High p53 and MAP1 light chain 3A co-expression predicts poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract. p53 and microtubule-associated protein 1 light chain 3A (LC3A) are regulators of apoptosis and autophagy and are expressed at high levels in a number of human tumors. The purpose of the current study was to evaluate the clinicopathological and prognostic significance of p53 and LC3A expression levels in esophageal squamous cell carcinomas (ESCCs). p53 and LC3A expression levels were measured by immunohistochemistry in 114 patients with stage II/III (T_{any} N+M_0 or $T_{3,4}$ N_{any} M_0) ESCCs treated with surgery followed by adjuvant concurrent chemoradiotherapy. The overexpression of p53 and LC3A was observed in 57 and 54% of ESCC samples, respectively. p53 staining was nuclear and LC3A was localized to the cytoplasm of tumor cells. p53 overexpression was more frequently observed in ESCCs with positive lymph nodes (P=0.017). Patients with ESCCs overexpressing p53 and LC3A were associated with a lower 5-year overall survival rate than those with low p53 and LC3A expression (18.0 vs. 54.4%; P=0.001). Univariate and multivariate analyses revealed that the overexpression of p53 or LC3A was not associated with poor patient outcome (P>0.05). However, patients with high levels of p53 and LC3A co-expression had poor clinical prognoses (P=0.027). Thus, p53 and LC3A co-expression is an independent prognostic marker for patients with ESCC.

Introduction

Esophageal carcinoma (EC) is a public health issue in China (1). It has a poor overall prognosis and treatment outcomes for patients with EC have not improved over the last few decades. Significant prognostic indicators include the extent of invasion and lymph node and distal metastases (2). However, specific patients with the same stage have different prognosis (3). Therefore, molecular markers to improve the outcome prediction for patients with EC must be identified.

Classified as type I programed cell death, apoptosis is involved in a number of cancer-related processes, including tumorigenesis, tumor progression and cellular responses to chemotherapy and radiotherapy (4-6). The well-studied tumor suppressor protein, p53, is key to apoptosis and cancer development. Wild-type p53 inhibits tumor growth by inducing apoptosis and inhibiting angiogenesis and metastasis; however, the *TP53* gene undergoes inactivating mutations in a variety of cancer types. More recently, p53 has been demonstrated to repress autophagy (7), indicating that apoptosis and autophagy may interact. Type II programmed cell death, or autophagy, is an evolutionarily conserved eukaryotic process important for maintaining homeostasis by recycling stable proteins and long-lived organelles (8). Autophagy is important in a number of physiological processes, including adaptation to hypoxia, prevention of tumorigenesis and antigen presentation (9-11). Although the role of autophagy in cancer is unclear, the current view is that it functions as a cytoprotective mechanism during tumor progression (12).

Previously, microtubule-associated protein 1 light chain 3A (LC3A), one of three microtubule-associated protein light chain isoforms, was revealed to be an essential component of autophagosomes (13). Using the immunohistochemical detection of LC3A protein as a marker of autophagic activity, autophagy was identified to be upregulated in urothelial cell carcinoma, non-small cell lung cancer and endometrial, colorectal and cutaneous squamous cell carcinomas (14-18). In addition, LC3A overexpression is linked to the aggressiveness of these tumors (16). However, the clinicopathological and prognostic significance of LC3A overexpression in esophageal squamous cell carcinomas (ESCCs) has not been established. Cancer-related *TP53* mutations are associated with the overexpression of inactive p53 (19). The combined prognostic effect of p53 and LC3A overexpression has not been investigated in patients with ESCC. In the current study, the expression of p53 and LC3A proteins was measured by immunohistochemistry and the prognostic significance of p53 and LC3A overexpression was assessed in patients with ESCC.

