Pathol. Oncol. Res. (2013) 19:771–777
DOI 10.1007/s12253-013-9642-0

RESEARCH

Prognostic Significance of Beclin-1 Expression in Laryngeal Squamous Cell Carcinoma

Li Huang · Shuang Wang · Shi-Sheng Li · Xin-Ming Yang

Received: 13 November 2012 / Accepted: 11 April 2013 / Published online: 19 May 2013

Abstract Beclin-1 plays a critical role in the regulation of autophagy, apoptosis, differentiation and the development and progression of cancer. The aim of the present investigation was to analyze the Beclin-1 protein expression and to assess its prognostic significance in tissue of laryngeal squamous cell carcinoma (LSCC). Beclin-1 protein expression in 82 primary laryngeal squamous cell carcinoma and 40 paracarcinoma non-tumor tissue samples was analyzed by immunohistochemistry and correlated with clinicopathological parameters and patients' outcome. The expression of Beclin-1 in tumor tissues was significantly lower than that in non-tumor tissues (P=0.035). Reduced Beclin-1 expression was significantly correlated with lymph node metastases (P=0.021). Kaplan–Meier survival estimates showed a significant correlation between Beclin-1 expression and patient's survival rate (log-rank P<0.05). Multivariate Cox proportional hazards model analysis confirmed that lymph node metastases (P=0.048) and Beclin-1 expression (P=0.029) were statistically significant, independent prognostic factors for LSCC. Our findings suggested that decreased Beclin-1 expression and lymph node metastases, as examined by immunohistochemistry, are both independent biomarker for poor prognosis of patients with LSCC.

Keywords Beclin-1 · Laryngeal squamous cell carcinoma · Metastasis · Prognosis

L. Huang
Department of Otolaryngology-Head and Neck Surgery,
West China Hospital, Sichuan University, 37, Guoxue Lane,
Chengdu 610041, China

S. Wang · S.-S. Li · X.-M. Yang (✉)
Department of Otolaryngology-Head and Neck Surgery,
The Second XiangYa Hospital, Central South University,
Changsha, Hunan 410011, China
e-mail: x16y2003@yahoo.com.cn

Introduction

Head and neck cancer describes a heterogeneous group of malignancies occurring in the upper aerodigestive tract, the majority (93 %) of which is squamous cell carcinoma [1]. Laryngeal squamous cell carcinoma (LSCC) is one of the most frequent cases of head and neck cancer, as it accounts for approximately 25 % of the malignant tumors involving in this area, and makes up around 5 % of systemic malignant tumors, to be responsible for 0.9 % of the total deaths from cancer. Despite therapeutic advances in cancer treatment, the overall 5-year survival of approximately 60 % among head and neck cancer patients has been virtually unchanged over the past 3 decades [2]. Moreover, the 5-year survival rate in patients with lymph node metastases is less than 50 % [3]. Locoregional recurrence, lymph node metastases and distant metastases are the factors that significantly affect the prognosis in LSCC patients [4]. However, these factors are not sufficient to predict the tumor's prognosis. Therefore, recent studies have focused on identifying molecular biomarkers potentially useful for the prediction of LSCC prognosis in an attempt to understand mechanisms driving aggressive tumor behavior and to identify patients who are at greatest risk for mortality.

Programmed cell death (PCD) is an essential and highly orchestrated process that plays a major role in morphogenesis and tissue homeostasis. Autophagy, a vacuolar process of cytoplasmic degradation, is characterized by accumulation of autophagic vacuoles, and is considered to be PCD type II. The exact role of autophagy in cancer remains controversial. Beclin-1 is a gene that indispensable for the first phases of autophagy. Silencing of the Beclin-1 gene with small interfering RNA (siRNA) is able to prevent downstream events of autophagy [5]. The Beclin-1 protein is monoallelically deleted in 75 % of ovarian cancers, 50 % of breast cancers, and 40 % of prostate cancers [6]. Moreover, Beclin-1−/− mutant mice die early in embryogenesis, and Beclin-1+/− mutant mice

suffer from a high incidence of spontaneous tumors, establishing that Beclin-1 is a critical component of mammalian autophagy and plays a role in autophagy of tumor suppression [7]. On the other hand, Ahn et al. [8] found that Beclin-1 is overexpressed in the malignant colorectal and gastric epithelial cells compared to the normal mucosal epithelial cells. These contradictory results prompt us to analyze Beclin-1 protein expression in LSCC. Wan Baoluo et al. demonstrated that Beclin-1 might play a role in carcinogenesis and development of laryngeal carcinoma for the decreased expression of Beclin-1 protein detected in LSCC, and there were significant differences in Beclin-1 expression regarding the tumor site, and the histological grade ($P<0.05$). To date, however, the prognostic significance of Beclin-1 expression in patients with LSCC is poorly understood.

