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ORIGINAL ARTICLE

Autophagy and Apoptosis Play Opposing Roles in Overall Survival of Esophageal Squamous Cell Carcinoma

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Abstract Esophageal cancer is among the most aggressive gastrointestinal tract malignancies, and squamous cell carcinoma is the most common subtype. Although both autophagy and apoptosis involve programmed cell death, autophagy also maintains cell survival by recycling cellular waste. The relationship between autophagy and apoptosis in esophageal squamous cell carcinoma (ESCC) is unclear. Autophagic and apoptotic markers of ESCC were detected by immunohistochemical staining (IHC) in 43 ESCC patients treated during 2007–2011. Chi-square test and Kaplan-Meier method were used to determine how clinicopathological parameters were related to IHC results for LC3B, Beclin-1 and caspase-3 (CASP-3). Correlations among Beclin-1, LC3B, and CASP-3 were analyzed by Spearman rho. The statistical analyses revealed no clinicopathological parameters that significantly correlated with expressions of Beclin-1, LC3B, and CASP-3. However, low CASP-3 expression and high LC3B expression revealed by IHC were predictors of a poor prognosis. Additionally, LC3B expression had a significant negative correlation with CASP-3 expression. Autophagy is antagonistic to apoptosis and predicts poor overall survival in ESCC.

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Introduction

Globally, squamous cell carcinoma (SCC) is the most common malignant tumor of the esophagus. It predominantly occurs in men (two to ten times more often than in women) with a peak incidence in the seventh decade of life. The incidence of SCC widely varies by geography and ethnicity. High rates (>50 per 100,000 population) of SCC have been reported in males in Zimbabwe and in both genders in various provinces of eastern China, northern Iran, and certain areas of Kazakhstan. In central Asia, high risk populations include those of Turkish or Mongolian origin, and rates in African-Americans are two- to threefold higher than those in Caucasian Americans [1]. Esophageal squamous cell carcinoma (ESCC), usually affects the middle third of the esophagus [1]. Major risk factors for ESCC include consumption of tobacco, alcohol and hot beverages. Common presenting symptoms of ESCC include dysphagia, weight loss, retrosternal or epigastric pain, and regurgitation caused by strictures [1]. Additionally, up to 3 % of ESCC patients have concurrent head and neck SCC [2].

A crucial regulatory mechanism of cell death and homeostasis is programmed cell death (PCD), which involves both apoptosis and autophagy. Apoptosis, or type I PCD, is a caspase-dependent process. Autophagy, or type II PCD, leads to bulk degradation of intracellular components induced by cellular starvation and other metabolic stresses [3].

The CASP-3 is synthesized as an inactive 32 kDa proenzyme. During apoptosis, CASP-3 is processed into its active form, which is composed of two subunits, p17–20 and p10–12. Activated CASP-3 is responsible for the cleavage of

poly(ADP-ribose) polymerase (PARP), actin and sterol regulatory element binding protein, which are associated with apoptosis [4].

Recent studies suggest that autophagy may play an important role not only in the regulation of cancer development and progression, but also in the response of cancer cells to anticancer therapy [5, 6]. Subsequent studies have also indicate that microtubule-associated protein 1 light chain 3 (LC3) [7] and Beclin-1 [8, 9] are essential markers of autophagy. Of the three LC3 isoforms (LC3A, LC3B, and LC3C), several lines of evidence show that LC3B is a good prognostic marker after chemotherapy (CT) for advanced breast cancer [10].

Although autophagy and apoptosis are markedly different processes, several signaling mediators regulate both autophagic and apoptotic mechanisms. Therefore, this study performed immunohistochemical analyses to investigate the roles of autophagy and apoptosis in ESCC.

Materials and Methods

Patients

The analysis included 43 ESCC patients (39 males and 4 females) who had received esophagectomy with lymph node dissection between 2007 and 2011 in Kaohsiung Medical University Hospital. Table 1 summarizes the clinicopathologic features of the patients. This study was approved by the Internal Review Board of Kaohsiung Medical University Hospital.

