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Original Article

Overexpression of cytoplasmic p62 protein is associated with poor prognosis in gastric adenocarcinoma

Zhengpeng Li[1], Yuan Li[1], Jian Liu[1], Liya Zhou[1], Yan Xue[1], Rongli Cui[1], Yan Cui[2], Jinlian Zhou[3], Jianzhong Zhang[3]

[1]Department of Gastroenterology, Peking University Third Hospital, Beijing, China; [2]Department of General Surgery, 306 Hospital of PLA, Beijing, China; [3]Department of Pathology, 306 Hospital of PLA, Beijing, China

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Abstract: SQSTM1/p62 (p62) is a multifunctional adapter protein implicated in selective autophagy, cell signaling pathways, and tumorigenesis. Recently, it has been shown that p62 was overexpressed in various malignancies and had implications in the prognosis. In this study, we aim to investigate the expression level of p62 in gastric adenocarcinoma (GA) and evaluate the relationships between its expression and clinical features as well as overall survivals of GA patients. Immunohistochemistry of tissue microarray was used to detect the p62 expression in 90 pairs of GA and nontumorous adjacent mucosa (NAM) samples. The results showed that p62 protein was located in the nucleus and/or cytoplasm in both GA and NAM tissues. The expression level of cytoplasmic p62 in GA tissues was significantly higher than in NAM tissues (P<0.001), while nuclear p62 showed lower expression in GA tissues (P=0.029). In addition, cytoplasmic overexpression of p62 was significantly associated with large tumor size (P=0.018), positive lymphatic invasion (P=0.011) and p62 cytoplasmic expression was different in various major histological types (P=0.036) of GA patients. Kaplan-Meier survival analysis showed that increased cytoplasmic expression of p62 protein was significantly correlated with lower overall survival rate in GA patients (P=0.030). Univariate and multivariate survival analyses indicated that increased cytoplasmic expression of p62 protein was significantly correlated with poor prognosis (P=0.037) but not an independent prognosis factor in GA patients (P=0.222). In conclusion, the results suggest that overexpression of cytoplasmic p62 may contribute to cancer progression and become a novel prognostic biomarker of GA.

Keywords: Gastric adenocarcinoma, SQSTM1/p62, immunohistochemistry, clinicopathologic features, prognosis

Introduction

Gastric cancer (GC) is one of the most frequently occurring malignant tumors worldwide, especially in China [1]. Host-associated factors especially chronic *Helicobacter pylori* infection as well as environmental factors contribute to disease development [2, 3], but molecular mechanisms involved in the oncogenesis and progression of GC have not yet been fully understood. At present, most patients who diagnosed with GC were at advanced stage and existing treatments were useless [4]. Therefore new prognostic markers and therapeutic targets for GC is urgently required.

Sequestosome 1 (SQSTM1/p62, hereafter referred to as p62) is a multifunctional adapter protein implicated in various cell signaling pathways for cell survival and cell death [5]. In addition, p62 is known to be one of the selective adapters of autophagy, a cellular degradation system, by which cytoplasmic components, organelles, and incorporated p62 protein are degraded [6]. With the progress in research on tumorigenesis, the importance of p62 in cancers is gradually recognized [7]. Besides, increasing evidences have shown that p62 was overexpressed and had implications in the prognosis in various malignant tumors [8-10], such as breast, liver, and lung cancer, etc. However, there are few reports regarding the role of p62 in GC [11]. In the present study, we aimed to investigate the expression levels of p62 in gastric adenocarcinoma (GA) and nontumorous adjacent mucosa (NAM) as GA cases accounts for most of GC patients, and then to

Table 1. Differences of p62 expression levels between GA and matched NAM tissues

Group	N	Cytoplasmic p62 expression		*P*	Nuclear p62 expression		*P*
		High n (%)	Low n (%)		High n (%)	Low n (%)	
GA	88	67 (76.1)	21 (23.9)	<0.001*	47 (53.4)	41 (46.6)	0.029*
NAM	88	29 (33.0)	59 (67.0)		63 (71.6)	25 (28.4)	

*Statistically significant difference by using McNemar Chi-square test (P<0.05).

evaluate its relationship to clinical features and prognosis of GA patients.

