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The "stone-like" pattern of LC3A expression and its clinicopathologic significance in hepatocellular carcinoma

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ABSTRACT

Autophagy is an evolutionarily conserved process that involves lysosomal degradations of cellular organelles. Microtubule-associated protein 1 light chain 3A (LC3A), an autophagic gene, is differentially expressed in human cancers. However, the relationship between LC3A expression and hepatocellular carcinoma (HCC) has not been investigated. Tissue microarray-based immunohistochemistry was used to examine the expression patterns of LC3A in HCC. The resulting data were analyzed using receiver operating characteristic curves, Spearman's rank correlation, Kaplan–Meier plots and Cox proportional hazards regression modeling. Two distinct patterns of LC3A expression were observed in HCC: "stone-like" structuring and diffuse cytoplasmic expression. High levels of LC3A expression were more frequently observed in HCC tissues compared to the adjacent non-tumorous tissue. Correlation analyses indicated that high expression of the "stone-like" LC3A was correlated with greater levels of serum AFP, poorer tumor differentiation and the presence of vascular invasion. Kaplan–Meier survival analysis showed a significant association between high expression of the "stone-like" LC3A and unfavorable prognosis ($P < 0.001$). Importantly, multivariate analysis ($P < 0.05$) identified the "stone-like" expression of LC3A in HCC as an independent prognostic factor. Collectively, our data provide compelling evidence that "stone-like" expression of LC3A plays an important role in HCC progression and may act as a biomarker of prognosis for patients with HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers. The incidence and mortality of HCC have recently increased [1]. Due to the high prevalence of hepatitis B virus (HBV) infection in Chinese populations, HBV-related HCC is a major fatal disease in China [2]. Early detection of HCC allows for curative or palliative treatment with surgical resection or transcatheter arterial chemoembolization [3]. Nevertheless, the prognosis of patients with HCC remains dismal despite recent advances in surgical techniques and medical treatments [4]. Therefore, the exploration of promising prognostic factors and therapies for HCC is of high clinical importance.

Autophagy is a degradation pathway that delivers cytoplasmic materials to lysosomes via double-membraned organelles (i.e., autophagosomes) that enclose a portion of the cytoplasm [5]. Autophagy has emerged as a homeostatic mechanism, which regulates the turnover of long-lived or damaged proteins and organelles. This process is also thought to buffer the metabolic stress induced under starvation conditions by forming intracellular constituents [6]. As is observed for other major cellular functions (such as division, differentiation and cell death), autophagy is perturbed in cancer cells [7]. Studies of the molecules involved in the control and execution steps of autophagy have highlighted the close relationship between autophagy and tumor progression. Microtubule-associated protein light-chain 3 (LC3) is a mammalian homolog of the yeast protein Atg8p and has been identified as a crucial component of autophagosomes. Three isoforms of LC3 (LC3A, LC3B and LC3C) were found to reside in the autophagosomal/autolysosomal membranes [8–10]. There is an increasing body of evidence that up-regulation of LC3A expression occurs in various human malignancies, including breast cancer, colorectal cancer, lung cancer, and cutaneous squamous cell carcinomas. The over-expression of this autophagic protein has been shown to be closely correlated with tumor aggressiveness and poor prognosis [11–15]. However, the expression pattern of LC3A and its prognostic significance in HCC have not been elucidated.

In the current study, tissue microarray-based immunohistochemistry (IHC) was utilized to examine the expression of LC3A

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in an HCC cohort. Receiver operating characteristic (ROC) curve analysis was conducted to define the cut-off value for separating LC3A expression into high- and low-expression groups. In addition, the clinical and prognostic significance of LC3A expression levels in HCC were discerned through statistical analyses.

