pigs.\textsuperscript{[7,8]} Previous researches focused on the role of MIF in the aspect of inflammatory immune response. Nowadays, increasing evidence indicate that MIF could exert its function of contribution to tumor microenvironment and immune cells. MIF is observed in a large variety of tumors, such as bladder,\textsuperscript{[9]} colon,\textsuperscript{[8]} and gastric cancers\textsuperscript{[10]} by contributing to formation of tumor microenvironment through regulating...
the responses of immune cells. Actually, MIF was over-expressed in the majority of the malignant tumor as well as it could promote the proliferation and growth of tumor cell with driving the influx of inflammation. On the other hand, MIF had the capabilities of preventing apoptosis of tumor cell via resisting p53 to translocate from the cytoplasm to the nucleus.

Autophagy is a homeostatic cellular process required for the recycling of proteins and damaged organelles. This process was characterized by cellular components enrolling into vesicle and delivery to lysosomes for degradation. It has been demonstrated to play crucial roles in the growth and metastasis of human cancer cells. More studies reported the deficiency of autophagy was able to increase cell survival under certain circumstances. Recently, a series of autophagy-related genes, as autophagy was able to increase cell survival under certain conditions.

Beclin1 might act as an important biochemical marker for autophagy.

To our best knowledge, there are few studies focusing on the relationship between MIF and ACC. Our previous data demonstrated that MIF was associated with the metastasis of salivary adenoid cystic carcinoma (SACC) cells. To further investigate the effects of MIF on SACC, we determined the expression of MIF and autophagy-related protein (i.e., LC3 and Beclin1) and the potential correlation with TNM stage. In this study, SACC patients received treatments were closely followed up, in order to evaluate the prognosis and the treatment regimen. Besides, the association between MIF and LC3II and P62 was also investigated, with an aim to investigate the correlation between MIF expression and the prognosis in SACC patients. At the same time, our study may lead to a better understanding of the treatment for SACC.

2. Materials and methods

2.1. Ethics statement

Each patient signed the informed consent. This study is carried out in line with the Declaration of Helsinki. The study protocols are approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University.

2.2. Sample collection

Human SACC specimens were obtained from 48 patients (male: 20; female: 28) admitted in the oral and maxillofacial region of the First Affiliated Hospital of Xinjiang Medical University from January 2003 to June 2015. Meanwhile, 15 normal salivary gland (NSG) specimens served as normal (or negative) control. Corresponding human oral squamous cell carcinoma (OSCC) specimens obtained from 15 patients (male: 9; female: 6) were used as positive control. SACC was confirmed by pathologic diagnosis. Patients underwent preoperative chemotherapy, hormone therapy, or radiotherapy were excluded from the study.

2.3. Immunohistochemical staining assays

The expression of MIF, LC3, and Beclin1 was detected by immunohistochemical staining. Briefly, all the samples were fixed by 4% formalin and then were embedded in paraffin. Slices (4 μm) were dewaxed and rinsed using tap water, distilled water, and PBS buffer. Then hydrogen peroxide (3%) was used to block the activity of endogenous peroxidase. To repair the antigen, the slices were heated with citric acid in a microwave. MIF, LC3, and Beclin1 levels were measured using a commercially available immunohistochemical staining of SP kit (ZK-9600, ZSGB-BIO Technology, Peking, China) according to the manufacturer’s instructions. For each slice, 3 fields were randomly selected and captured with BH-2 micro camera system under a magnification of 200× by 2 investigators blinded to this study. The immunoreactivity of MIF, LC3, and Beclin1 was assessed according to a score that added the intensity of staining to the proportion of positive cells as previously described. The intensity of staining was graded as

1. weak,
2. moderate,
3. strong.

The proportion of positive cells was divided into 5 grades as ≤10%, 11% to 25%, 26% to 50%, 51% to 80%, and >80%.

2.4. Follow-up

The patients were followed up by communication via telephone or email. Fifty-eight SACC patients were followed up until recurrence or death. Nine cases were lost in the follow-up. Cases with no recurrence were followed up until January 1, 2016. Palindroma was defined as the recurrence of local tumor confirmed by pathology after discharge.

2.5. Statistics analysis

Data analysis was carried out using the Statistical Package for Social Sciences (IBM SPSS Statistics 17.0, NY). All data were presented as mean±standard deviation (SD). Pearson correlation, 1-way analysis of variance (ANOVA) and multiple linear regression analysis were used to statistic data. The Kaplan–Meier method was utilized to compare the prognosis among patients. Graphs were prepared with Prism version 6 (Graph Pad Software Inc., LaJolla, CA). P<0.05 was considered to be statistically significant.