Known prognostic factors for squamous cell carcinoma of the head and neck include stage, tumor size and lymph node metastasis, tumor grade and surgical tumor margin positivity [9, 10]. In the present study, we examined the expression levels of Beclin-1 protein in LSCC and paracarcinoma (paired) non-tumor tissues by immunohistochemistry. Meanwhile, the clinicopathological significance of Beclin-1 expression in LSCC was also assessed. The primary objective of this study was to evaluate the prognostic value of Beclin-1 in LSCC by assessing its association with overall survival, as well as known prognostic factors for the disease.

Materials and Methods

Patients and Tissue Samples

Tissue samples from 82 cases of LSCC were used in the present study. All of the tumors were surgically resected in the department of otolaryngology and head and neck surgery of Second XiangYa Hospital in Central South University from January 2005 to January 2007. All 82 patients met the following inclusion criteria: no history of previous malignancies; primary squamous cell carcinoma of the larynx only; and no history of radiotherapy or chemotherapy. In addition, 40 paracarcinoma (paired) non-tumor tissue samples removed in total or partial laryngectomy were included as control. For each case, 2 investigators reviewed all of the original hematoxylin and eosin stained sections.

The clinicopathological variables were evaluated, including age, sex, the primary tumor (pT), nodal (pN), the TNM stage group, tumor site and the histological grade. The age of the patients ranged between 40 and 77 years (median age, 60.20 years; SD=7.56 years). The 82 cases comprised 77 men and 5 women. Lymph node metastasis was classified as present (43 cases) or absent (39 cases). The TNM stage was classified in accordance with the International Union Against Cancer 2002 tumor-node-metastasis (TNM) staging system [11]. Tumor site was classified as supraglottic (26 cases), glottic (45 cases) and subglottic (11 cases). Tumor histological differentiation was categorized according to the World Health Organization system, as well differentiated (42 cases), moderately differentiated (26 cases) or poorly differentiated (14 cases). Clinicopathological characteristics of LSCC are collected and detailed in Table 1.

All the patients with incomplete clinical follow-up data were excluded from the study. Overall survival was defined as the time from operation to death or was censored at the last known alive data [12]. The mean patient follow-up duration was 41.87 months (SD=18.46 months; range, 6–60 months). Among a total of 82 patients, 48 (58.54 %) patients died of disease and 34 (41.46 %) patients remained alive on the day of starting this study. Among the 48 died patients, local recurrence occurred in 18 cases (37.5 %), regional lymph node occurred in 27 cases (56.25 %), distant metastasis occurred in 3 cases (6.25 %), including 1 to the brain, 2 to lung. Among the 34 alive patients, there were 27 cases (79.41 %) survive without recurrence, local recurrence occurred in 5 cases (14.71 %), regional lymph node occurred

Table 1 Correlation between Beclin-1 expression and clinicopathological parameters

	All cases	Beclin-1 protein expression		*P*-value
		Positive	Negative	
Age				
</=60	44	20	24	
>60	38	24	14	0.083
Sex				
Female	5	4	1	
Male	77	40	37	0.229
pT				
$T_{1–2}$	30	17	13	
$T_{3–4}$	52	27	25	0.427
Lymph node				
N_0	39	26	13	
N_+	43	18	25	0.021
Tumor stage				
I–II	22	14	8	
III–IV	60	30	30	0.199
Tumor site				
Subglottic	26	12	14	
Glottic	45	26	19	
Supraglottic	11	6	5	0.485
Histological grade				
Well differentiated	42	19	23	
Moderately differentiated	26	16	10	
Poorly differentiated	14	9	5	0.142

in 2 cases (5.88 %). Institute Research Medical Ethics Committee of Second XiangYa Hospital of Central South University granted approval for this study.