2. Materials and methods

2.1. Patients and tissue specimens

For this study, paraffin-embedded pathological specimens from 125 patients with HCC were obtained from the archives of the Department of Pathology, Sun Yat-sen University Cancer Center, Guangzhou, China, between July 2005 and May 2008. The cases were selected based on the following characteristics: pathological diagnosis of HCC; primary and curative resection for tumor without preoperative or postoperative anticancer treatment; and the availability of resection tissue and follow-up data. The HCC cohort included 108 (86.4%) men and 17 (13.6%) women with a mean age of 47.7 years. The average follow-up time was 26.79 months (median, 28.0 months; range, 1.0–61 months). The clinicopathologic features detailed in Table 1 include age, sex, hepatitis history, serum alpha-fetoprotein (AFP) level, the presence of cirrhosis, the number of tumors, tumor size, level of tumor differentiation, tumor stage, the extent of vascular invasion and relapse occurrence. Tumor differentiation was determined based on the criteria proposed by Edmonson and Steiner [16]. Tumor stage was defined according to the tumor-node-metastasis (TNM) classification system from the American Joint Committee on Cancer/International Union Against Cancer [17]. The Institute Research Medical Ethics Committee of Sun Yat-sen University Cancer Center granted approval for this study.

2.2. Tissue microarray (TMA) construction

Tissue microarrays were constructed as described previously [2]. Briefly, formalin-fixed, paraffin-embedded tissue blocks and the corresponding H&E-stained slides were overlaid for TMA sampling. A senior pathologist (M.-Y. Cai) reviewed the slides to identify and mark representative tumor areas. Triplicate cylinders (0.6 mm diameter) were punched from the representative tumor areas and from adjacent non-malignant liver tissue from the individual donor's tissue blocks; these cylinders were then re-embedded into a recipient paraffin block at defined positions using a tissue arraying instrument (Beecher Instruments, Silver Spring, MD).

2.3. Immunohistochemistry (IHC)

The TMA block was cut into 4-μm sections and processed for IHC in accordance with a previously described protocol [18]. The TMA slide was subjected the following steps: drying overnight at

Table 1
The correlation between the two patterns of LC3A reactivity and patients' clinicopathological features in primary hepatocellular carcinomas.

Variable	All cases	Stone-like		Diffuse cytoplasmic	
		High expression	*P* value[a]	High expression	*P* value[a]
Age (years)			0.769		0.659
⩽47.7[b]	61	34 (55.7%)		30 (49.2%)	
>47.7	64	34 (53.1%)		34 (53.1%)	
Sex			0.694		0.877
Male	108	58 (53.7%)		55 (50.9%)	
Female	17	10 (58.9%)		9 (52.9%)	
HBV			0.095		0.717
Positive	106	61 (57.5%)		55 (51.9%)	
Negative	19	7 (36.8%)		9 (47.4%)	
AFP (ng/ml)			<0.001		0.809
⩽20	69	26 (37.7%)		36 (52.2%)	
>20	56	42 (75.0%)		28 (50.0%)	
Liver cirrhosis			0.604		0.571
Yes	87	46 (52.9%)		46 (52.9%)	
No	38	22 (57.9%)		18 (47.4%)	
Tumor size (cm)			0.219		0.009
⩽5	76	38 (50.0%)		46 (60.5%)	
>5	49	30 (61.2%)		18 (36.7%)	
Tumor multiplicity			0.793		0.179
Single	87	48 (55.2%)		48 (55.2%)	
Multiple	38	20 (52.6%)		16 (42.1%)	
Differentiation			0.003		0.447
Well-moderate	86	39 (45.3%)		46 (53.5%)	
Poor-undifferentiated	39	29 (74.4%)		18 (46.2%)	
Stage			0.181		0.128
I–II	62	30 (48.4%)		36 (58.1%)	
III–IV	63	38 (60.3%)		28 (44.4%)	
Vascular invasion			0.018		0.809
Yes	56	37 (66.1%)		28 (50.0%)	
No	69	31 (44.9%)		36 (52.2%)	
Relapse			0.413		0.851
Yes	42	25 (59.5%)		22 (52.4%)	
No	83	43 (51.8%)		42 (50.6%)	

[a] Chi-square test.
[b] Mean age; HBV, hepatitis B virus; AFP, alpha-fetoprotein.