3. Results

3.1. Patients’ characteristics

In total, 48 cases (21–80 yrs; mean, 52.81±12.92 yrs) were included. For the histology, cribriform was the predominant type (45.8%, Table 1). In regards to the TNM staging, the majority was classified into I/II grade (58.3%), followed by III/IV grade (37.5%). Among the involved organs, parotid gland was mostly affected (22.9%). Meanwhile, 21 cases (43.8%) showed a maximum diameter of 2–4 cm.

3.2. Kaplan–Meier survival analysis

The patients were followed up with duration of 2 to 130 months (mean, 35.37±10.31 months). Forty-one patients (85.4%)
underwent surgery adjuvant radiotherapy, while the other 7 patients (14.6%) underwent surgery resection alone. In patients received the combination of surgery and radiotherapy, Kaplan-Meier survival curves showed the overall 3-year, 5 year-, and 10 year-survival rates were 83.9%, 69.9%, and 46.6%, respectively (Fig. 1). In the patients only received surgery, the overall 3-year and 5-year survival rates were 71.4% and 23.8%, respectively. Compared with the cases received surgery and radiotherapy, the long-term survival rate showed decrease in the counterparts only received surgery \( (P = .025) \).

### 3.3. Expression of MIF, LC3, and Beclin1 in ACC and SCC patients

The immunohistochemical staining results of MIF, LC3, and Beclin1 were shown in Figure 2. Over-expression of MIF, LC3, and Beclin1 were detected in all SACC samples and SCC samples.

### 3.4. Concentrations of MIF, LC3, and Beclin1 in SACC specimens of different histology

Significant decrease was noticed in the MIF in solid samples compared with that of cribriform \( (424.43 \pm 161.69 \text{ vs } 200.46 \pm 72.00, P < .001, \text{Fig. 3}) \). Significant increase was observed in LC3 expression in cribriform samples compared with tubular types \( (619.21 \pm 66.00 \text{ vs } 249.22 \pm 57.68, P = .009) \) and solid type \( (619.21 \pm 66.00 \text{ vs } 230.12 \pm 57.12, P < .001) \). Similarly, the expression of Beclin1 showed significant decrease in cribriform compared with that of tubular type \( (284.84 \pm 75.64 \text{ vs } 183.63 \pm 39.06, P = .008) \) and solid \( (284.84 \pm 75.64 \text{ vs } 170.62 \pm 64.41, P < .001) \), respectively.

### 3.5. Expression of MIF, LC3, and Beclin1 in SACC specimens of different TNM grade

Expression of Beclin1 and LC3 were tended to decrease in SACC cases of stage III or IV compared with these of stage 0, I, or II, respectively \( (P < .05, \text{Fig. 4}) \). On the contrary, the expression of MIF in the patients of stage III or IV showed significant increase compared with the counterparts of stage 0, I, or II, respectively \( (P < .05) \).

### 3.6. Pearson correlation among MIF, LC3, and Beclin1

A significant positive correlation was observed between Beclin1 and LC3 \( (r^2 = .684, P < .001) \). Besides, MIF was negatively correlated with Beclin1 and LC3 \( (r^2 = .332, P = .034; r^2 = .394, P = .011, \text{Fig. 5}) \).
Figure 2. Immunohistochemical staining of MIF, LC3, and Beclin1 in SACC and SCC patients. The images were observed under a magnification of 400×. LC3 = light-chain 3, MIF = migration inhibitory factor, SACC = salivary adenoid cystic carcinoma, SCC = squamous cell carcinoma.

Figure 3. Expression of MIF, LC3, and Beclin1 in different pathological types in SACC patients. LC3 = light-chain 3, MIF = migration inhibitory factor, SACC = salivary adenoid cystic carcinoma.
3.7. Multiple linear regression analysis between MIF and LC3 or Beclin1

Multiple linear regression analysis revealed the correlation between MIF and LC3 or Beclin1 (Table 2). Stepwise regression was performed using LC3 and Beclin1 as independent variables and MIF as a dependent variable. The equation was as follows: 
\[ \hat{Y} = 709.048 - 0.156x_{LC3} - 1.058x_{Beclin1} (R^2 = 0.822) \]. Student t test was used to estimate the parameters of the regression coefficients and the hypothesis test. Beclin1 was negatively correlated with MIF \((P = .003)\).