Immunohistochemical staining (IHC)

Blocks of tissue fixed in formalin and embedded in paraffin were sectioned into thicknesses of 3 μm, deparaffinized, rehydrated, then autoclaved at 121 °C for 10 min in pH 6.0 Target Retrieval solution (Dako, Glostrup, Denmark) to retrieve antigens. Endogenous peroxidase in the sections was blocked by 5 min incubation in 3 % hydrogen peroxide at room temperature. After washing with a Tris buffer solution, the sections were incubated with 1:200 dilution of rabbit polyclonal anti-human LC3B, Beclin-1, and CASP-3 antibodies for 1 h at room temperature. The sections were incubated with secondary antibody conjugated with horseradish peroxidase (REAL EnVision, Dako) for 30 min at room temperature. Finally, the slides were incubated in 3,3-diaminobenzidine for 5 min, counterstained with Mayer's hematoxylin for 90 s, and mounted. The stains for Beclin-1, LC3B and CASP-3 were scored as follows: negative = 0 (≦10 % of tumor cells stained by the antibody); weak positive = 1 (11–30 % of tumor cells stained by the antibody); positive = 2 (31–70 % of tumor cells stained by the antibody); strong positive = 3 (71 %-100 % of tumor cells stained by the antibody) [11].

Table 1 Expressions of Beclin-1 proteins according to clinicopathological parameters

	Total	Beclin-1		
		Low	High	*P*
Age				0.622
≥ 65	33	17 (51.5 %)	16 (48.5 %)	
< 65	10	6 (60.0 %)	4 (40.0 %)	
Gender				0.412
M	39	21 (53.8 %)	18 (46.2 %)	
F	4	2 (50.0 %)	2 (50.0 %)	
Recurrence				0.052
Yes	27	14 (51.9 %)	13 (48.1 %)	
No	16	9 (56.3 %)	7 (43.8 %)	
Metastasis				0.183
Yes	24	12 (50.0 %)	12 (50.0 %)	
No	19	11 (57.9 %)	8 (42.1 %)	
pT				0.338
pT1	7	3 (42.9 %)	4 (57.1 %)	
pT2	11	5 (45.5 %)	6 (54.5 %)	
pT3	20	11 (55.0 %)	9 (45.0 %)	
pT4	5	4 (80.0 %)	1 (20.0 %)	
pN				0.875
pN0	19	7 (36.8 %)	12 (63.2 %)	
pN1	15	9 (60.0 %)	6 (40.0 %)	
pN2	9	7 (77.8 %)	2 (22.2 %)	
pM				0.622
pM0	26	13 (50.0 %)	13 (50.0 %)	
pM1	17	10 (58.8 %)	7 (41.2 %)	
Stage				0.321
I and II	25	13 (52.0 %)	12 (48.0 %)	
III and IV	18	11 (61.1 %)	7 (38.9 %)	
Therapy				0.244
Yes	20	11 (55.0 %)	9 (45.0 %)	
No	23	13 (56.5 %)	10 (43.5 %)	
Tumor size				0.321
≥ 0.5 cm^3	21	9 (42.9 %)	12 (57.1 %)	
< 0.5 cm^3	22	15 (68.2 %)	7 (31.8 %)	
Tobacco				0.559
Yes	12	6 (50.0 %)	6 (50.0 %)	
No	31	18 (58.1 %)	13 (41.9 %)	
Alcohol				0.363
Yes	16	8 (50.0 %)	8 (50.0 %)	
No	27	16 (59.3 %)	11 (40.7 %)	
Areca				0.285
Yes	24	13 (54.2 %)	11 (45.8 %)	
No	19	11 (57.9 %)	8 (42.1 %)	