37 °C, deparaffinization in xylene, rehydration through a graded alcohol series, immersion in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, and antigen-retrieval by pressure cooking for 3 min in citrate buffer (pH = 6.0). Then, the slide was preincubated with 10% normal goat serum at room temperature for 30 min to reduce nonspecific reactivity. Subsequently, the slide was incubated with rabbit polyclonal anti-LC3A antibody (Abcam, Cambridge, MA, 1:100 dilution) and stored overnight at 4 °C. The slide was next washed with PBS (2 × 5 min) and then incubated with a secondary antibody (Envision; Dako, Glostrup, Denmark) for 1 h at room temperature. Thereafter, the section was washed with PBS twice for 5 min and stained with 3,3-diaminobenzidine (DAB). Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. A negative control was obtained by replacing the primary antibody with normal murine IgG. Verified, positive immunostaining slides were used as positive controls.

2.4. IHC evaluation

Two distinct patterns ("stone-like" and diffuse cytoplasmic) of LC3A expression in HCC were identified, as described in previous studies [11]. Briefly, the "stone-like" structure was evaluated by counting in all of the available fields in a spot at 400× magnification and expressed as the mean value of the triplicate experiments. The diffuse cytoplasmic pattern was assessed as follows: Each TMA spot was assigned an intensity score from 0–3 (I0, I1–3). Then, the proportion of tumor cells with that intensity was divided by the total number of tumor cells and recorded in 5% increments from 0 to 100 (P0, P1–3). The final *H* score (range 0–300) was determined by adding the sum of the scores obtained for each intensity and the proportion of the area stained (H score = I1 × P1 + I2 × P2 + I3 × P3). Expression of the two patterns of LC3A was assessed by two independent pathologists (M.-Y. Cai and S.-Y. Xi), who were blinded to the clinicopathological data.

2.5. Selection of cutoff score

The ROC curve analysis was used for the selection of a LC3A cutoff score (for both the "stone-like" and diffuse cytoplasmic patterns) by a 0, 1-criterion [19,20]. Briefly, the sensitivity and specificity for the evaluated outcome were plotted to generate ROC curves (Fig. 1). The score localized closest to the point (i.e., 0.0, 1.0) at the maximum sensitivity and specificity was selected as the cutoff score to determine the greatest number of tumors that were correctly classified as having or not having the outcome. To facilitate the ROC curve analysis, the clinicopathologic features were dichotomized as follows: AFP level (⩽20 ng/ml vs. >20 ng/ml), tumor size (⩽5 cm vs. >5 cm), tumor multiplicity (single vs. multiple), tumor grade (well-moderately vs. poorly-undifferentiated), stage (I + II vs. III + IV), vascular invasion (absence vs. presence), relapse (absence vs. presence) and survival status [death vs. others (censored, alive or death from other causes)].

2.6. Statistical analysis

Statistical analysis was performed with the SPSS statistical software package (standard version 16.0; SPSS, Chicago, IL). ROC curve analysis was applied to determine the cutoff score for high expression of LC3A. The correlation between the expression patterns of LC3A and the clinicopathologic features of the HCC patients was evaluated by a $\chi 2$-test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. Survival curves were obtained using the Kaplan–Meier method. Differences were considered significant if the *P*-value from a two-tailed test was <0.05.

3. Results

3.1. LC3A immunostaining patterns in HCC

The expression patterns of LC3A in liver tissues were analyzed by IHC staining of the 125 pairs of HCC and the corresponding adja-

