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Original Article

Immunohistochemical assessment of autophagic protein LC3B and p62 levels in glioma patients

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Abstract: Glioma is a serious malignant central nervous system disease. Autophagy is a basic cellular catabolic mechanism maintaining the cellular homeostasis through degradation of unnecessary molecules and components and reusing them. Autophagy also promotes development, progression and anticancer therapy resistance of many types of human cancers. In this study, we detected the expression of two autophagic protein LC3B and p62 in 81 glioma tissues by immunohistochemistry analysis. LC3B and p62 was highly expressed in high-grade glioma tissues, compared with low-grade glioma tissues. High levels of LC3B and p62 protein were also associated with advanced tumor stages, worse relapse-free survival (RFS) and overall survival (OS) in glioma patients, but not with patients' age, gender or KPS. Additionally, there was a statistically positive correlation between the expression of LC3B and p62 in glioma tissues. Therefore, we determined LC3B and p62 which contributed to autophagy behavior promoted development and poor prognosis of malignant gliomas. Therapeutic methods based on autophagy or targeting LC3B or p62 may be considered as a potential therapeutic strategy to retard progression of malignant gliomas.

Keywords: LC3B, p62, autophagy, glioma

Introduction

Malignant glioma remains the most common disease causing death in patients with intracranial tumors, which a more than 70% of primary malignant central nervous system tumors [1-3]. Surgical resection with adjunctive radiation therapy or chemotherapy is the only option for malignant glioma patients, which invariably exhibits a bad prognosis. The median survival of patients with gliomas is less than 1 year [1, 2, 4]. Several mechanisms tried to explain the etiology of gliomas, such as embryonic brain abnormalities, genetic factors, chemical factors, etc., but have not got a convincing consensus [2]. Exploring the molecular pathogenesis of the disease to develop novel targeted therapies is necessary and urgent.

Autophagy is a cellular renewal process, which degrades the unnecessary cellular molecules and components and then utilizes the breakdown molecules and components maintaining the cellular homeostasis [1, 5-8]. Autophagic activity is a good mechanism for cell survival in response to different environments [1, 8, 9]. Recently, more and more studies reported the important role of autophagy in development and progression of human cancers [1, 10]. Moreover, recent studies showed autophagy also determines the response of cancer cells to anticancer therapy [11-14]. Specially, autophagy was demonstrated to participate in malignant behavior of gliomas [1, 2, 11].

As previous reported, microtubule associated protein 1 light chain 3 (LC3) and p62/SQSTM1 (p62) protein are essential markers for autophagy [11, 15-18]. LC3A, LC3B, and LC3C are three isoforms of LC3. LC3B and p62 were reported to be significant in predicting the prognosis of patients with breast cancer or oral squamous cell carcinoma, etc. [15, 19]. But it was still unknown about the roles of LC3B and p62 in glioma.

Here, we collected 81 tissues of malignant gliomas and evaluated the expression of LC3B and

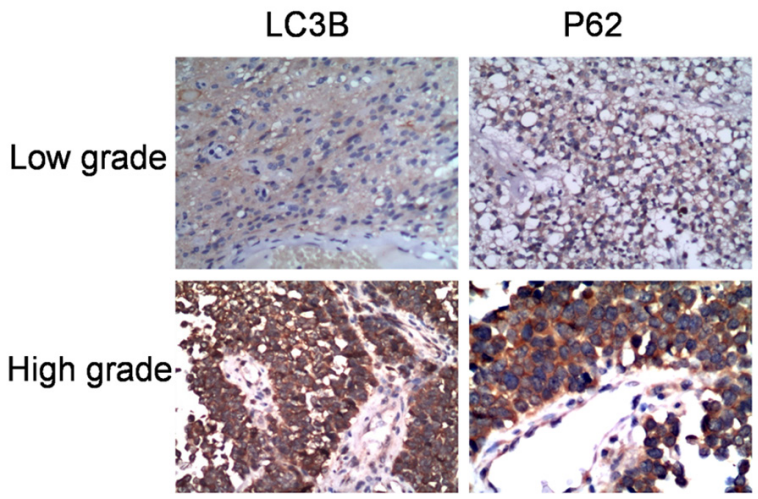

Figure 1. LC3B and p62 protein expressed more in high-grade glioma tissues than in low-grade glioma tissues. Expression of LC3B and p62 proteins in glioma tissues was detected using Immunohistochemistry method. Representative pictures were selected to show the difference of LC3B and p62 in high-grade and low-grade glioma cases. Both LC3B and p62 expression were high in high-grade glioma tissues, and were low in low-grade glioma tissues.