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Table I. p53 and LC3A expression and clinicopathological characteristics.

		p53			LC3A		
Parameters	Cases	High	Low	P-value	High	Low	P-value
Age, years							
≤60	46	20	26	0.077	22	24	0.592
>60	68	41	27		36	32	
Gender							
Male	101	54	47	0.979	50	51	0.414
Female	13	7	6		8	5	
Weight loss							
≤10%	28	13	15	0.387	15	13	0.743
>10%	86	48	38		43	43	
Location							
Ut/Mt	61	36	25	0.206	35	26	0.136
Lt	53	25	28		23	30	
Histology							
Well	49	24	25	0.400	22	27	0.268
Mod/poor	65	37	28		36	29	
T stage							
T1-2	37	15	22	0.092	18	19	0.741
T3-4	77	46	31		40	37	
N stage							
N0	55	29	26	0.017	24	31	0.135
N+	59	32	27		34	25	
Clinical stage							
II	48	26	22	0.904	25	23	0.826
III	66	35	31		33	33	

Well/mod/poor, well, moderately and poorly differentiated squamous cell carcinoma; Ut, upper thoracic; Mt, middle thoracic; Lt, lower thoracic; N0, negative lymph node metastasis; N^+, positive lymph node metastasis; LC3A, microtubule-associated proteins 1A/1B light chain 3.

Materials and methods

Patients and tissue specimens. From our clinical archives, 114 patients with stage II/III (T_{any} N+M_0 or $T_{3,4}$ N_{any} M_0) ESCC were assessed. Patient were treated with surgery followed by adjuvant concurrent chemoradiotherapy (CRT) in the Department of Oncology at the Affiliated Hospital of Binzhou Medical College (Shandong, China) between January 2006 and December 2008. The study was approved by the ethics committee of the Affiliated Hospital of Binzhou Medical College (Shandong, China). Prior to surgery, all patients underwent computed tomography staging of regional lesions and metastases of the neck, thorax and abdomen. Characteristics of the 114 patients, including gender, cell differentiation, weight loss, T, N and clinical stages and location, are presented in Table I. The median patient age was 58.1 years (range, 40-71 years). Clinical staging was assessed according to the 2002 TNM Staging of the International Union Against Cancer (20). Informed consent was obtained from all patients. Following surgery, tissue specimens obtained from all the patients were fixed in formalin and embedded in paraffin. Patients did not undergo chemotherapy or radiotherapy prior to surgery. The median follow-up was 57.5 months (range, 4-70 months) and survival information was available for 107 patients.

Treatment. All patients underwent radical surgical tumor resection. Patients with upper thoracic esophageal malignancies were subjected to transthoracic esophagectomy with three-field lymphadenectomy and patients with middle/lower thoracic ESCC underwent two-field lymphadenectomy. Following surgery, adjuvant CRT consisted of three-dimensional conformal radiotherapy with 49.2 Gy (range, 40-50 Gy) concurrent with two adjuvant cycles of cisplatin (25 mg/m^2; days 1-3) and fluorouracil (600 mg/m^2; days 1-3) chemotherapy.

Immunohistochemistry. Polyclonal antibodies against LC3A (1:80; Cell Signaling Technology, Inc., Danvers, MA, USA) and p53 (1:100; Dako, Carpinteria, CA, USA) were obtained. Immunohistological analysis of p53 and LC3A was performed using 3-μm-thick formalin-fixed paraffin-embedded esophageal tumor sections. Sections were deparaffinized with

xylene, rehydrated using graded alcohol solutions and then heat-induced epitope retrieval was performed in 0.01 M citrate buffer (pH 6.0) in a microwave oven. Tissue sections (T_{any} $N+M_0$ or $T_{3,4}$ N_{any} M_0) were incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Non-specific antibody binding was blocked by incubation with Protein Block (Dako) for 5 min at room temperature. Sections were then incubated overnight at 4˚C with primary polyclonal antibody, washed in phosphate-buffered saline (PBS) and then incubated with secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 30 min at room temperature. Following washing with PBS, sections were incubated with 3,3'-diaminobenzidine for 5 min and counterstained with hematoxylin. PBS was used as a negative control for the primary antibody; no staining was detected.

Immunohistochemical analysis of p53 and LC3A expression. For scoring, five random fields were selected from each tissue section and the mean score for each slide was used for subsequent analyses. Assessment of p53 expression was based on the percentage of tumor cells revealing p53 immunoreactivity and immunointensity. Tumor staining was classified into three categories; >10, 10-50 and >50% of cells with positive staining. p53 immunointensity was classified into two categories; no or weak immunostaining and marked immunostaining. Tumors with >50% of cells revealing marked p53 and LC3A immunostaining were defined as exhibiting high p53 and LC3A expression, respectively. Scoring of immunohistochemical staining was performed by two independent pathologists blind to the clinicopathological status of the samples.