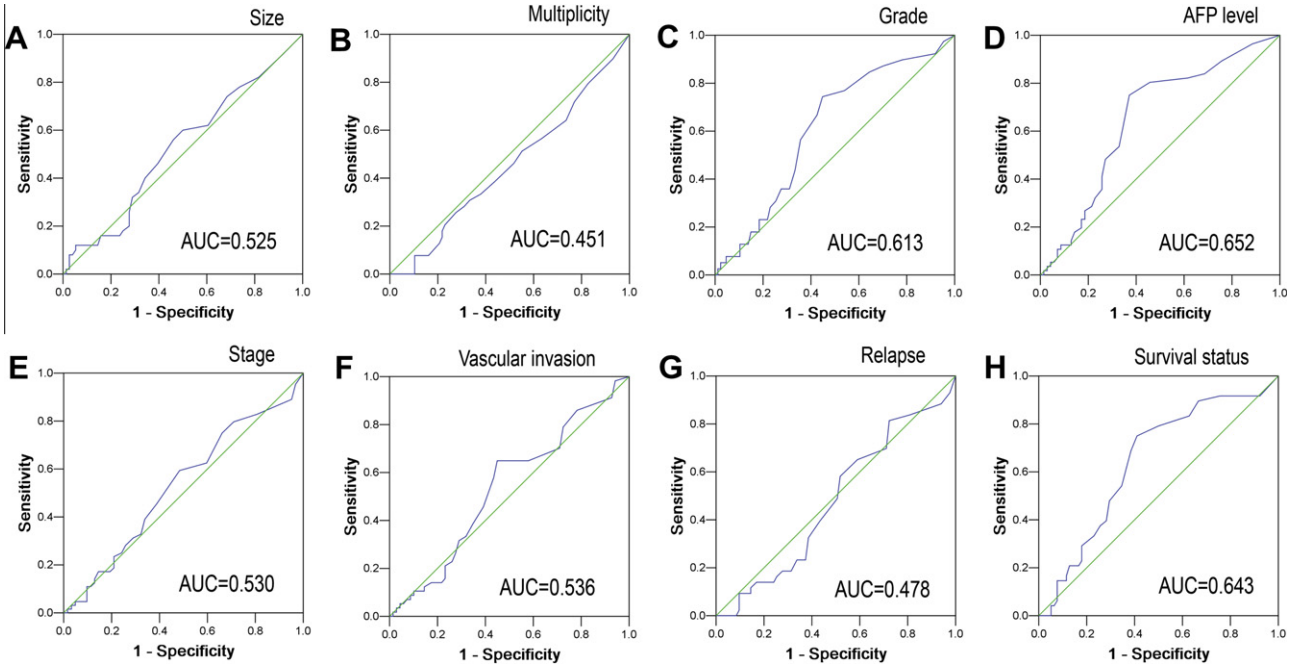

Fig. 1. ROC curve analysis was conducted to determine the cutoff value for high LC3A expression. The sensitivity and specificity for each outcome were plotted: tumor size (A), tumor multiplicity (B), tumor differentiation (C), serum AFP level (D), clinical stage (E), vascular invasion (F), tumor relapse (G), and survival status (H).

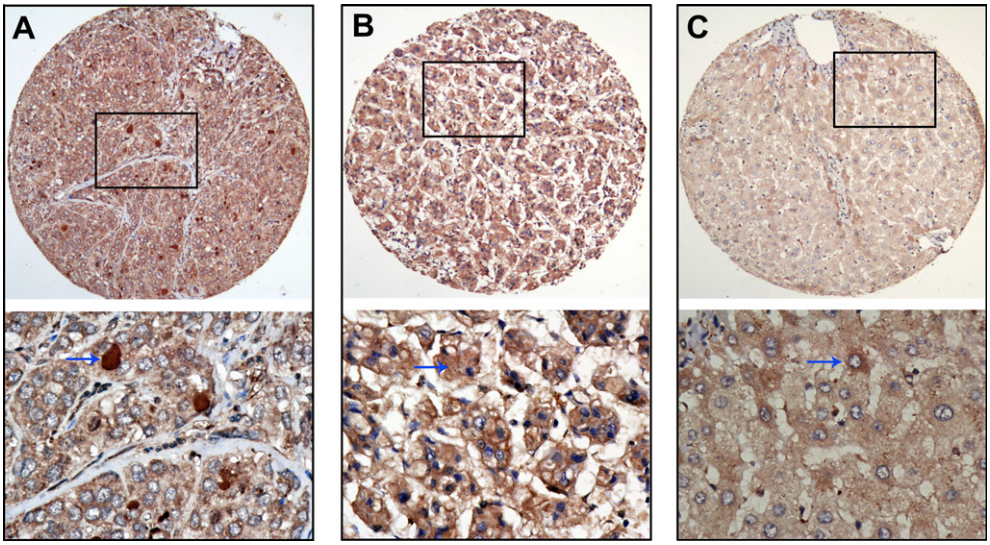

Fig. 2. The distinct patterns of LC3A immunostaining in HCC and noncancerous liver tissues. (A). The "stone-like" pattern of LC3A in a HCC case showing the immunoreactivity as a block mass (upper panel, ×100; lower panel, ×400). (B). The diffuse cytoplasmic pattern of LC3A observed in a HCC case. This shows that the positive staining of LC3A was diffusely distributed in cytoplasm (upper panel, ×100; lower panel, ×400). (C). Diffuse cytoplasmic expression of LC3A shown in the adjacent noncancerous tissue (upper panel, ×100; lower panel, ×400).

cent liver tissues. Positive staining of the LC3A protein was observed in most of the HCC cases in two distinct patterns: a "stone-like" structure or a diffuse cytoplasmic distribution.