Materials and methods

Samples of tissue microarray

Formalin-fixed, paraffin-embedded GA and NAM samples of 90 patients were obtained from the Biobank of National Engineering Center for Biochip at Shanghai. Besides, the retrospective study was approved by the Ethics Committees of National Engineering Center for Biochip at Shanghai and informed consent forms were obtained before the operation. All patients who underwent gastric surgical resection from August 2008 to March 2009 were pathologically diagnosed with GA and without any presurgery treatment. After surgery, patients were followed up until September 2014 without loss and database of clinical variables was established. The pathological grades and major histological types were classified according to Japanese Gastric Cancer Association criteria in 2010 while the clinical stages were updated according to American Joint Committee on Cancer guidelines in 2010. Overall survival was measured from time of definitive operation to death for GA progression and all the clinicopathological features are described in **Table 2**. The tissue microarray was produced by Shanghai outdo biotech company (HStm-Ade180Sur-06) and secondary diagnosis was confirmed by HE staining.

Immunohistochemistry (IHC) assay

The PV6000 Detection System (ZSGB, BJ, China) was used to detect the expression of p62 in GA and matched NAM. Tissue microarray was taken out from the refrigerator for rewarming and was heated at 60°C for one hour to melt the seal wax away from the surface. The slides were deparaffinized in xylene twice for 20 minutes, and rehydrated using graded ethanol (100%, 95% and 70%) to water for every 5 minutes. After the retrieval of antigens by boiling with pressure cooker at 100°C in 0.01 M citrate buffer (pH 6.0) for 2 minutes, the slides were treated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase. Primary antibody to p62 (ABCAM, CA, UK) was added at dilutions of 1:1000 in 37°C incubator for 2 hours. Subsequently, a HRP labeled secondary antibody was applied and was incubated at 37°C for 30 minutes. Finally, the slides were stained with DAB and counterstained with hematoxylin. Positive as well as negative control slides were included in every experiment and 0.01M PBS (pH 7.4) was used as washing buffer.

Evaluation of immunostaining score

All the immunostained sections were evaluated by 3 pathologists who were blinded to the clinical data. Five views per section were randomly examined, and 100 tumor cells were observed per view at magnification of × 400. In present study, p62 was expressed in the nucleus as well as cytoplasm of cancer cells. The positive rate of every case was obtained by calculating the percentage of positively stained cells in each section. Percentage scores of p62 staining were assigned as 0: <1%; 1: 1-10%; 2: 11-50%; 3: 51-75%; 4: 76-100%. The intensity of p62 staining were scored as 0, 1, 2 and 3 if negative, weak, moderate, or marked, respectively. Scores from each section were multiplied to give a final score of 0 to 12, and the cases were finally determined, based on scores ≥4, as having high cytoplasmic or nuclear expression, respectively.

Statistical analyses

McNemar chi-square test was used to analyze the difference of p62 expression in GA and NAM tissues. Chi-square test or Fisher's exact test was used to analyze the relationship between p62 expression and clinical features. The Kaplan-Meier and log-rank test were used to analyze the survival rates. Cox proportional hazard model was used to determine factors related to patient survival. P<0.05 was regarded as statistically significant. All statistical analyses were performed using SPSS 18.0.