Statistical analysis. Statistical analysis was performed using SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). p53 and LC3A expression levels were defined as categorical variables. Differences between groups were estimated using the Chi-square test. Patient survival was assessed using Kaplan-Meier curves and differences in survival between groups of patients were analyzed using the log-rank test. Cox's proportional hazards model was used for multivariable analysis. $P<0.05$ was considered to indicate a statistically significant difference. The primary endpoint was overall survival, which was defined as the time (in months) between the date of therapy and the date of the last follow-up or mortality.

Results

p53 and LC3A expression in archival ESCC samples. Immunohistochemical analysis identified that 53.5 (61/114) and 50.9% (58/114) of tumors exhibited p53 and LC3A overexpression, respectively. In addition, p53 was largely localized to the nuclei of tumor cells (Fig. 1A and B), while LC3A expression was cytoplasmic (Fig. 1C and D). In contrast to previous reports, LC3A expression was not observed in perinuclear, 'stone-like' structures (SLS) in ESCC samples (17).

p53 and LC3A overexpression correlates with ESCC clinical aggressiveness. The correlation between p53 and LC3A expression and various clinicopathological parameters are presented in Table I. p53 overexpression was more frequently observed in lymph node-positive than in lymph node-negative tumors (P=0.017). There were no associations between p53 immunostaining and other factors, including gender, weight loss, age and tumor location. Similarly, no clinicopathological characteristics were found to correlate with LC3A immunoreactivity.

High p53 and LC3A co-expression inversely correlates with patient survival. Having identified correlations between p53 and LC3A expression and clinicopathological parameters, the correlation between p53 and LC3A expression and patient survival was determined. Among the clinicopathological char-

Table II. Univariate Cox analysis of 5-year overall survival rate following surgery in 107 patients with ESCC.

Parameters	Cases	HR	95% CI	P-value
Age, years				
≤60	43	1.000		0.325
>60	64	1.340	0.841-2.408	
Gender				
Male	95	1.000		0.412
Female	12	0.820	0.475-1.316	
Weight loss				
≤10%	25	1.000		0.253
>10%	82	1.297	0.739-2.054	
Location				
Ut/Mt	57	1.000		0.844
Lt	50	0.902	0.307-3.238	
Histology				
Well	44	1.000		0.471
Mod/poor	63	1.313	0.712-2.951	
T stage				
T1-2	36	1.000		0.298
T3-4	71	1.165	0.414-3.977	
N stage				
N0	51	1.000		0.000
N+	57	4.624	1.153-10.356	
Clinical stage				
II	45	1.000		0.044
III	62	3.072	1.315-5.924	
LC3A				
High	54	1.000		0.063
Low	53	0.653	0.280-1.561	
p53				
High	59	1.000		0.156
Low	48	0.705	0.232-1.748	

HR, hazard ratio; well/mod/poor, well, moderately and poorly differentiated squamous cell carcinoma; Ut, upper thoracic; Mt, middle thoracic; Lt, lower thoracic; N0, negative lymph node metastasis; N^+, positive lymph node metastasis; LC3A, microtubule-associated proteins 1A/1B light chain 3; ESCC, esophageal squamous cell carcinoma.

Table III. Multivariable analysis of factors associated with survival.

A, High p53 and LC3A co-expression, analyzed as two independent factors.

Variable	Hazard ratio	95% CI	P-value
T stage (T3,4)	1.5	0.709-3.152	0.234
N stage (N+)	4.2	1.104-9.613	0.000
Clinical stage (III)	8.3	1.825-19.463	0.013
p53 (high)	0.8	0.254-3.182	0.597
LC3A (high)	1.7	0.571-3.467	0.435

B, High p53 and LC3A co-expression, analyzed as a single factor.