The "stone-like" structure was typically enclosed within a cytoplasmic vacuole. This structure was recognizable as a large, rounded, densely staining, amorphous or laminated structure (Fig. 2A). The autophagosomes containing such structures varied greatly in size but were usually large. The number of "stone-like" structures in each TMA spot was counted and ranged from 1 to 34, with a mean of 5.4.

The diffuse cytoplasmic pattern showed a finely/granular reactivity in the cytoplasm and was frequently present in both cancerous and noncancerous hepatic cells (Fig. 2B and C). The mean *H* scores of LC3A expression in cancerous and noncancerous tissues were 146.5 and 93.5, respectively. The difference in these *H* scores was statistically significant ($P < 0.001$).

3.2. Selection of a cutoff score for the high LC3A expression in HCC

ROC analysis was applied to determine the cutoff score and a optimal cutoff for high LC3A expression in HCC was identified. The ROC curves for each clinicopathologic parameter show the value on the curve closest to the point (i.e., 0.0, 1.0) that maximizes both the sensitivity and the specificity for the outcome [19]. Tumors with scores above the obtained cutoff value were considered as having high expression. This scoring maximized the number of tumors that were correctly classified as having or not having the clinical outcome. Data about the corresponding area under the curve (AUC) for LC3A (including the "stone-like" and the diffuse cytoplasmic patterns) were collected (Table 2). ROC curve analysis of the LC3A "stone-like" pattern for the AFP level had the shortest distance from the curve to the point (i.e., 0.0, 1.0) (Fig. 1). Therefore, the cutoff score for high expression of "stone-like" LC3A was defined when the counts were above three. Similarly, tumors designated as having high LC3A expression of the diffuse cytoplasmic pattern were those with *H* scores above a value of 133.

3.3. The correlation of between LC3A expression and the clinicopathologic features of the HCC patients

The correlation between both patterns of LC3A expression and the clinicopathologic features of the HCC patients was determined. The results showed that the high expression of "stone-like" LC3A was positively correlated with the serum AFP level ($P < 0.0001$), tumor differentiation ($P = 0.003$) and vascular invasion ($P = 0.018$) (Table 1). In addition, the high LC3A expression of the diffuse cytoplasmic pattern was negatively related to tumor size ($P = 0.009$, Table 1).

3.4. The relationship between LC3A expression and HCC patient survival: Univariate survival analysis

The representativeness of the HCCs in this study was tested using established prognostic factors of the patients' survival. Kap-

Table 2
Area under the curve (AUC) of receiver operating characteristic curve of LC3A distinct patterns for each clinicopathological feature.

Variable	Stone-like		Diffuse cytoplasmic	
	AUC (95% CI)	*P* value	AUC (95% CI)	*P* value
AFP	0.652 (0.555–0.750)	0.003	0.489 (0.388–0.591)	0.836
Tumor size	0.525 (0.422–0.628)	0.636	0.387 (0.287–0.486)	0.029
Tumor multiplicity	0.451 (0.343–0.559)	0.384	0.445 (0.344–0.546)	0.286
Differentiation	0.613 (0.509–0.716)	0.044	0.468 (0.367–0.570)	0.544
Vascular invasion	0.536 (0.434–0.638)	0.488	0.489 (0.388–0.591)	0.836
Stage	0.530 (0.428–0.631)	0.566	0.432 (0.331–0.533)	0.189
Status	0.643 (0.545–0.742)	0.007	0.483 (0.381–0.585)	0.743
Relapse	0.478 (0.374–0.582)	0.682	0.508 (0.406–0.610)	0.878

CI indicates confidence interval.

Table 3
Univariate and multivariate analysis of different prognostic factors in 125 patients with hepatocellular carcinoma.