Table 2. Relationship between p62 expression and clinicopathologic features in GA

Variables	N	Cytoplasmic p62 expression		*P*	Nuclear p62 expression		*P*
		High n (%)	Low n (%)		High n (%)	Low n (%)	
Sex				0.418			0.921
Male	52	38 (73.1)	14 (26.9)		28 (53.8)	24 (46.2)	
Femal	36	29 (80.6)	7 (19.4)		19 (52.8)	17 (47.2)	
Age				0.176			0.060
≥60 years	53	43 (81.1)	10 (18.9)		24 (45.3)	29 (54.7)	
<60 years	35	24 (68.6)	11 (31.4)		23 (65.7)	12 (34.3)	
Tumor size				0.018*			0.568
≥5 cm	53	45 (84.9)	8 (15.1)		27 (50.9)	26 (49.1)	
<5 cm	35	22 (62.9)	13 (37.1)		20 (57.1)	15 (42.9)	
T stage				1.000			0.570
T1+T2	13	10 (76.9)	3 (23.1)		6 (46.2)	7 (53.8)	
T3+T4	75	57 (76.0)	18 (24.0)		41 (54.7)	34 (45.3)	
N stage				0.312			0.537
N0	22	15 (68.2)	7 (31.8)		13 (59.1)	9 (40.1)	
N1+N3+N3	66	52 (78.8)	14 (21.2)		34 (51.5)	32 (48.5)	
M stage				1.000[a]			1.000
M0	84	64 (76.2)	20 (23.8)		45 (53.6)	39 (46.4)	
M1	4	3 (75.0)	1 (25.0)		2 (50.0)	2 (50.0)	
Clinical stage				0.138			0.419
1+2	34	23 (67.6)	11 (32.4)		20 (58.8)	14 (41.2)	
3+4	54	44 (81.5)	10 (18.5)		27(50.0)	27 (50.0)	
Pathological grade				0.562			0.542
I+II	21	15 (71.4)	6 (28.6)		10 (47.6)	11 (52.4)	
III	67	52 (77.6)	15 (22.4)		37 (55.2)	30 (44.8)	
Lymphovascular invasion				0.011*			0.532
Negative	65	45 (69.2)	20 (30.8)		36 (55.4)	29 (44.6)	
Positive	23	22 (95.7)	1 (4.3)		11 (47.8)	12 (52.2)	
Nerve invasion				0.593			0.625
Negative	79	59 (74.7)	20(25.3)		41 (51.9)	38 (48.1)	
Positive	9	8 (88.9)	1 (11.1)		6 (66.7)	3 (33.3)	
Histological type				0.036[a]*			0.395[a]
TA	28	20 (71.4)	8 (28.6)		12 (42.9)	16 (57.1)	
PA	10	8 (80.0)	2 (20.0)		4 (40.0)	6 (60.0)	
PDA	40	33 (82.5)	7 (17.5)		24 (60.0)	16 (40.0)	
MA	5	5 (100.0)	0 (0.0)		4 (80.0)	1 (20.0)	
SRCC	5	1 (20.0)	4 (80.0)		3 (60.0)	2 (40.0)	

TA: tubular adenocarcinoma; PA: papillary adenocarcinoma; PDA: poorly differentiated adenocarcinoma; MA: mucinous adenocarcinoma; SRCC: signet ring cell carcinoma. *Statistically significant difference ($P<0.05$) by using Fisher's exact test (a) or Chi-square test (others).

Results

Expression levels of p62 in GA and matched NAM tissues

There were 90 GA cases enrolled in our study but 2 cases of them were eliminated after IHC array because of section shedding. The IHC staining results showed that p62 protein was located in the nucleus and/or cytoplasm in both GA and NAM tissues (**Figure 1**). Interestingly, the staining intensity of cytoplasmic p62 in GA tissues seemed to be stronger than NAM tissues. In addition, p62-positive