P values were determined by Chi-square analysis

Table 2 Expressions of LC3B proteins according to clinicopathological parameters

	Total	LC3B		
		Low	High	*P*
Age				0.64
≥ 65	33	19 (57.6 %)	14 (42.4 %)	
< 65	10	6 (60.0 %)	4 (40.0 %)	
Gender				0.559
M	39	23 (59.0 %)	16 (41.0 %)	
F	4	2 (50.0 %)	2 (50.0 %)	
Recurrence				0.531
Yes	27	13 (48.1 %)	14 (51.9 %)	
No	16	12 (75.0 %)	4 (25.0 %)	
Metastasis				0.43
Yes	24	15 (62.5 %)	9 (37.5 %)	
No	19	10 (52.6 %)	9 (47.4 %)	
pT				0.503
pT1	7	5 (71.4 %)	2 (28.6 %)	
pT2	11	4 (36.4 %)	7 (63.6 %)	
pT3	20	12 (60.0 %)	8 (40.0 %)	
pT4	5	4 (80.0 %)	1 (20.0 %)	
pN				0.598
pN0	19	12 (63.2 %)	7 (36.8 %)	
pN1	15	6 (40.0 %)	9 (60.0 %)	
pN2	9	7 (77.8 %)	2 (22.2 %)	
pM				0.657
pM0	26	11 (42.3 %)	15 (57.7 %)	
pM1	17	14 (82.4 %)	3 (17.6 %)	
Stage				0.423
I and II	25	11 (44.0 %)	14 (56.0 %)	
III and IV	18	14 (77.8 %)	4 (22.2 %)	
Therapy				0.307
Yes	20	9 (45.0 %)	11 (55.0 %)	
No	23	16 (69.6 %)	7 (30.4 %)	
Tumor size				0.577
≥ 0.5 cm^3	21	10 (47.6 %)	11 (52.4 %)	
< 0.5 cm^3	22	15 (68.2 %)	7 (31.8 %)	
Tobacco				0.514
Yes	12	12 (100.0 %)	0 (0.0 %)	
No	31	13 (41.9 %)	18 (58.1 %)	
Alcohol				0.278
Yes	16	10 (62.5 %)	6 (37.5 %)	
No	27	15 (55.6 %)	12 (44.4 %)	
Areca				0.341
Yes	24	15 (62.5 %)	9 (37.5 %)	
No	19	10 (52.6 %)	9 (47.4 %)	

P values were determined by Chi-square analysis

Table 3 Expressions of caspase-3 proteins according to clinicopathological parameters

	Total	Caspase-3		
		Low	High	*P*
Age				0.571
≥ 65	33	18 (54.5 %)	15 (45.5 %)	
< 65	10	6 (60.0 %)	4 (40.0 %)	
Gender				0.471
M	39	21 (53.8 %)	18 (46.2 %)	
F	4	3 (75.0 %)	1 (25.0 %)	
Recurrence				0.593
Yes	27	17 (63.0 %)	10 (37.0 %)	
No	16	7 (43.8 %)	9 (56.3 %)	
Metastasis				0.507
Yes	24	13 (54.2 %)	11 (45.8 %)	
No	19	11 (57.9 %)	8 (42.1 %)	
pT				0.693
pT1	7	4 (57.1 %)	3 (42.9 %)	
pT2	11	7 (63.6 %)	4 (36.4 %)	
pT3	20	10 (50.0 %)	10 (50.0 %)	
pT4	5	3 (60.0 %)	2 (40.0 %)	
pN				0.07
pN0	19	10 (52.6 %)	9 (47.4 %)	
pN1	15	8 (53.3 %)	7 (46.7 %)	
pN2	9	6 (66.7 %)	3 (33.3 %)	
pM				0.545
pM0	26	16 (61.5 %)	10 (38.5 %)	
pM1	17	8 (47.1 %)	9 (52.9 %)	
Stage				0.367
I and II	25	15 (60.0 %)	10 (40.0 %)	
III and IV	18	9 (50.0 %)	9 (50.0 %)	
Therapy				0.526
Yes	20	10 (50.0 %)	10 (50.0 %)	
No	23	14 (60.9 %)	9 (39.1 %)	
Tumor size				0.367
≥ 0.5 cm^3	21	10 (47.6 %)	11 (52.4 %)	
< 0.5 cm^3	22	14 (63.6 %)	8 (36.4 %)	
Tobacco				0.114
Yes	12	6 (50.0 %)	6 (50.0 %)	
No	31	18 (58.1 %)	13 (41.9 %)	
Alcohol				0.638
Yes	16	10 (62.5 %)	6 (37.5 %)	
No	27	14 (51.9 %)	13 (48.1 %)	
Areca				0.369
Yes	24	12 (50.0 %)	12 (50.0 %)	
No	19	12 (63.2 %)	7 (36.8 %)	

P values were determined by Chi-square analysis

For statistical analysis, scores of 0 and 1 were defined as low-level expression, and scores of 2 and 3 were defined as high-level expression [12]. All LC3B, Beclin-1, and CASP-3 specimens showed cytoplasmic staining.

Statistical Analysis

Social Sciences for Windows, Version 19.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. For each clinicopathological parameter, Chi-square test was used to identify correlations with expressions of LC3B, Beclin-1, and CASP-3. The survival rate was analyzed by the Kaplan-Meier method with log-rank test. Spearman rho was used to analyzed correlations between Beclin-1, LC3B, and CASP-3. A p value of less than 0.05 was considered statistically significant.