Immunohistochemistry

Consecutive 4-μm tissue sections were cut from the paraffin blocks of both laryngeal carcinoma tissues and paired non-tumor tissues. After dewaxing, one set of the samples slides was stained with hematoxylin and eosin (H&E), another set for immunohistochemical detection of Beclin-1 expression. Negative control experiments were conducted by replacing the primary antibody with phosphate-buffered saline. Antigen retrieval was performed and the sections were cooled and treated with peroxidase-blocking reagent for 20 min, rinsed by PBS (PH=7.4) for 3 times, and treated with sufficient primary antibody (Beclin-1, No. 2026-1, the United States Epitomics Company's products) for 14~16 h in 4 °C, the density is 1:50. Sections were rinsed again and treated for 15 min with polymerized HRP-anti mouse/rabbit IgG (Maxvision tm). Sections were rinsed again and treated with visualization reagent solution (DAB) for 3~5 min. Sections were counterstained with hematoxylin, and then mounted in Permount. Controls without primary antibody and positive control tissues were included in all experiments to ensure the staining quality.

Evaluation of the Immunohistochemical Staining

After immunohistochemical stain, Beclin-1 was detected in both non-tumor tissues and LSCC tissues. The immunoreactivity of the granulosa-lutein cells in the Beclin-1 expression were analyzed with a semi-quantitative scoring method. The score was calculated according to the intensity and proportion of the immunoreactivity. The intensity score was assigned: 0 (negative staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining), as was a proportion score: 0 (none), 1 (less than 30 % of tumor cells), or 2 (over 30 % of tumor cells). The intensity score and proportion score were multiplied to yield the total score, which was considered negative between 0 and 1 and positive between 2 and 6 [13].

Statistical Analysis

Statistical evaluation was performed using the SPSS software package (SPSS 16.0, SPSS Inc., Chicago, USA). The Chi-square test was used to analyze the correlation between Beclin-1 expression and clinicopathological features. The relationship between Beclin-1 protein expression and LSCC patients' overall survival was assessed using Kaplan–Meier estimates. Differences in survival rate between subgroups were compared using the log-rank test. A Cox proportional hazard model was used to assess factors independently associated with prognosis. A p-value <0.05 was considered to be statistically significant.

Results

Beclin-1 Expression in Primary Carcinomas and Non-Tumor Tissues

The expression of Beclin-1, as determined by immunohistochemical staining, appeared as fine granular and diffuse cytoplasmic staining with sporadic nuclear staining, was stronger in non-tumor tissues. In primary carcinomas, the ratio of negative expression of Beclin-1 is higher than that in non-tumor tissues, and Beclin-1 expression is downward (Fig. 1). Beclin-1 was negatively expressed in primary carcinomas of 38 cases (46.34 %), positively expressed of 44 cases (53.66 %). In the non-tumor tissues, Beclin-1 was negatively expressed in the non-tumor tissues of 11 cases (27.5 %), positively expressed of 29 cases (72.5 %). There were significant differences between the primary carcinoma compared with the non-tumor tissues (P=0.035).

Correlations of Beclin-1 Expression and Clinicopathological Features

While analyzing clinicopathological factors, lymph node metastasis showed statistically significant correlations with Beclin-1 expression. Namely, negative expression of Beclin-1 was associated with increased lymph node metastasis (P=0.021); however, there was no significant difference between Beclin-1 expression and other characteristics (age, sex, the primary tumor, the TNM stage group, tumor site and the histological grade) (Table 1).

Correlations of Beclin-1 Expression and Survival

Based on Beclin-1 protein immunohistochemistry results, the 82 LSCC patients were divided into two groups: 38 patients in the Beclin-1 negative expression group and 44 patients in the Beclin-1 positive expression group. During the follow-up period, 48 patients died within 5 years after surgery. Among these 48 patients, 28 had Beclin-1 expression negatively, 20 had expression positively. Using Kaplan–Meier analysis, we found that Beclin-1 expression negatively correlated with a lower survival rate (log-rank test, P=0.005; Fig. 2).