Variable	Hazard ratio	95% CI	P-value
T stage (T3,4)	1.2	0.227-3.041	0.485
N stage (N+)	3.1	1.562-8.275	0.000
Clinical stage (III)	5.6	1.647-14.264	0.009
p53 (high)/LC3A (high)	2.8	1.536-6.183	0.027

LC3A, microtubule-associated proteins 1A/1B light chain 3; N^+, positive lymph node metastasis.

acteristics, N stage (N_0) and clinical stage (II) were identified to significantly correlate with survival (N_0 vs. N_+, P=0.000; II vs. III, P=0.044; Table II). The overall 5-year survival rate of 54 patients with high p53 expression was not observed to differ significantly from that of 53 patients with low p53 expression (P>0.05). Similarly, there was no correlation between LC3A expression and the 5-year survival rate (P>0.05). Next, 107 patients were divided into four subgroups according to their p53 and LC3A status (high p53/high LC3A, n=27; high p53/low LC3A, n=32; low p53/high LC3A, n=27; and low p53/low LC3A, n=21). Kaplan-Meier curves revealed that low p53 and LC3A co-expression in primary ESCCs is associated with longer overall patient survival times, whereas high p53 and LC3A co-expression is associated with shorter patient survival time (median of 45 vs. 28 months, respectively; log-rank test, P=0.001; Fig. 2). The 5-year survival rate of patients with high p53 and LC3A co-expression was 18.0%, while that of patients with low p53 and LC3A co-expression was 54.4% (P=0.001). Thus, high expression of p53 and LC3A in ESCCs is linked to poor patient prognosis.

High p53 and LC3A co-expression is a poor prognostic factor for ESCC patients. To determine whether p53 and LC3A represent prognostic factors of ESCC, overall patient survival was examined using Cox regression proportional hazard analysis on prognostic factors (T, N and clinical stage and p53 and LC3A status) in 107 ESCC patients. High levels of p53 and LC3A expression were not identified as independent prognostic factors (P>0.05; Table III, part A); however, N and clinical stages

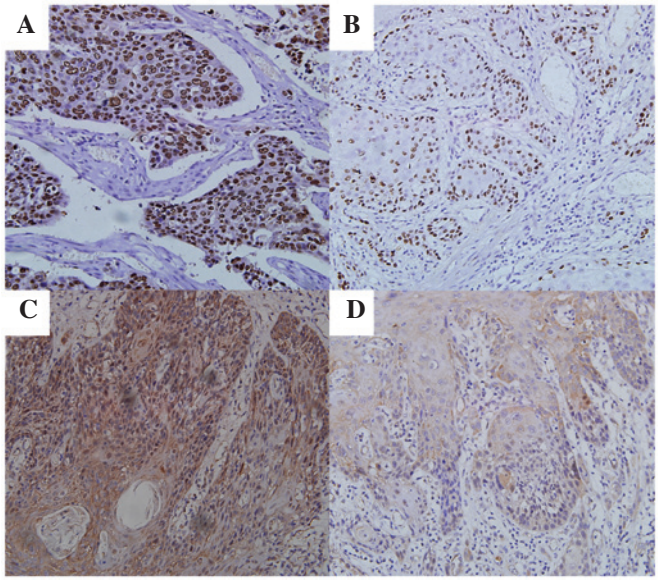

Figure 1. Immunohistochemical analysis of p53 and LC3A expression in ESCC tissues. Tissue sections revealing expression of (A) high and (B) low p53 and (C) high and (D) low LC3A expression. Magnification, x200. LC3A, microtubule-associated proteins 1A/1B light chain 3; ESCC, esophageal squamous cell carcinoma.