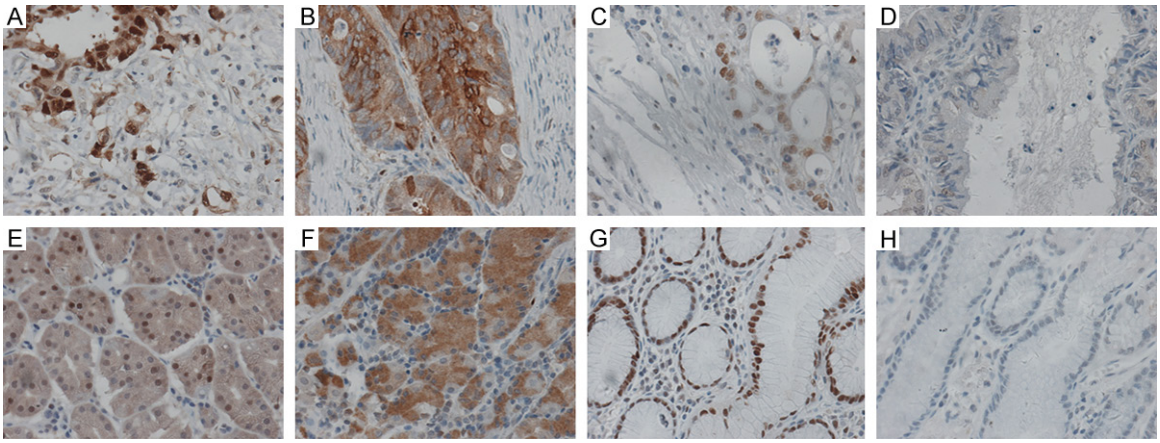

Figure 1. IHC staining of p62 expression in GA and NAM tissues. A and E. Representative images of GA and NAM tissues with high p62 expression both in the cytoplasm and nucleus. B and F. Representative images of gastric GA and NAM tissues with high p62 expression only in the cytoplasm. C and G. Representative images of GA and NAM tissues with high p62 expression only in the nucleus. D and H. Representative images of GA and NAM tissues with low p62 expression both in the cytoplasm and nucleus. (Original magnification, × 400).

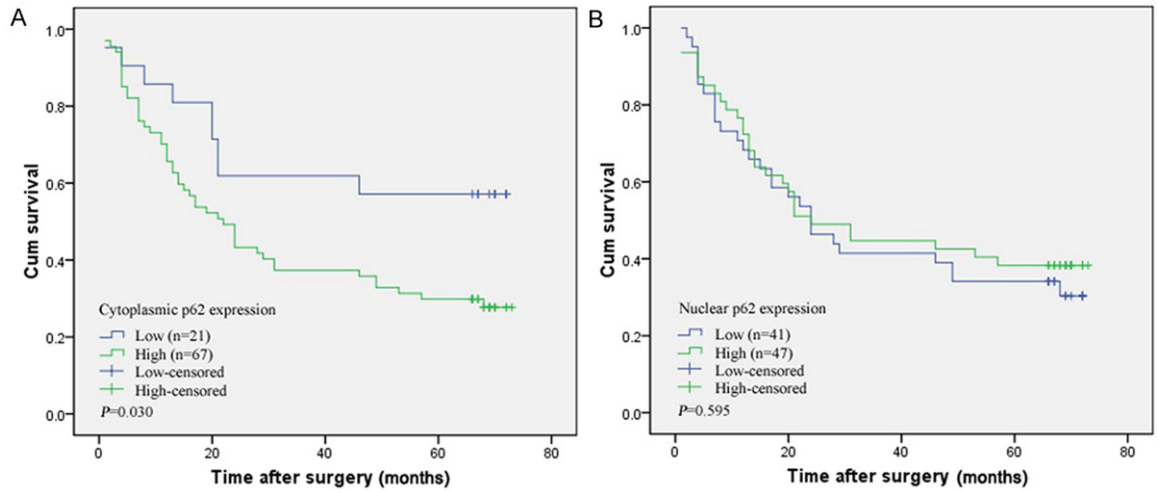

Figure 2. Kaplan-Meier curves for cumulative survival rates of GA patients according to p62 expression. A. GA patients with high cytoplasmic p62 expression have poor cumulative survival rate compared to GA patients with low cytoplasmic p62 expression; B. Cumulative survival rates of GA patients with high or low nuclear p62 expression are not different.

aggregate-like structures can be observed in part of GA tissues (**Figure 1A**) while rarely seen in NAM tissues. The expression level of cytoplasmic p62 in GA was significantly higher than in matched NAM (76.1% vs 33.0%, $P<0.001$), while nuclear p62 showed lower expression in GA tissues (53.4% vs 71.6%, $P=0.029$) (**Table 1**).