The Cox proportional hazards regression model was used to assess the relative influence of clinicopathological parameters and Beclin-1 expression on the disease prognosis. In the univariate Cox analysis, lymph node metastases (P=0.011), tumor stage (P=0.028), and Beclin-1 expression (P=0.006) were statistically correlated with prognosis (Table 2). Multivariate Cox proportional hazards model analysis confirmed that

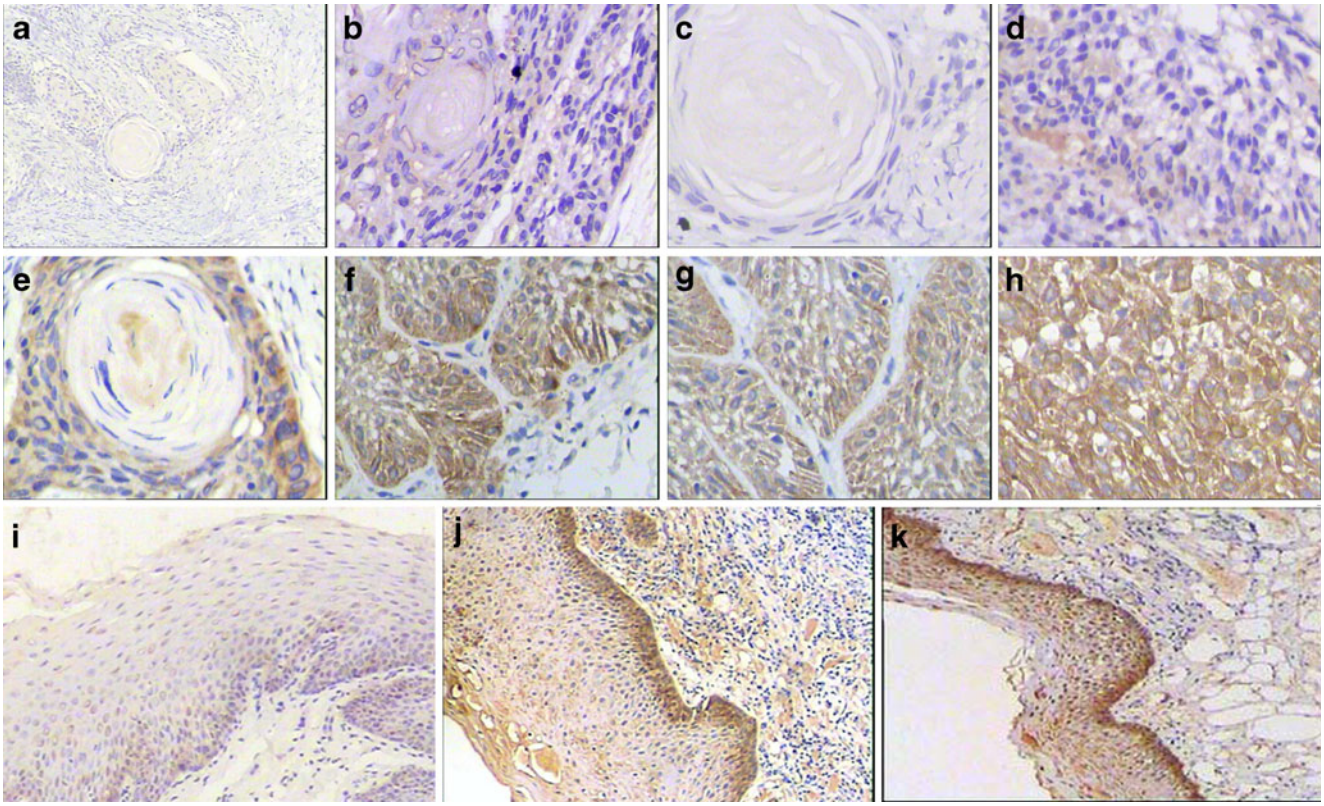

Fig. 1 Representative immunohistochemical staining for Beclin-1 in primary human LSCC tissues and adjacent noncarcinoma epithelial tissues. Negative expression of Beclin-1 in LSCC tissues (**a**, **b**, **c**, **d**). Positive expression of Beclin-1 in LSCC tissues (**e**, **f**, **g**, **h**). Negative expression of Beclin-1 in adjacent non-carcinoma epithelial tissues (**i**). Positive expression of Beclin-1 in adjacent non-carcinoma epithelial tissues (**j**, **k**). (original magnification **a**, **i**–**k**, ×100; **b**–**h**, ×400)

lymph node metastases (P=0.048) and Beclin-1 expression (P=0.029) were both independent prognostic factors for LSCC. Other parameters cannot predict disease prognosis separately.

