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Expression and prognostic significance of the DNA damage response pathway and autophagy markers in gastric cancer

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Gastric cancer (GC) is a leading cause of mortality and morbidity worldwide. We assessed the expression patterns of DNA damage response (DDR)-related markers, including ATM, CHK2, p-p53 (S15), Rad51, and BRCA2 and autophagy-related proteins including p62 and Beclin-1 in 153 GC specimens using immunohistochemistry staining. GC tissues showed lower levels of ATM, CHK2, p-p53, BRCA2, and higher levels of Rad51 compared to adjacent normal tissues. The autophagy-related protein p62 was upregulated, whereas Beclin-1 was downregulated in human GC groups. Additionally, different statuses of DDR pathways and autophagy characterized by protein expression were associated with overall survival. Our results indicated that the impairment of DNA damage and autophagy may be implicated in gastric cancer progression and its clinical prognosis.

Key words: DNA damage response, autophagy, gastric carcinoma, immunohistochemistry

Gastric cancer (GC) remains one of the leading causes of mortality and morbidity worldwide. Based on the GLOBOCAN 2018 estimates, GC is the fifth most commonly diagnosed malignancy and the third deadliest cancer [1]. Eastern Asian countries (such as China, Japan, and Korea) have a higher incidence of GC than Western countries. The geographic differences in gastric cancer incidence are attributed in part to the diet and *Helicobacter pylori* (*H. pylori)* infection [2]. Several genetic and environmental factors are involved in the development of gastric carcinoma. *H. pylori*, a helix-shaped gram-negative bacterium, is the strongest known risk factor for GC [3]. Stomach cancer is most frequently diagnosed in advanced stages and therefore has lower 5-year survival rates. Although a large number of dysregulated genes have been identified to be involved in the development of gastric carcinoma, the molecular mechanism remains unknown.

The human genome has been estimated to undergo several thousands of DNA lesions per cell every day. The integrity and stability of the genome are continuously monitored by a complicated DDR cellular network. DNA damage can be generated by environmental stressors, such as chemical agents and radiation [4]. Among several types of DNA lesions, DNA double-strand breaks (DSBs) are the most deleterious types of damage. Phosphorylated H2AX (γH2AX) has been identified as a marker of DSBs. Accumulating evidence has shown that γH2AX is aberrantly expressed in a variety of tumors [5]. In our previous study, the levels of the DSBs marker γH2AX were significantly higher in GC tissues than in adjacent normal tissues [6]. The failure of DNA lesion repair is responsible for the accumulation of DNA lesions, which leads to the accumulation of mutations that ultimately drive tumorigenesis [7].

The DDR signaling pathway orchestrated by ATM and ATR kinases coordinates several cellular processes, including DNA repair, cell-cycle checkpoints, and cell death. The upstream kinases ATM and ATR are activated by DSBs and induce activation of the checkpoint kinases CHK1 and CHK2, which initiates cell-cycle arrest and DNA repair. DSBs are mainly repaired by either homologous recombination (HR) or non-homologous end-joining (NHEJ) [8]. Our previous studies have indicated that DNA-PKcs and Ku70/80, the critical markers of NHEJ, were remarkably overexpressed in human GC tissues [9, 10]. In this study, we aimed to deter-

mine the effect of DNA HR repair on the development of gastric carcinoma by assessing the expression of the critical recombinase proteins Rad51 and BRCA2.

Recently, DNA damage has been linked to autophagy, which is an evolutionarily conserved intracellular degradation process by which cytoplasmic constituents are delivered to the lysosome for digestion [11]. In response to numerous cellular stresses, such as increased levels of reactive oxygen species (ROS) and pathogen infection, several types of DNA lesions are induced and then autophagy is stimulated [12]. The activation of autophagy is important for maintaining the integrity of the human genome by modulating the DDR pathway such as DNA repair, cell cycle checkpoints, and cell death [13]. Emerging evidence has shown that the loss of autophagy can impair DNA repair and give rise to genome instability [14]. A conserved set of autophagy-related genes (ATG) are involved in the formation of autophagosomes that engulf cargo destined for degradation. P62/SQSTM1, a well-known selective substrate for autophagy, directly interacts with LC3 for autophagosome formation. Because p62 is efficiently degraded by autophagy, decreased levels of p62 are widely used as a predictor of autophagic activity. Autophagy has a multifaceted role in the progression of tumors, either suppressing or promoting tumor growth and aggressiveness, depending on the tumor type. Recent studies have shown that p62 is overexpressed in various cancer tissues such as colorectal cancer, breast cancer, and lung carcinoma. Furthermore, the accumulation of p62 by the inhibition of autophagy has been reported to contribute to tumorigenesis and poor prognosis [15]. In addition, Beclin-1, the mammalian orthologue of yeast Atg6, is also a well-known key regulator of autophagy. Several studies have observed the loss of Beclin-1 in many cancer types [16]. However, there is sparse information on the expression of the autophagy markers p62 and Beclin-1 in gastric carcinoma.