p62. We found an increased expression of both LC3B and p62 in high-grade gliomas tissues, and high expression of LC3B or p62 was associated with patient worse relapse-free survival (RFS) and overall survival (OS). Moreover, a positive correlation was observed in the expression of LC3B and p62 in gliomas, which indicated that LC3B and p62 might have a combined effect to promote tumorigenesis and progression of gliomas. Therefore, we provided evidence that autophagy related protein LC3B and p62 can be used as new therapeutic targets for glioma therapy.

Materials and methods

Patients and tissue samples

81 glioma cases were retrieved from archive of the Department of Pathology, Anhui Medical University, who underwent surgery at the Second Affiliated Hospital of Anhui Medical University (Hefei, P. R. China) between 2009 and 2013. All of the patients were not treated with adjuvant therapy before surgical resection. We followed up these patients for more than 3 years. Tumors were graded according to Edmondson-Steiner grading system and staged according to American Joint Committee on Cancer staging system [20, 21]. The current protocol for use of tissue samples was approved by the Institutional Review Board, and written informed consent was obtained from each patient.

Immunohistochemistry

Immunohistochemistry analysis was performed as described earlier [22]. Immunohistochemistry was performed using the following antibodies: mouse monoclonal antibodies against LC3B (1:100, Cell Signaling Biotechnology, Danvers, MA, USA) and p62 (1:200, Abcam, Cambridge, UK).

Assessment of immunohistochemical staining

Two experienced pathologists reviewed and scored the stained slides under an Olympus microscope (Olympus America Inc., Melville, NY) independently. Before scored, they had got no information of the patients' identities or clinical status. As in previous studies [23, 24], we used two-tier grading system to analyze the LC3B and p62 staining levels and classified them to low expression group versus high expression group.

Respectively, LC3B-stained tissues were scored in three 200× fields, when positive cells were above 50%, the case was graded as LC3B high; when below 50%, the case was graded as LC3B low. For p62 staining, we also scored the cased in three 200× fields, but we used 30% to distinguish the p62 high level and p62 low level.

Statistical analysis

All statistical analyses were performed using SPSS software for Windows (version 13.0; SPSS, Chicago, IL, USA). Pearson's chi-square test was used to study the relationship of LC3B expression and p62 expression in glioma tissues. We constructed Kaplan-Meier curves to determine patients' relapse-free survival (RFS) and overall survival (OS) according to LC3B expression level or p62 expression level respectively. The statistical differences in survival among subgroups were compared using log-

Table 1. Association of LC3B and p62 expression with clinicopathological parameters from glioma patients

		LC3B expression (n (%))		P62 expression (n (%))	
Parameter	*n*	High	*P*	High	*P*
Age (years)					
<50	52	40 (76.9)	0.804	22 (42.3)	0.935
≥50	29	23 (79.3)		12 (41.4)	
Gender					
Female	32	25 (78.1)	0.952	10 (31.3)	0.114
Male	49	38 (77.6)		24 (49.0)	
KPS					
<80	28	23 (82.1)	0.492	12 (42.9)	0.907
≥80	53	40 (75.5)		22 (41.5)	
Grade					
I-II	38	25 (65.8)	0.015	9 (23.7)	0.002
III-IV	43	38 (88.4)		25 (58.1)	

rank test. Spearman's coefficient was calculated to determine the association between expression of LC3B and P62 proteins. $P < 0.05$ was considered as statistically significant.

Results

Association of LC3B and p62 expression with clinicopathological parameters from glioma patients

Recent studies have reported the important role of autophagy in diverse kind of human cancers including glioma, which not only regulated cancer development and progression, but also contributed to resistance to radiation therapy/chemotherapy [1]. Many autophagy related proteins were demonstrated to affect cancer behavior, such as LC3A, LC3B, p62, Beclin 1, ULK1, ULK2, XIAP, etc. Especially, these proteins also played an important role in glioma [3]. In our study, we selected LC3B and p62 for further study in 81 glioma tissues. Firstly, we did immunohistochemistry analysis of LC3B and p62 in all of the tissues. Remarkably, the expression of both LC3B and p62 was higher in high-grade glioma than that in low-grade glioma tissues (**Figure 1**). Furthermore, to investigate the relationship between LC3B and p62 levels and other important clinicopathological parameters in glioma patients, we analyzed the association of LC3B and p62 expression with patients' age, gender, KPS, and tumor grade. As shown in the **Table 1**, LC3B protein level was significantly associated with advanced tumor stages (P=0.015). Similarly, p62 protein level was also significantly associated with advanced tumor stages (P=0.002). But there was no significant association between expression level of LC3B and p62 and patients' age, gender or KPS (**Table 1**).