Results

Correlations of expressions of Beclin-1, LC3B, and CASP-3 protein with clinicopathological parameters

IHC results for Beclin-1, LC3B, and CASP-3 were separately analyzed to determine how protein expressions were related to clinicopathological parameters such as age, gender, smoking, drinking, betel nut chewing, tumor size, recurrence, metastasis, tumor node metastasis stage, and adjuvant therapy (radiation therapy (RT) and/or CT). Tables 1, 2 and 3 show that the chi-square analysis of these parameters revealed no significant correlations with expressions of Beclin-1, LC3B, and CASP-3 protein, respectively. The Spearman rho analysis showed that CASP-3 had no significant correlation with Beclin-1 ($p = 0.512$) but had a significant negative correlation with LC3B ($r = -0.416$, $p = 0.012$). Figure 1 shows that the IHC results revealed a negative association between LC3B and CASP-3.

Expressions of Beclin-1, LC3B, and CASP-3 as prognostic indicators in ESCC

The median overall survival was 12.88 months in patients with high Beclin-1 expression and 12.1 months in those with low Beclin-1 expression. Figure 2a shows that Beclin-1 protein expression had no significant correlation with overall survival ($p = 0.824$). The median overall survival time was 8.63 months in patients with high LC3B expression and 15.5 months in those with low LC3B expression. Figure 2b shows that high LC3B expression was significantly associated with a poor prognosis ($p = 0.032$). Conversely, patients with high CASP-3 expression had a median overall survival time of 16.7 months, and those with low CASP-3 expression had a median overall survival time of 7.13 months. Figure 2c shows that low CASP-3 expression indicated by IHC staining was a significant predictor of a poor prognosis ($p<0.01$).

Discussion

In Taiwan, esophageal cancer is among the most aggressive malignancies of the gastrointestinal tract and was the ninth leading cause of cancer death in 2012. Its mortality rate is high (6.8 per population of 100,000) and is still increasing [13], probably due to increasing consumption of cigarettes, alcohol, and betel nut. Additionally, ESCC is the most common histological subtype of esophageal cancer in Taiwan. Finally, the incidence of ESCC in Taiwan, especially in males, is also increasing, which contrasts with World Health Organization data for 2010 showing that the global incidence of ESCC is decreasing [1].

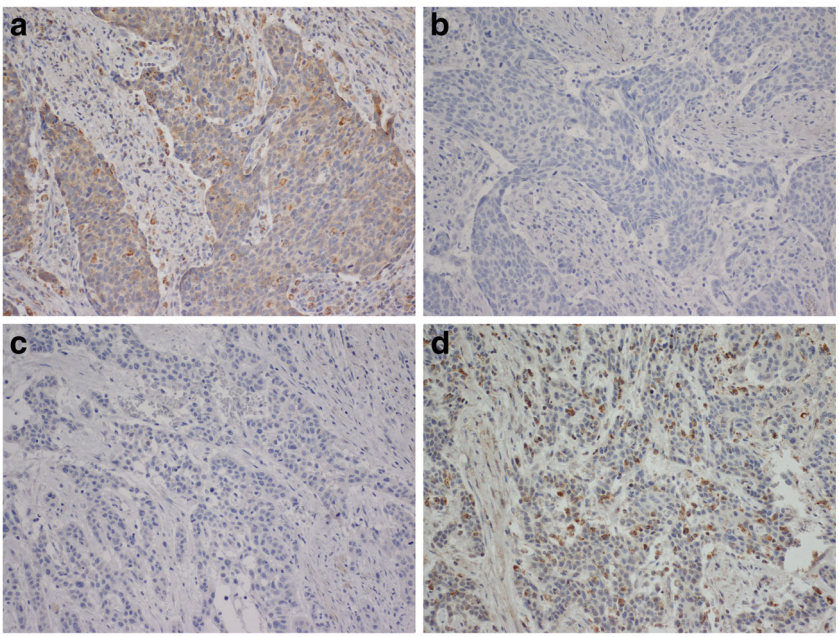

Fig. 1 Immunohistochemical staining results for esophageal squamous cell carcinoma in representative patients showing the opposing roles of LC3B and caspase-3 (CASP-3). **a** High LC3B expression and **b** low caspase-3 in one patient. **c** Low LC3B expression and **d** high CASP-3 expression in another patient. Magnification ×200 for both LC3B and CASP-3

This study performed IHC analyses of expressions of LC3B and Beclin-1 proteins, two important members of autophagy-related proteins involved in the autophagic pathway, in 43 ESCC patients. The results showed that high LC3B expression correlated with poor overall survival, which is consistent with previous studies [14].