In this study, we evaluated the expression of the DDR pathway and autophagy-related proteins in human stomach carcinoma tissues by immunohistochemistry. In addition, the correlation between the expression of these markers, clinicopathological characteristics, and the survival outcomes of gastric cancer patients was assessed. Also, we explored the influence of the DDR pathway and autophagy on stomach tumors.

Patients and methods

Gastric specimens. A total of 153 human GC tissue samples and their matched adjacent non-tumorous specimens were available for immunohistochemical analysis. Patients who underwent gastrectomy were recruited from the First Affiliated Hospital of Nanchang University from January 2007 to March 2012. These GC patients included 100 males and 53 females, and their median age was 58 years. None of the patients had received preoperative chemotherapy or radiotherapy. The GC patients were followed up over a five-year period. The overall survival of GC patients was calculated as the time from diagnosis to the date of death. The study protocol and exemption of informed consent were approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University.

Immunohistochemistry staining. All surgical specimens from the patients were fixed in 10% formalin and embedded in paraffin. These formalin-fixed paraffin-embedded (FFPE) sections were sliced into 4 μm thick sections. Immunohistochemistry staining was performed to detect the expression of DDR and autophagy-related markers. The sections were deparaffinized with xylene and rehydrated in a graded alcohol series. The activity of endogenous peroxidase activity was blocked by 3% fresh H_2O_2 for 10 min. Microwave heating of the section was performed to expose antigenic sites for antibody binding. Next, the sections were incubated with primary antibodies at 4°C overnight and then washed with PBS three times. The antibodies and their sources were as follows: ATM (ab78), Phospho-ATM Ser1981 (ab36810), CHK2 (ab8108), Phospho-CHK2 Thr68 (ab3846), Phospho-p53 Ser15 (ab1431), Rad51 (ab88572), BRCA2 (ab27976), and Beclin-1 (ab62557) from Abcam (Cambridge, MA, USA); p62 (AP21836) from Abgent (San Diego, CA, USA). The sections were then incubated with secondary antibody (PV-6000, Zhongshan Golden Bridge Biotechnology, Beijing, China) at 37°C for 30 min. For visualization, the HRP/DAB Detection Kit (ZLI-9030) was used according to the manufacturer's instructions. Finally, the sections were counterstained with hematoxylin and mounted with coverslips. Each sample was strained at least 1 time.

Immunohistochemical assessment. The scoring of immunohistochemical staining patterns for all proteins was performed as described previously [6]. All slides were evaluated and scored by two experienced pathologists. The staining intensity was scored on a scale of 0–3 (0, no staining; 1, weak staining; 2, intermediate staining; and 3, strong staining). The proportion of positive cells was scored on a scale of 0–4 (0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, 75–100%). The sum of the scores was determined by multiplying the intensity scores by the extent of positive cells. Finally, the protein levels were stratified as negative (–, 0 score), weakly positive (+, 1–4 score), moderately positive (++, 5–8 score), or strongly positive (+++, 9–12 score) and scored for intensity (scaled 0–3) and frequency (scaled 0–4) (Tables 1 and 2; Supplementary Tables S1 and S2) [17].

Western blot analysis. Tissues were lysed in RIPA lysis buffer with protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were detected by the BCA assay kit (Thermo Fisher Scientific). Proteins separated by SDS–polyacrylamide gel and transferred to nitrocellulose membranes. After blocking with 5% milk, the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies of p62 (7695S) and Beclin-1 (4122S) were from Cell Signaling Technologies (Beverly, MA, USA). GAPDH was used as an internal

Table 1. Clinicopathological association of DNA repair proteins expression in patients with GC.