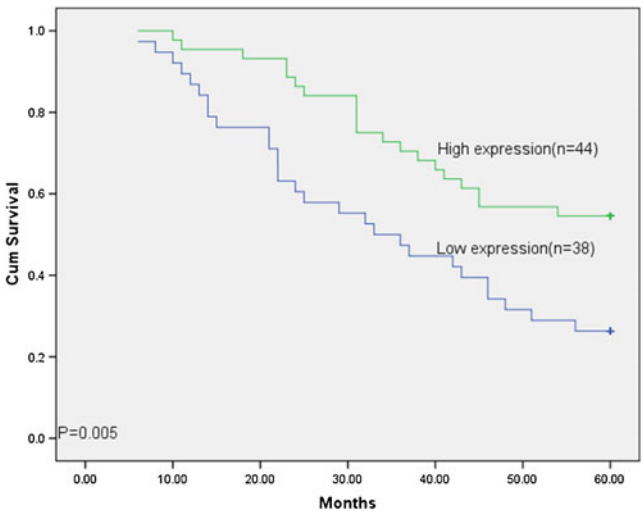

Fig. 2 Kaplan–Meier survival curves showing 5-year survival rate of patients with high or low Beclin-1 expression. (p=0.005; log-rank test)

Discussion

The problem of autophagy in cancer cells causing death or protection of cells is controversial. Several studies showed that carcinogenesis is associated with decreased levels of autophagy [8]. Mathew et al. reported that compromised autophagy can increase DNA damage and genomic instability which can promote tumorigenesis [14]. 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 under nutrient-limiting conditions [15]. Amaravadi [16] presented results supporting the idea that autophagy can protect tumor cells from cell death stimuli. Given that various stress conditions, such as hypoxia and high acidity, autophagy may assume a dominant role in survival and growth of tumor cells. Beclin-1 can play a role in recruiting proteins from the cytosol for autophagic degradation or in supplying the autophagic pathway with membrane components. Lots of observations indicate that expression of Beclin-1 may play a role in the tumorigenesis and/or progression of human cancers [17].

In this study, we detected the expression of Beclin-1 by immunohistochemistry in 40 specimens of non-tumor tissues

Table 2 Cox proportional hazards regression model analysis: correlations of clinicopathological parameters, Beclin-1 expression and prognosis

Clinicopathologic variables	Univariate analysis		Multivariate analysis	
	Exp(B)	*P*	Exp(B)	*P*
Age				
</=60				
>60	–	0.926	–	–
Sex				
Female				
Male	–	0.458	–	–
pT				
$T_{1–2}$				
$T_{3–4}$	–	0.204	–	–
N0				
N_+	2.146	0.011	1.837	0.048
Tumor stage				
I–II				
III–IV	2.342	0.028	–	0.284
Tumor site				
Supraglottic				
Glottic/subglottic		0.300	–	–
Histological grade				
Well differentiated				
Moderately/poorly differentiated	–	0.999	–	–
Beclin-1 expression				
Low expression				
High expression	−0.670	0.006	−0.720	0.029

and 82 specimens of LSCC tissues. We found that the positive expression of Beclin-1 is significantly higher in the samples of non-tumor tissues than those in LSCC tissues (72.5 % versus 53.66 %, $p=0.035$), confirming the finding of the previous study [9]. Autophagy is relatively defective in LSCC cells, indicating a tumor suppressive role for autophagy. The exact mechanism of this role is still unclear. However, researchers have proposed several hypotheses. First, autophagy suppresses tumorigenesis by limiting necrosis and inflammation in tumor microenvironment. When autophagy and apoptosis in tumors are inhibited, metabolic stress specifically induces necrosis, followed by chronic inflammatory response, which contributes to tumor initiation and progression. Second, autophagy produces ATP at minimum amount levels required for DNA repair. Third, damaged organelles, unfolded proteins, and reactive oxygen species (ROS) may be removed from cells by autophagy, which indirectly limits genomic instability [18]. Further studies are warranted to determine the exact role of autophagy in carcinogenesis and its mechanisms.

In addition to tumorigenesis, autophagy has important roles in different cancer stages including tumor progression and metastasis [19]. Previous studies showed that Beclin-1 deficiency was related to the aggressiveness of brain tumors and immortalized kidney and mammary epithelial cells [20]. Low growth rate and decreased invasive and metastatic ability are, therefore, presumed for Beclin-1 overexpression in LSCC. The correlations between Beclin-1 expression and various clinicopathological characteristics were analyzed. Reduced Beclin-1 expression in specimens of LSCC was significantly correlated with increased lymph node metastases ($p=0.021$). Decreased expression of Beclin-1 was closely associated with a metastatic phenotypic feature.