Variable	All cases	Univariate analysis[a]		Multivariate analysis[b]	
		Mean survival (months)	*P* value	HR (95% CI)	*P* value
Age at surgery (years)			0.796		
⩽47.9[c]	61	36.5			
>47.9	64	34.6			
Sex			0.581		
Male	108	35.5			
Female	17	37.9			
Hepatitis history			0.978		
Yes	106	36.1			
No	19	35.0			
AFP (ng/ml)			<0.001		0.016
⩽20	69	45.1		1.0	
>20	56	22.2		2.647 (1.195–5.866)	
Liver cirrhosis			0.932		
Yes	87	36.1			
No	38	35.0			
Tumor size (cm)			<0.001		0.060
⩽5	76	41.3		1.0	
>5	49	27.8		1.824 (0.975–3.411)	
Tumor multiplicity			<0.001		0.039
Single	87	41.6		1.0	
Multiple	38	24.7		2.007 (1.037–3.881)	
Differentiation			0.081		
Well-moderate	86	37.5			
Poor-undifferentiated	39	31.2			
Stage			<0.001		0.194
I–II	62	45.8		1.0	
III–IV	63	27.7		1.328 (0.866–2.036)	
Vascular invasion			<0.001		0.361
Yes	56	25.9		2.368 (1.085–2.036)	
No	69	43.5		1.0	
Relapse			<0.001		0.110
Yes	42	27.8		1.930 (0.861–4.324)	
No	83	41.1		1.0	
Stone-like			<0.001		0.019
Low expression	57	45.0		1.0	
High expression	68	25.8		2.483 (1.162–5.304)	
Diffuse cytoplasmic			0.312		
Low expression	61	33.8			
High expression	64	37.3			

[a] Log-rank test.
[b] Cox regression model.
[c] Mean age; HR, hazards ratio; CI, confidence interval; AFP, alpha-fetoprotein.

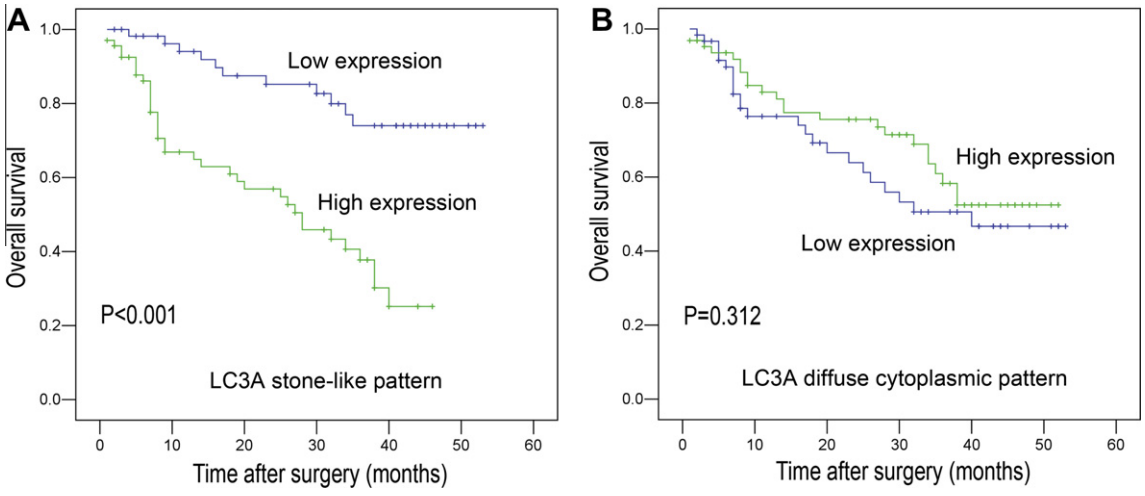

Fig. 3. Kaplan–Meier survival analysis of LC3A expression in patients with HCC (log-rank test). (A). The "stone-like" pattern of expression and the probability of survival of all patients with HCC: low expression, $n = 57$, high expression, $n = 68$. (B). The diffuse cytoplasmic pattern and the probability of survival of all patients with HCC: low expression, $n = 61$, high expression, $n = 64$.