Characteristics	n	Overall score of Rad51 expression						Overall score of BRCA2 expression					
		–, n	+, n	++, n	+++, n	%[a]	p-value	–, n	+, n	++, n	+++, n	%[a]	p-value
Gender													
Male	100	8	43	37	12	92.0	>0.05	65	27	5	3	35.0	>0.05
Female	53	5	28	17	3	90.6		38	11	3	1	28.3	
Age (years)													
≥55	91	8	44	27	12	91.2	>0.05	62	22	5	2	31.9	>0.05
<55	62	5	27	27	3	91.9		41	16	3	2	33.9	
Location													
Antrum	78	10	35	24	9	87.2	>0.05	57	16	2	3	26.9	>0.05
Body and cardia	75	3	36	30	6	96.0		46	22	6	1	38.7	
c (Borrmann)													
I+II	76	9	34	26	7	88.2	>0.05	54	18	3	1	28.9	>0.05
III+IV	77	4	37	28	8	94.8		49	20	5	3	36.4	
Differentiation													
Well and moderately	85	9	49	21	6	89.4	<0.01	59	19	4	3	30.6	>0.05
Poorly and undifferentiated	68	4	14	23	27	94.1		44	19	4	1	35.3	
Invasive depth													
Submucosa and muscularis	38	5	19	12	2	86.8	>0.05	29	7	2	0	23.7	>0.05
Below subserosa	115	8	50	43	14	93.0		74	31	6	4	35.7	
TNM													
I+II	62	5	37	16	4	91.9	<0.05	48	10	4	0	22.6	>0.05
III+IV	91	8	34	38	11	91.2		57	26	4	4	37.4	
Lymph node metastasis													
With	119	8	52	44	15	93.3	<0.05	79	30	6	4	33.6	>0.05
Without	34	5	20	9	0	85.3		24	8	2	0	29.4	

Note: [a] percentage of immunostaining

control. The membranes were then incubated with secondary antibodies and visualized by the BioRad-ChemiDoc CR+ system. Each sample was performed at least 1 time.

Statistical analysis. All statistical analyses were performed using SPSS 20.0 software. The data are presented as the mean ± standard deviation or percentage. Group comparisons were performed using the Kruskal-Wallis test (≥2 groups) or Mann-Whitney U test (2 groups). A p-value ≤0.5 was considered significant.

Results

DDR and HR-related proteins ATM, CHK2, p-P53, BRCA2 are downregulated, and Rad51 is upregulated in gastric carcinoma. Our previous studies have shown that the DSBs biomarker γH2AX is overexpressed in human GC tissues [6]. Cell-cycle checkpoints can be activated to induce cell cycle arrest and initiate DNA repair mechanisms in response to DSBs. To determine the clinical relevance of the checkpoint kinase ATM-CHK2 pathway in the development of human GC, we first assessed the expression patterns of ATM (S1981) phosphorylation, CHK2 (T68) phosphorylation, total ATM, CHK2, and p53 (S15) phosphorylation using immunohistochemistry staining in tumor tissues compared with their adjacent normal tissues. The representative results of the immunohistochemistry staining patterns for ATM, CHK2, and p53 (S15) are shown in Figure 1A. ATM and CHK2 staining were exclusively nuclear with no cytoplasmic expression in cancer tissues. Compared with the adjacent noncancerous tissues, the expression levels of ATM and CHK2 were significantly decreased in stomach carcinoma tissues (Figure 1B). Additionally, the expression of p53 at Ser15 appeared to be localized in the nucleus. The gastric carcinoma tissues showed significantly decreased phosphorylated levels of p53 compared to control tissues (Figure 1B). Furthermore, statistical analyses were performed to examine the association of the phosphorylation of ATM at S1981, the phosphorylation of CHK2 at T68, total ATM, CHK2, and the phosphorylation of p53 at Ser15 with the clinicopathological characteristics of patients with GC. High expression of ATM was significantly associated with poor differentiation ($p<0.05$) and advanced TNM stage ($p<0.05$), but was not correlated with other clinical parameters, including sex, age, tumor location, tumor types, invasive depth, and lymph node metastasis. Intriguingly, phosphorylated ATM (S1981) showed different characteristics. The phosphorylation of ATM was associated with age ($p<0.05$), tumor location ($p<0.05$), tumor differentiation ($p<0.01$), TNM stage ($p<0.01$), and lymph node metastasis ($p<0.05$). In addition, CHK2 and its phosphorylated expression at T68 were markedly associ-