The overall survival of the patients was significantly related to the UICC stage, in agreement with previous reports that the initial classification (N stage) is significant prognostic factors for survival in LSCC [21]. This premise was further substantiated by Kaplan–Meier analysis results: the survival rate of the Beclin-1 positive group was better than that in the Beclin-1 negative group ($p=0.005$). This result is consistent with those of previous studies indicating that Beclin-1 is a novel favorable predictor of outcome for several solid tumors, such as breast, ovarian, brain, liver, and colon cancers [22, 23].

The prognostic significance of Beclin-1 had been studied in several types of solid tumors. In colon cancer, higher expression of Beclin-1 was reported to carry a better prognosis [12]. Similar results were also found in hepatocellular carcinoma [24]. To our knowledge, no previous studies have evaluated the prognostic value of Beclin-1 in LSCC patients. We fitted this expression level into the Cox regression model, together with other, more conventional clinicopathological parameters. It is widely accepted that lymph node metastasis is a useable prognostic predictor of LSCC [25]. Additionally, the incidence of local recurrence and risk of distant metastases increase as the tumor burden in the neck increases [26]. These premises were confirmed by our study, as univariate Cox analysis results: lymph node metastases, tumor stage and Beclin-1 expression were statistically correlated with prognosis. As the grade of the malignancy increases, so does the risk of blood/lymphatic vessel invasion and distant metastases, cancers of the larynx with reduced Beclin-1 expression tend to metastasize earlier and are more often diagnosed at the advanced tumor stage, all these reasons may lead to a worse prognosis in reduced Beclin-1 expression of LSCC. In the multivariate Cox proportional hazards model analysis, lymph node metastases and Beclin-1 expression were statistically significant, independent predictors of prognosis. The Exp (B) value of Beclin-1 expression was negative, which showed this parameter was protective factors in the prognosis, high Beclin-1 expression tumors might have better prognosis than low Beclin-1 expression tumors.

High Beclin-1 protein expression was favorable prognostic indicator for LSCC. It does not conform to the study of Wan et

al., who reported that overexpression of Beclin-1 was predictor of poor prognosis in nasopharyngeal carcinoma [27]. This inconsistent role of Beclin-1 in clinical outcome may have resulted from the different properties of the tumor types studied and different therapeutic regimens. Beclin-1 is a known tumor suppressor gene, but its function may be altered under conditions (such as starvation, low oxygen, and hormonal stimulation) of an accelerated autophagic activity, which provides additional energy to proliferate cells by recycling defective organelles and long-lived cytoplasmic proteins. In a previous study, both underexpression and overexpression of Beclin-1 in colorectal carcinomas were linked with poor postoperative outcome [28]. In addition, it has been reported that Beclin-1 expression in breast cancer is related to aggressive features of the cancer cells, but a significant prognostic implication was not reached [29]. These studies revealed a biphasic action of the Beclin-1 protein. In this study, we found that decreased expression of Beclin-1 was linked with poor prognosis of LSCC. Apparently, the role of Beclin-1 in human tumors might be much more complex than what it is generally thought. In further studies of this issue, direct detection, induction, and inhibition of autophagy and expression of Beclin-1 that regulate it will be indispensible to verifying the molecular mechanism.

Conclusion

It is concluded that Beclin-1 has a significant impact on growth and prognosis of human LSCC. The expression of Beclin-1 is down-regulated in LSCC. Low Beclin-1 expression was linked with increased lymph node metastases and poor prognosis, thus Beclin-1 appears to be a promising molecule in LSCC targeting therapy. Further studies are necessary in order to clarify the impact of Beclin-1 and autophagy related proteins on the prognosis of human malignancies.

Acknowledgments We thank Dr Ting Zhang for collecting patients' survival data. We thank Daiqiang Li and Songqing Fan (Department of Pathology, Second Xiangya Hospital, Central South University) and Yunjun Deng (Central Laboratory of Medical Research, Second Xiangya Hospital, Central South University) for their evaluation of these clinical samples.

The authors have no funding, financial relationships, or conflicts of interest to disclose.

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Dr Xinming Yang takes responsibility for the integrity of the content of the paper.