4. Discussion

ACC is usually featured by perineural invasion and multiple local recurrences. The incidence of regional lymph node metastases is comparatively lower, but some patients may present hematogenous metastasis in lung, bone or liver, as well as neuroinvasion.\(^1\) Nowadays, the molecular biology and potential prognostic of ACC have been considered as a new hot topic in that field.\(^19\) In this study, we aim to investigate the regulation and expression of MIF and autophagy-related proteins including Beclin1 and LC3 in SACC.

Immunohistochemical assay demonstrated that the level of MIF was significantly higher in SACC of solid type compared to that of cribriform and tubular types. In contrast, Beclin1 and LC3 were down-regulated in SACC of solid histology compared to that in the other types. Moreover, MIF showed an increased tendency in cases with advanced TNM stages. Whereas, a tendency of reduce (down-regulation) was seen in Beclin1 and LC3 in cases with advanced stages. Furthermore, we also analyzed the relationship among the MIF, LC3, and Beclin1, which indicated that MIF was negatively correlated with Beclin1 and LC3, respectively. In addition, Beclin1 was positively correlated with LC3. The survival rate in patients only received surgery resection was significantly lower than that received surgery combined with radiotherapy. According to the Kaplan–Meier analysis, the overall 3-, 5-, and 10-year survival rates of cases received surgery combined with radiotherapy were 83.9\%, 69.9\%, and 46.6\%, respectively.

Little is known about the potential roles of MIF in SACC cases. Currently, abundantly studies have been focusing on the relationship between MIF and different cancers. For example, up-regulated expression of MIF was reported in various tumors, such as brain tumor, colon carcinoma, and prostate cancer.\(^8,20,21\) Claudia et al.\(^22\) showed that MIF might be responsible for recruiting neutrophils to head and neck cancers by CXCR2 and increasing neutrophil cell survival by inducing release of protumor proteins. Besides, MIF was considered to be responsible for activating MAPK/ERK and PI3K/AKT signaling pathways. Moreover, during the pathogenesis of tumor, MIF has been implicated in inhibiting p53 function, one of the most effective tumor suppressors, which then contributing to tumor cell proliferation.\(^21,23\)

To date, there are 3 histological types available for ACC including cribriform, tubular, and solid types. In our study, we
found MIF showed the highest concentration in solid SACC patients that usually show poor prognosis after treatment.\(^{[11]}\) Furthermore, MIF is up-regulated in late TNM stages, which suggested that MIF may play a crucial role in the pathogenesis of SACC.

ACC is associated with tumor cell autophagy. In this study, we aim to explore an expression of autophagy-related gene (i.e., Beclin1 and LC3) in SACC patients. Beclin1 gene is likely the mammalian homolog of the yeast Atg6 gene involving in the autophagy. It is an indispensable factor to recruit proteins from the cytoplasm for autophagic program.\(^{[24]}\) Qiu et al study suggested that Beclin1 was significantly down-regulated in human hepatocellular carcinoma (HCC).\(^{[23]}\) Recently, several studies have indicated that the high expression of Beclin1 is significantly correlated with poor prognosis of ACC.\(^{[26,27]}\) Meanwhile, we determined the level of LC3 as it played important roles in the formation of mature autophagic vacuoles. LC3 is conjugated to N-acyl-phosphatidyethanolamine and found in autophagic vacuole bilayer.\(^{[28]}\) These results were in line with our findings that Beclin1 and LC3 were down-regulated in solid histological type or late TNM stages. Simultaneously, a negative correlation was noticed between LC3 and MIF.

SACC patients usually present poor survival rate due to local recurrence. On this basis, it is necessary to perform surgery tumor resection with radiotherapy as adjuvant option. In this study, patients received adjuvant radiotherapy treatment showed significant higher survival rates compare to those cases who received single surgery. In line with a previous study\(^{[19]}\) reporting a 5-, 10-, 20-year survival rates of 68%, 52%, and 28% in ACC patients, our result showed a similar tendency. However, radiotherapy is an expensive treatment and is linked to the increased risk of complications, such as impaired bowel function, incontinence, and sexual dysfunction. Thus, it is necessary to explore a new treatment option for SACC.\(^{[29]}\)

### 5. Conclusions

In summary, up-regulation of MIF was noticed among SACC patients. Anti-MIF is a promising treatment option for SACC. This study also suggests that MIF might be a risk factor for SACC patients, whereas LC3 and Beclin1 might be an effective strategy for treatment of SACC. We presented a theoretical and experimental study of the molecular pathogenesis of SACC.

### Author contributions

Li CX drafted the article; Liu H revised it critically for important intellectual content; Chen QL, Tian ZQ contributed to analysis and interpretation of data; Lin QZ, Wang B agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors finally approved the version to be published.

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