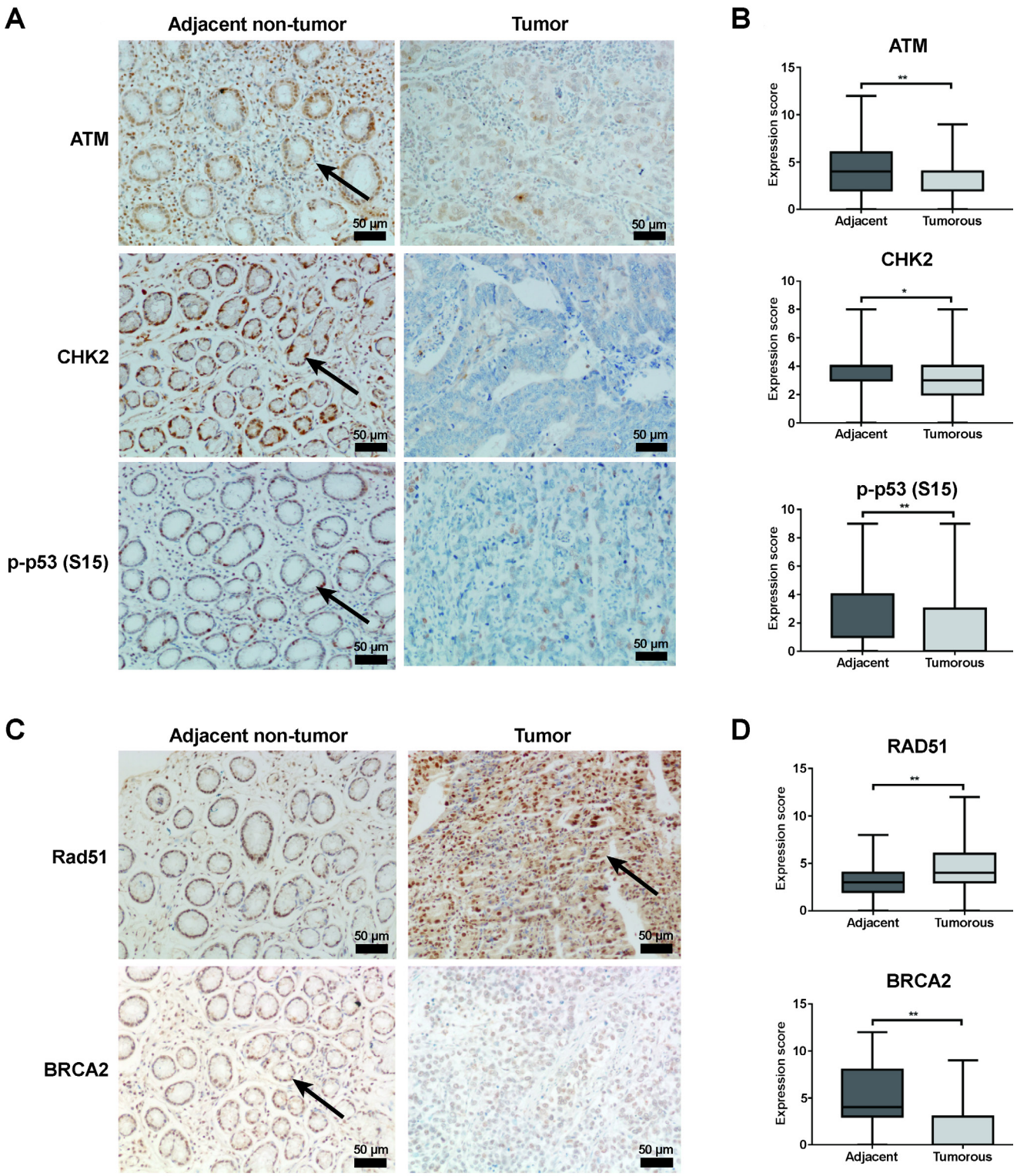

Figure 1. The expression levels of DDR-related proteins in GC. A) Representative immunohistochemistry staining images of ATM, CHK2, and p-P53 (S15) in human GC tissues and adjacent normal tissues. (ATM, CHK2, and p-P53 are stained in yellow) B) Quantitative comparison of immunohistochemical staining for ATM, CHK2, and p-P53 (S15) between normal and tumor tissues. C) Representative immunohistochemistry staining results of Rad51 and BRCA2 in GC tissues. D) Comparison of the immunohistochemical scores of Rad51 and BRCA2 between GC tissues and adjacent non-tumor tissues.