Relationships between p62 expression and clinical features of patients with GA

We further examined the relationships between p62 expression and clinical features of patients with GA. The results showed that cytoplasmic overexpression of p62 was significantly associated with large tumor size (84.9% vs 62.9%, $P=0.018$), positive lymphatic invasion (95.7% vs 69.2%, $P=0.011$) and was different in various major histological types ($P=0.036$) of GA patients. Meanwhile, cytoplasmic expression of p62 in GA tissues showed no association with clinical features of GA patients including sex, age, histological grades, T stage, N stage, M stage, clinical stage, pathological grade and nerve invasion. Interestingly, there was no significant correlation between nuclear p62 ex-

Table 3. Cox proportional hazard analyses for overall survival of GA patients

Variables	Univariate analysis		*P*	Multivariate analysis		*P*
	HR	95% CI		HR	95% CI	
Tumor size			0.013*			0.613
≥5 cm vs <5 cm	0.485	0.274-0.856		0.841	0.431-1.643	
T stage			0.007*			0.388
T1+T2 vs T3+T4	0.203	0.06-0.650		0.551	0.142-2.135	
N stage			<0.001*			0.484
N0 vs N1+N3+N3	0.121	0.043-0.336		0.603	0.147-2.482	
M stage			0.012*			0.271
M0 vs M1	0.259	0.091-0.742		0.518	0.160-1.673	
Clinical stage			<0.001*			0.004*
1+2 vs 3+4	9.391	4.333-20.350		5.235	1.696-16.158	
Pathological grade			0.021*			0.223
I+II vs III	2.316	1.132-4.738		1.737	0.714-4.226	
Lymphovascular invasion			<0.001*			0.218
Negative vs Positive	0.569	0.431-0.752		0.681	0.370-1.254	
Histological type			0.049*			0.309
TA vs PA	0.415	0.121-1.423	0.162	0.330	0.093-1.173	0.087
TA vs PDA	1.389	0.755-2.554	0.291	0.505	0.245-1.038	0.063
TA vs MA	3.299	1.197-9.090	0.021*	0.759	0.241-2.392	0.638
TA vs SRCC	0.946	0.275-3.247	0.929	0.704	0.182-2.724	0.611
Cytoplasmic p62 expression			0.037*			0.222
High vs Low	0.684	0.479-0.977		0.582	0.244-1.388	

TA: tubular adenocarcinoma; PA: papillary adenocarcinoma; PDA: poorly differentiated adenocarcinoma; MA: mucinous adenocarcinoma; SRCC: signet ring cell carcinoma. HR: hazard ratio; CI: confidence interval. *Statistically significant difference ($P<0.05$).

pression in GA tissues and clinical features of GA patients (**Table 2**).

Impact of p62 expression on the prognosis of patients with GA

To further examine the impact of p62 expression on the overall survival rates of GA patients, we employed the Kaplan-Meier analysis to plot the survival curves of all 88 GA patients, and statistical significance was assessed using the log-rank test. Increased cytoplasmic expression of p62 protein was significantly correlated with lower overall survival rate in GA patients ($P=0.030$), while nuclear expression of p62 protein has no relation with overall survival rates in GA patients ($P=0.595$) although it seemed that GA patients with low p62 nuclear expression may have higher overall survival rate (**Figure 2**).