In some cases, autophagy is considered a form of PCD. In cancer patients, however, the main role of autophagy is to improve cell survival in stressful metabolic environments [15]. Many studies show that inhibited autophagy sensitizes cancer cells to DNA-damaging anti-cancer reagents. For example, experiments performed in human colorectal cancer cells show that, when autophagy is inhibited by 3-methyladenine (3-MA) and Atg7 siRNA, cytotoxicity induced by 5-fluorouracil (5-FU) is enhanced [16]. Suppression of autophagy also enhances the therapeutic efficacy of cisplatin and 5-FU in esophageal and colon cancer cells, respectively [16, 17]. Additionally, siRNA-mediated silencing of autophagy-related genes such as Beclin-1, Atg3, and Atg4b sensitizes resistant cancer cells to ionizing radiation [18]. These studies suggest that autophagy may be a mechanism through which cancer cells acquire resistance to radiotherapy and CT.

Many studies agree that CASP-3 expression is associated with prognosis in many malignancies. CASP-3 protein expression substantially differs between primary ESCC patients with metastatic lymph nodes and those with primary tumors. In patients who undergo resection for ESCC, CASP-3 expression is an independent predictor of poor survival in those with lymph node metastasis but is associated with improved survival in those with primary tumors. Although further studies are needed, their findings suggest that CASP-3 protein expression may be a useful prognostic indicator in ESSC patients with metastatic tumors. However, further studies of CASP-3 are needed to improve understanding of the role of this familiar biomarker in ESCC [19].

In our study, Kaplan-Meier analysis showed that low CASP-3 expression and high LC3B expression were