Prognostic significance of LC3B and p62 expression in glioma patients

For further study, we want to know the association of LC3B and p62 expression with RFS and OS in these glioma patients. Kaplan-Meier curves were prepared and stratified with LC3 and p62 expression respectively (**Figure 2**). Obviously, patients with high LC3B protein level had a much lower mean 3-yr RFS and OS than the patients with low LC3B level (P=0.044 and P=0.022). On the other hand, patients with high p62 protein level had a lower mean 3-yr OS than the patients with low p62 level (P=0.001), whereas the difference of RFS was not significant (P=0.594).

Correlation of LC3B and p62 expression in glioma patients

Next, we analyzed the correlation of LC3B and p62 expression in these glioma patients. As showed in **Table 2**, there was a statistically positive correlation between LC3B and p62 protein level (P=0.013). Pearson's correlation coefficient was 0.274.

Discussion

In this study, we documented that the autophagy related proteins, LC3B and p62, were positively correlated to the disease progression of glioma. There was a significant association of LC3B and p62 protein level with higher stage and worse prognosis in patients with glioma, respectively. Here, we made comprehensive analysis of LC3B and p62 with clinicopathological parameters from glioma patients, and this has an important medical value and social significance.

Autophagy is a basic cellular catabolic mechanism maintaining the cellular homeostasis through degradation of unnecessary molecules