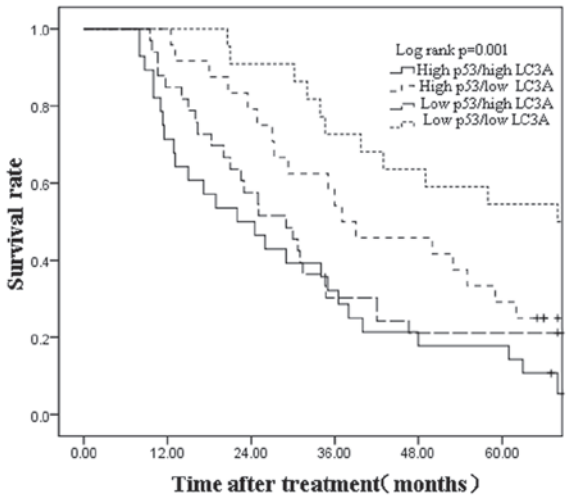

Figure 2. p53 and LC3A expression levels affect the overall survival of patients with ESCC. Kaplan-Meier curves for 107 patients reveal significant differences in the 5-year overall survival rates among the four groups (high p53/high LC3A, n=27; high p53/low LC3A, n=32; low p53/high LC3A, n=27; and low p53/low LC3A, n=21). Patients with high LC3A/high p53 expression had poor overall survival. Patients with low p53/low LC3A expression reveal higher overall survival (P=0.001). LC3A, microtubule-associated proteins 1A/1B light chain 3; ESCC, esophageal squamous cell carcinoma.

were revealed to be independent prognostic factors (P=0.006 and P=0.013, respectively). However, when defined as a single factor, high p53/high LC3A co-expression was determined as an independent prognostic factor (P=0.027; Table III, part B).

Discussion

In the present study, the expression of LC3A and mutant p53 was measured by immunohistochemistry. Results indicated

that high p53 expression was linked to ESCC lymph node metastasis, but did not correlate with the overall survival of patients with ESCC. However, when high levels of p53 and LC3A co-expression were considered as a single factor, it was linked to poor prognosis.

The *TP53* gene is commonly mutated in human tumors (21,22); however, associations between mutant p53 expression and patient prognosis remain unclear. *TP53* mutations often lead to a loss of tumor suppressor activity and an extended half-life; therefore, the accumulation of mutant p53 protein is detectable by immunohistochemistry (23). Previous studies have demonstrated that p53 expression correlates with tumor stage, lymph node metastasis and overall survival in EC (2,23-24). Consistent with these studies, p53 overexpression was found to be more frequent in lymph node-positive ESCCs in the current study. By contrast, additional studies have reported that p53 expression is not linked to lymph node metastasis and survival (25-26). These inconsistencies may be caused by the use of various experimental conditions, classification standards and patient populations between studies.

LC3A is a key mediator of autophagy and specific studies have indicated that the immunohistochemical determination of LC3A expression is a useful prognostic indicator (15-17). Fujii *et al* (27) demonstrated that high LC3A expression correlates with reduced disease-free and overall patient survival. In addition, more recent studies have found that LC3A exhibits three expression patterns in numerous human malignancies; diffuse cytoplasmic, cytoplasmic/juxtanuclear and SLS (14-18). High numbers of SLS have been linked to poor patient outcome, whereas the remaining two expression patterns have demonstrated no prognostic significance. In the present study, only diffuse cytoplasmic expression was observed in ESCCs. A previous study reported that LC3A expression was restricted to the cytoplasm in melanomas (28) and that LC3A overexpression does not correlate with patient survival. We therefore hypothesized that LC3A expression patterns and clinical significance are tissue-specific.

The programmed cell death pathways, apoptosis and autophagy, do not function independently. Environmental stressors, including hypoxia and irradiation, induce apoptosis and autophagy, and molecular cross-talk exists between these pathways. p53 induces apoptosis through transactivation of Bcl-2 family proteins and inhibits autophagy through a direct interaction with RB1CC/FIP200 (7). In addition, the autophagy component, hAtg7, is a p53 regulator and in the absence of hAtg7, the proapoptotic activity of p53 increases, while p53-mediated cell cycle arrest decreases (29). In addition, other molecules, including mTOR and Atg5, are important for cross-talk between various cell death signaling pathways (30,31), thus highlighting the complex regulation of these processes. In the majority of cases, the regulation of apoptosis and autophagy occurs in opposite directions; however, both are inhibited by activation of the PI3K/Akt/mTOR pathway (32). Therefore, we hypothesized that inactivating mutations in p53 simultaneously decrease apoptosis and increase autophagy, thus promoting tumor cell survival. This hypothesis is consistent with results of the current study, which revealed that high p53 and LC3A co-expression is linked to poor prognosis in patients with ESCC.

In conclusion, observations of this study indicate that p53 expression is linked to lymph node metastasis and that high levels of p53 and LC3A co-expression correlate with poor patient prognosis. Thus, immunohistochemical analysis of p53 and LC3A co-expression represents a promising prognostic indicator for this disease. However, these results require validation in further, large-cohort, prospective studies.

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