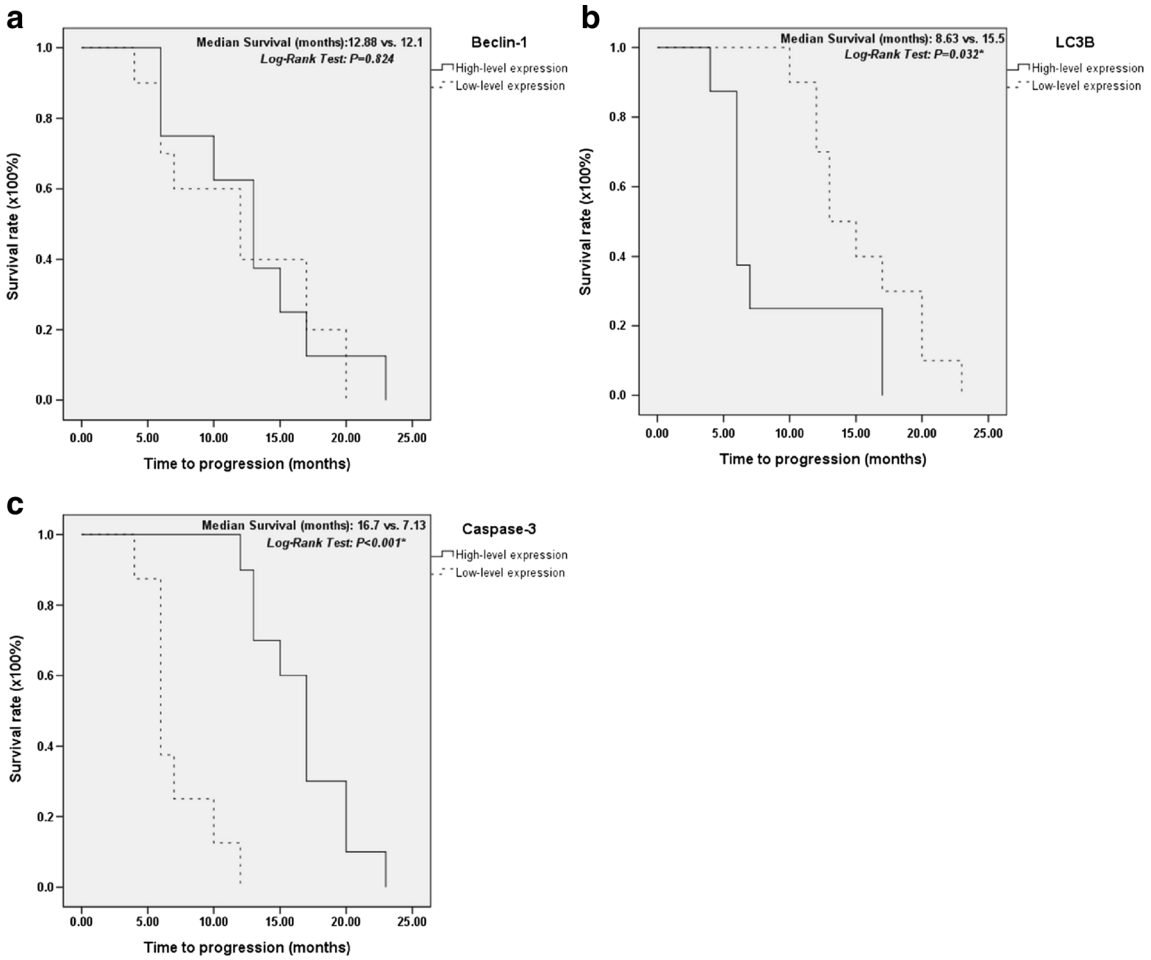

Fig. 2 Relationship between Beclin-1, LC3B and caspase-3 (CASP-3) protein expressions and overall survival of esophageal squamous cell carcinoma patients. **a** High and low expressions of Beclin-1 showed no significant correlations with overall survival (p = 0.824). **b** Overall survival was significantly higher in patients with low LC3B expression than in those with high LC3B expression (p = 0.032). **c** Overall survival was significantly higher in patients with high CASP-3 expression than in those with low caspase-3 expression (p < 0.001). * A p value less than 0.05 was considered statistically significant

predictors of poor prognosis. Spearman rho analysis revealed a significant negative correlation between LC3B expression and CASP-3 expression. These results suggest that autophagy and apoptosis may play opposing roles in overall survival of ESCC. Both apoptosis and autophagy are regulated by the mTOR complex, Bcl-2 family proteins, Beclin-1, DAPK, JNK, LC3, p62, and Atg5, which suggests the existence of crosstalk among these processes [20]. Besides promoting autophagy, Atg5 enhances susceptibility to apoptotic stimuli [20] that sensitize tumor cells to CT. Yousefi et al. [21] reported that silencing this gene partially increases resistance to anticancer therapy. Additionally, autophagy induced by anticancer drugs such as cisplatin differs between normal and tumor cells in a dose-dependent and time-dependent manner [22]. Recent reports indicate that, upon activation by autophagosomes, caspase-8 interacts with autophagic factors such as p62 and ATG5; specifically, the caspase-8/FADD complex associates with ATG5 on ATG16- and LC3-positive structures, which suggests that the autophagosomal membrane functions as a platform for formation of a dual-armed Death-Inducing Signaling Complex that facilitates activation of caspase-8 and initiation of apoptosis [23]. Conversely, studies show that the proteolytic Beclin-1 C-terminal fragment induces the release of apoptotic factors from mitochondria. Therefore, through cleavage and inactivation of Beclin-1, caspases inhibit autophagy and enhance apoptosis [24]. These observations are a strong indication that autophagy is independent of apoptosis and vice versa [23] and indicate the need for further detailed studies of these processes [25]. When the apoptotic pathway is suppressed, autophagy is an adaptive response that switches from a protective role to a death-promoting role through crosstalk with the apoptotic pathway. [26, 27]. From the above we can speculate that corsstalk among autophagy and apoptosis in tumor cells correlates with clinical outcomes in cancer patients, and LC3B and CASP-3 proteins can be used as prognostic markers. Autophagy might play a role as a self-protective mechanism in esophageal cancer cells, and its inhibition could be a novel strategy for the adjuvant therapy of esophageal cancer.

Our study has certain limitations. For example, the patient number was too small to identify clinicopathological parameters that significantly correlated with expressions of the Beclin-1, LC3B, and CASP-3 proteins. Further studies are needed in a larger population. Additionally, this study only performed IHC analyses. Further studies are needed to confirm the results using semi-quantitative methods or Western blot.

Conclusion

This study of ESCC patients showed that high LC3B expression and low CASP-3 expression were associated with poor overall survival. Additionally, expressions of LC3B and CASP-3 revealed a significant inverse correlation. Autophagy and apoptosis may have opposing roles in the development of ESCC tumors. Expressions of LC3B and CASP-3 may be useful markers for predicting outcome in patients with ESCC.

Compliance with Ethical Standards

Conflict of Interest The authors of this study have no conflicts of interest to declare.

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