Univariate analysis showed that tumor size, T stage, N stage, M stage, clinical stage, pathological grade, lymphatic invasion, histological type and cytoplasmic p62 expression significantly correlated with prognosis of GA patients ($P=0.013$, $P=0.007$, $P<0.001$, $P=0.012$, $P<0.001$, $P=0.021$, $P<0.001$, $P=0.049$, $P=0.037$, respectively). When multivariate analysis was performed using these nine factors, only advanced clinical stage was significantly correlated with poor prognosis ($P=0.004$) (**Table 3**). These findings from IHC analysis suggest that high expression of p62 protein in the cytoplasm may be a molecular marker for poor prognosis of patients with GA.

Discussion

As is known, p62 is a multifunctional adapter protein that contains different kinds of protein-protein interaction domains and participates in various cell signaling pathways through its ability to recruit and oligomerize important signaling molecules in cytosolic speckles for cell survival and cell death such as NF-κB, mTOR and Nrf2 pathways [5]. In addition, p62 is known to

be one of the selective adapters as well as substrates of autophagy, an important degradation pathway which can determine cell survival or death [6]. Not surprisingly, p62 is required for tumorigenesis and tumor progression owing to its roles as a key molecule in cell proliferation, nutrient sensing, induction of oxidative detoxifying proteins, and as a modulator of mitotic transit and genomic stability [12-16]. Furthermore, increasing evidences have shown that p62 was overexpressed and played important roles in various malignant tumors such as breast, liver, and lung cancer [8-10]. However, there are few reports regarding the role of p62 in gastric cancer [11].

In the current study, we found that p62 protein was located in the nucleus and/or cytoplasm in both GA and NAM tissues. The expression level of cytoplasmic p62 in GA was significantly higher than in matched NAM and p62-positive aggregate-like structures can be observed in part of GA tissues while rarely seen in NAM tissues. In addition, cytoplasmic overexpression of p62 was significantly associated with large tumor size, positive lymphatic invasion and was different in various major histological types of GA patients. Survival analyses showed increased cytoplasmic expression of p62 protein was significantly correlated with poor prognosis but not an independent prognosis factor in GA patients. Like other studies of p62 in various tumors, the results demonstrate that cytoplasmic p62 expression may be involved not only in tumor growth but also in tumor invasion for GA and may have prognostic value for GA patients. These evidences suggest that high expression level of cytoplasmic p62 may contribute to tumorigenesis and tumor malignancy. The possible mechanisms may be as follows. Firstly, p62 overexpression results in activation of NF-kB which is an important pathway for tumorigenesis and tumor progression in GC [12]. Furthermore, p62 participates in autophagy and activation of mTOR pathway which is a central regulator of cell growth as well as autophagy [13, 14]. At last, p62 can activate Nrf2 to induct oxidative detoxifying proteins which is important to tumor survival [15]. In spite of this, further studies for definite mechanisms are needed.

However we found that nuclear p62 showed lower expression in GA tissues but had no relationship with clinicopathologic features of GA patients. What's more, GA patients with decreased nuclear expression of p62 protein seemed to have higher overall survival rate but there was no statistical difference. This suggests that localization of p62 protein in the nucleus may attenuate the malignancy of GA but further study is needed. Previous studies showed p62 protein was shuttled between the nuclear and cytosolic compartments by its signals for nuclear localization and export to participate in the protection of genome stability [17]. The possible mechanism may be that nuclear p62 can interact with the promyelocytic leukemia bodies involved in the regulation of gene transcription, DNA damage response and etc [18].

In conclusion, the results suggest that the subcellular distribution of p62 is characterized by localization in the cytoplasm or nucleus. Cytoplasmic p62 overexpression may contribute to cancer progression in GA and become a novel prognostic biomarker for GA patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liya Zhou, Department of Gastroenterology, Peking University Third Hospital, No. 49 Huayuan North Road, Haidian District, Beijing 100191, China. Tel: 8610-82266719; Fax: 8610-62034716; E-mail: zhoumed@sohu.com

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