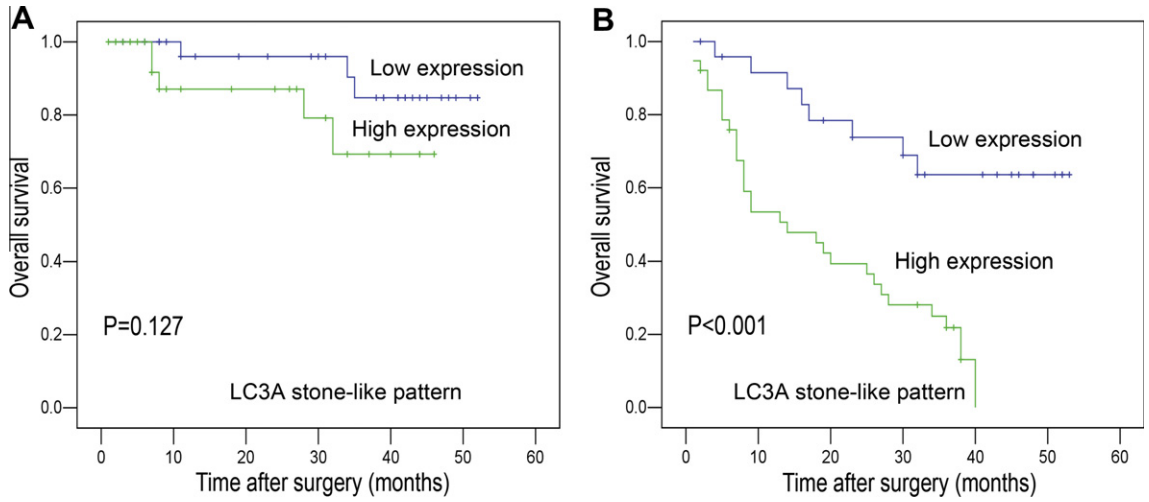

Fig. 4. Kaplan–Meier survival analysis of "stone-like" LC3A expression in a subset of HCC patients with different clinical stages (log-rank test). (A). Probability of survival of the "stone-like" pattern in HCC patients at stage I–II: low expression, $n = 32$, high expression, $n = 30$. (B). Probability of survival of the "stone-like" pattern in HCC patients at stage III–IV: low expression, $n = 25$, high expression, $n = 38$.

lan–Meier analysis demonstrated a significant impact of the well-known clinicopathologic prognostic factors on patient survival; these factors included serum AFP levels ($P < 0.001$), tumor size ($P < 0.001$), tumor multiplicity ($P < 0.001$), clinical stage ($P < 0.001$), vascular invasion ($P < 0.001$) and relapse ($P < 0.001$) (Table 3). Survival assessment revealed that high LC3A expression of the "stone-like" expression pattern correlated with adverse disease-specific survival of the HCC patients ($P < 0.001$, Fig. 3A and Table 3). No significant association between LC3A expression of the diffuse cytoplasmic pattern and survival was found in the HCC patients ($P > 0.05$, Fig. 3B and Table 3).

Stratified survival analysis was also performed with regard to the "stone-like" LC3A expression in subsets of the HCC patients at different clinical stages (Fig. 4A and B). The results indicated that the "stone-like" LC3A expression was a potential prognostic factor in patients at stage III–IV ($P < 0.001$, Fig. 4B).

3.5. Independent prognostic factors of HCC: Multivariate Cox regression analysis

Because the variables observed to have prognostic influence by univariate analysis may covary, the "stone-like" LC3A expression results were subjected to multivariate analysis (Table 3). Our results showed that "stone-like" LC3A was an independent prognostic factor [Hazard Ratio (HR) 2.483; 95% confidence interval (95% CI) 1.162–5.304; $P = 0.019$] (Table 3). Other clinicopathologic features that were significant in the univariate analysis were also examined by multivariate analysis. AFP level and tumor multiplicity were both found to be independent prognostic predictors for overall survival ($P < 0.05$, Table 3).

4. Discussion

Carcinogenesis and tumor progression are influenced by disturbances in the molecular machinery of cells, including the machinery involved in autophagy [21]. LC3, a subunit of the neuronal microtubule-associated protein (MAP), was found to be homologous to Apg8p/Aut7p, which is essential for yeast autophagy and is correlated with autophagosome membranes [7,21,22]. Recently, an antibody to LC3A has been introduced into laboratory practice and has been used to reveal the autophagic activity of LC3A [11]. In light of the key role of LC3A in autophagy, we performed TMA-based IHC to examine the expression of LC3A in HCC and determine its potential impact on HCC tumorigenesis and prognosis.