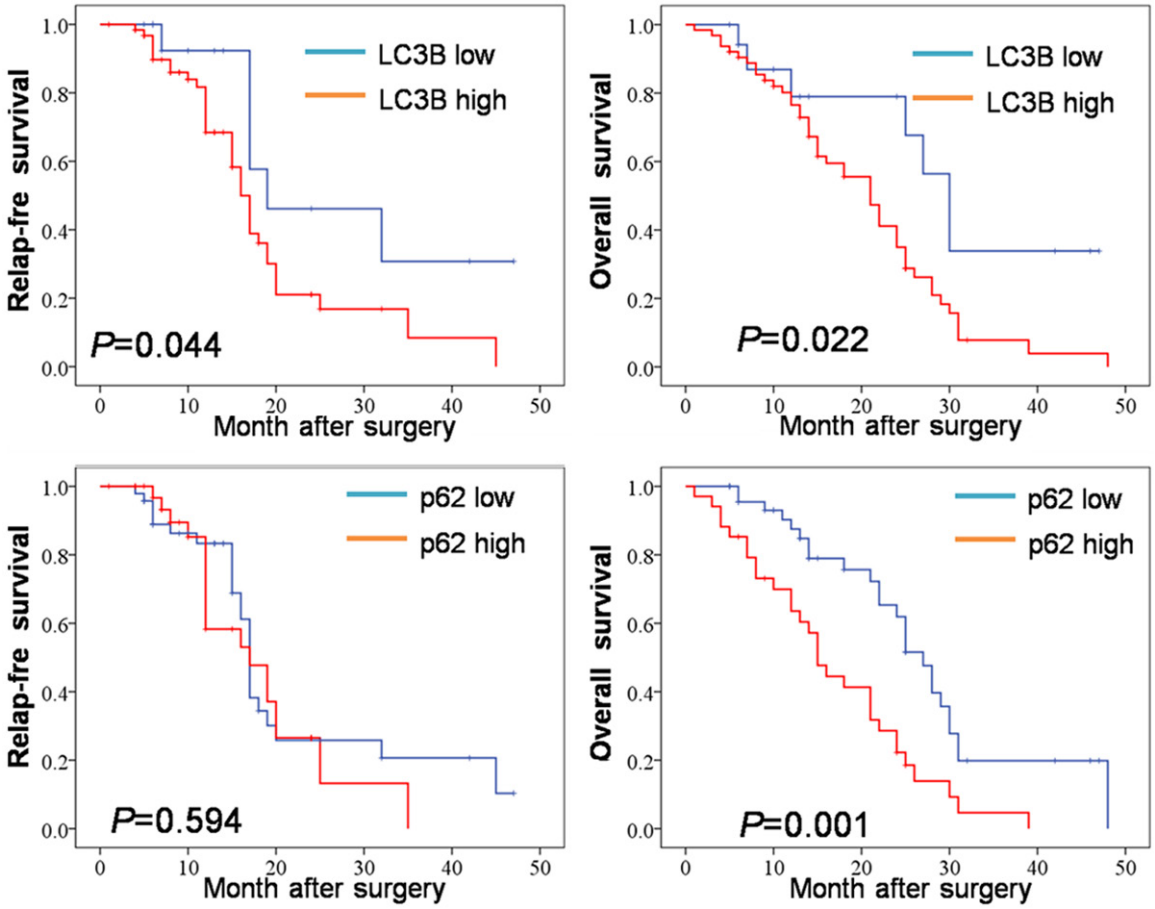

Figure 2. Relapse-free and overall survival curves stratified by LC3B or p62 level. Glioma patients with high expression of LC3B or p62 were associated with worse relapse-free survival and overall survival.

Table 2. Correlations of LC3B and p62 expression in glioma patients were analyzed

Correlations		LC_s	P62_s
LC_s	Pearson Correlation	1	.274*
	Sig. (2-tailed)		.013
	N	81	81
P62_s	Pearson Correlation	.274*	1
	Sig. (2-tailed)	.013	
	N	81	81

*. Correlation is significant at the 0.05 level (2-tailed).

and components and reusing them [1, 3, 5-7, 25]. It is a good pathway of cells to protect themselves against metabolic stress by autophagy [26-29]. Recent studies showed that cancer cells also use autophagy to adapt stressful conditions, so autophagy could promote development, progression and anticancer therapy resistance of many kinds of human cancers [1, 10-14, 30]. As reported previously, LC3 is associated with the membrane of autophagosomes participating in autophagy, and LC3 is widely used as an autophagy marker to study autophagy related behavior. Moreover, LC3 can be tested using LC3 antibody instead of other methods in clinical tissue specimens recently [11, 15-18, 24, 31]. Many studies revealed high expression of LC3 associates with poor prognosis in diverse cancer patients through regulating the activity of autophagy [32-35]. In malignant gliomas, a few studies documented the association of autophagy with cancer aggravation [1, 2, 11]. However, to our knowledge, there was no study systematically investigating the relation between the expression of LC3 and clinical parameters of glioma patients. LC3 consists of three isoforms: LC3A, LC3B, and LC3C [15, 19]. In immunohistochemical testing, LC3A shows three patterns: diffuse cytoplasmic, cytoplasmic/juxtanuclear, and stone-like pattern, and which pattern represented the functional LC3A involved in autophagy remains

confusing [15, 24, 36, 37]. In all tissues, LC3C is much lower than that of LC3A and LC3B. So LC3B was selected to study the relation between LC3 and clinical behavior of glioma patients here, which has a high expression level and has a defined punctate pattern by immunohistochemical testing participating in autophagy. Results in our study showed high level of LC3B associated with glioma progression and worse prognosis of patients, which inferred autophagy played an activating role to promote cancer progression in glioma patients.

Rather than tested only one marker, we used another autophagy marker p62 to evaluate the role of autophagy in glioma patients. P62 is an important substrate of autophagy, which is widely used as a marker to determine autophagy procedure [15, 38]. Our data documented high level of p62 in glioma tissues represented high grade, worse prognosis of patients. These results were accordant with other studies which reported that increased expression of p62 was associated with poor prognosis and tumor development in many types of human cancers [39-42]. In our research, we found expression of LC3B and p62 were positive correlated in the progression of glioma. As known previously, LC3 participated in constructing of autophagosomes, and p62 participated in autophagic degradation as an important substrate [15, 38]. So, our result suggests that LC3B and p62 might involve in the whole procedure of autophagy and impact the development and prognosis of malignant gliomas.

In a word, this is the first study systematically analyzed the expression of LC3B and p62 in glioma patients. High level of autophagic protein LC3B and p62 represented aggressive behaviors of malignant gliomas and poor prognosis. Autophagy played an important role in gliomas. Therapeutic methods based on autophagy or targeting autophagy related genes offered a new idea to treat gliomas.

Disclosure of conflict of interest

None.

Abbreviations

IHC, Immunohistochemistry; LC3, microtubule associated protein 1 light chain 3; OS, overall survival; RFS, relapse-free survival.

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