Autophagy enables cancer cells to survive the dramatically harsh and stressful conditions arising during chemotherapy [23]. Autophagy is also thought to exhibit its anticancer activity by allowing the residual or metastasized cancer cells to survive cytotoxic stress [24]. Studies are accumulating that indicate that the inhibition of autophagy might be an efficient strategy for cancer eradication. Vazquez-Martin et al. showed that knockdown of LC3 expression via shRNA resulted in resensitization to Trastuzumab treatment in breast cancer [25]. Bellodi and colleagues reported that the inhibition of autophagy using either pharmacological inhibitors or siRNAs of essential autophagy genes potentiated cell death in CML cells [26]. Recently, Shi et al. suggested the combination of autophagy modulation and molecular targeted therapy as a promising therapeutic strategy in HCC treatment [27]. Collectively, strategies targeting autophagy, especially *in vivo*, for HCC treatment deserve further investigation.

In the present study, two distinct patterns of LC3A immunostaining ("stone-like" structure and diffuse cytoplasmic distribution) were microscopically identified in most HCC tissues. Our data also showed that high LC3A expression was more frequently observed in HCC tissues than in the adjacent liver tissues. Consistent with our findings, LC3A was found to be abundantly expressed in breast and gastrointestinal cancers [28,29]. The diffuse cytoplasmic pattern of LC3A was observed in both HCC and noncancerous cells, whereas the "stone-like" LC3A pattern was only present in cancer cells. The diffuse cytoplasmic pattern may indicate the production of soluble LC3A, which is a possible marker for basal autophagic activity [11]. Furthermore, the expression of diffuse cytoplasmic LC3A in cancerous and noncancerous tissues was significantly different, suggesting that autophagic activity might be enhanced in HCC tissue. In contrast, the "stone-like" expression pattern is found in the most aggressive carcinomas and is considered a tumor-cell-restricted response.

In this study, our data indicated that the high expression of "stone-like" LC3A in HCC is positively correlated with serum AFP levels, tumor differentiation and vascular invasion; this correlation suggests that LC3A is involved in tumor progression. In line with our results, the "stone-like" pattern of LC3A expression was reported to be associated with the malignant phenotypes of breast, colorectal and lung cancers [11,13,14]. Sivridis et al. showed that

the peri-nuclear pattern of LC3A, which was rarely observed in our study, was related to increased ER and PR expression and tumor size in breast cancer [11]. Previous studies also revealed that the presence of high "stone-like" LC3A expression was significantly associated with the development of distant metastases [13]. In addition, Fujii et al. reported that the up-regulated expression of LC3A was linked to increased tumor size and extensive necrosis in patients with pancreatic adenocarcinoma [30]. These data provided evidence that high "stone-like" expression of LC3A might play an important role in the tumorigenic process of different human cancers, including HCC.

The most important finding of the present study is the prognostic significance of the "stone-like" LC3A expression pattern in HCC. Strikingly, HCC patients with high expression of LC3A with a "stone-like" pattern survived for shorter periods than those with low LC3A expression. With regard to the prognostic impact of LC3 on other human cancers, contradictory results have been reported. High expression of LC3 with a diffuse cytoplasmic pattern was correlated with poor outcome in pancreatic carcinoma but favorable prognosis in glioblastoma [30,31]. Although the correlation of LC3 expression in cancer and patient survival has been the focus of a large number of studies, the underlying mechanism by which LC3 affects prognosis remains elusive and will require future investigation.

In conclusion, two distinct patterns of LC3A expression ("stone-like" structure and diffuse cytoplasmic distribution) were recognized in human HCC tissues. The high "stone-like" expression of LC3A in HCC was correlated with higher serum AFP level, poor tumor differentiation and the presence of vascular invasion. This result suggests that the "stone-like" pattern of LC3A expression may be important in the acquisition of the malignant phenotype. Importantly, high LC3A expression with a "stone-like" pattern predicted unfavorable postoperative survival in HCC patients.

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