Table 2. Clinicopathological association of Beclin-1 and p62 expression in patients with GC.

Characteristics	n	Overall score of p62 expression						Overall score of Beclin-1 expression					
		–, n	+, n	++, n	+++, n	%[a]	p-value	–, n	+, n	++, n	+++, n	%[a]	p-value
Gender													
Male	100	36	50	13	1	64.0	>0.05	34	60	6	0	66.0	>0.05
Female	53	20	25	7	1	62.3		26	16	11	0	50.9	
Age (years)													
≥55	91	40	38	13	0	56.0	<0.05	31	51	9	0	65.9	>0.05
<55	62	16	36	8	2	74.2		29	27	7	0	54.8	
Location													
Antrum	78	31	36	10	1	60.3	>0.05	35	37	6	0	55.1	>0.05
Body and cardia	75	25	39	10	1	66.7		25	40	10	0	66.7	
Gross type (Borrmann)													
I+II	76	29	35	10	2	61.8	>0.05	29	43	4	0	61.8	>0.05
III+IV	77	27	40	10	0	64.9		31	34	12	0	59.7	
Differentiation													
Well and moderately	85	33	39	12	1	61.2	>0.05	37	43	5	0	56.5	>0.05
Poorly and undifferentiated	68	23	35	9	1	66.2		23	34	11	0	66.2	
Invasive depth													
Submucosa and muscularis	38	14	19	4	1	63.2	>0.05	15	19	4	0	60.5	>0.05
Below subserosa	115	42	56	16	1	63.5		45	57	13	0	60.9	
TNM													
I+II	62	24	28	8	1	59.7	>0.05	25	32	5	0	59.7	>0.05
III+IV	91	32	46	12	1	64.8		35	45	11	0	61.5	
Lymph node metastasis													
With	119	43	59	16	1	63.9	>0.05	45	59	15	0	62.2	>0.05
Without	35	13	14	5	1	57.1		15	17	2	0	54.3	

Note: [a] percentage of immunostaining

ated with poor tumor differentiation ($p<0.01$). There was no significant difference between phosphorylated p53 and the pathological features (Supplementary Tables S1, S2).

In response to dangerous DSBs, DDR kinases are activated to initiate DNA repair to maintain genomic stability. Given the aberrant expression of NHEJ markers in GC tissues, we further evaluated the effect of HR, which is another major DNA repair pathway in GC progression. The expression of the central markers of HR, Rad51 and BRCA2, was examined in human GC tissues. Representative results indicated that Rad51 and BRCA2 were attributed in the nucleus in the majority of gastric carcinomas (Figure 1C). The accumulation of Rad51 has been found in human GC tissues compared with adjacent normal tissues. In contrast, there was a decrease in the expression of BRCA2 in cancer tissues (Figure 1D). Furthermore, the expression of Rad51 and BRCA2 was analyzed to explore their relationship with clinicopathological features. Our results showed that the overexpression of Rad51 was associated with poorly differentiated tumors ($p<0.01$) (Table 1). These data suggest that abnormal DDR kinases and HR repair may result in the failure of DNA repair and the accumulation of DNA mutations in GC initiation and progression.

Autophagy-related protein p62 is upregulated and Beclin-1 is downregulated in human GC tissues. Autophagy has been reported to be linked to DNA damage. The activation of autophagy is responsible for the functional outcomes of the DDR signaling pathways, such as DNA repair and cell-cycle checkpoints. To define the autophagy status in GC progression, we analyzed the expression of autophagy-related proteins including p62 and Beclin-1 in human GC tissues. As shown in Figure 2A, Beclin-1 and p62 were mainly distributed both in the cytoplasm and nucleus of cancer cells. Compared to the adjacent normal tissues, the expression of the autophagy substrate p62 significantly accumulated in neoplastic tissues (Figure 2B). In contrast, the loss of Beclin-1 was observed more frequently in most stomach tumor cases (Figure 2C). These findings were further supported by western blot analysis (Figure 2F). These data suggested that the accumulation of p62 and reduction of Beclin-1 may indicate a loss of autophagy, which is involved in the tumorigenesis and progression of GC. The relationship between the levels of p62 and Beclin-1 levels and the clinicopathological features of the tumors are shown in Supplementary Table S2. There was no significant association between the p62 and Beclin-1 expression

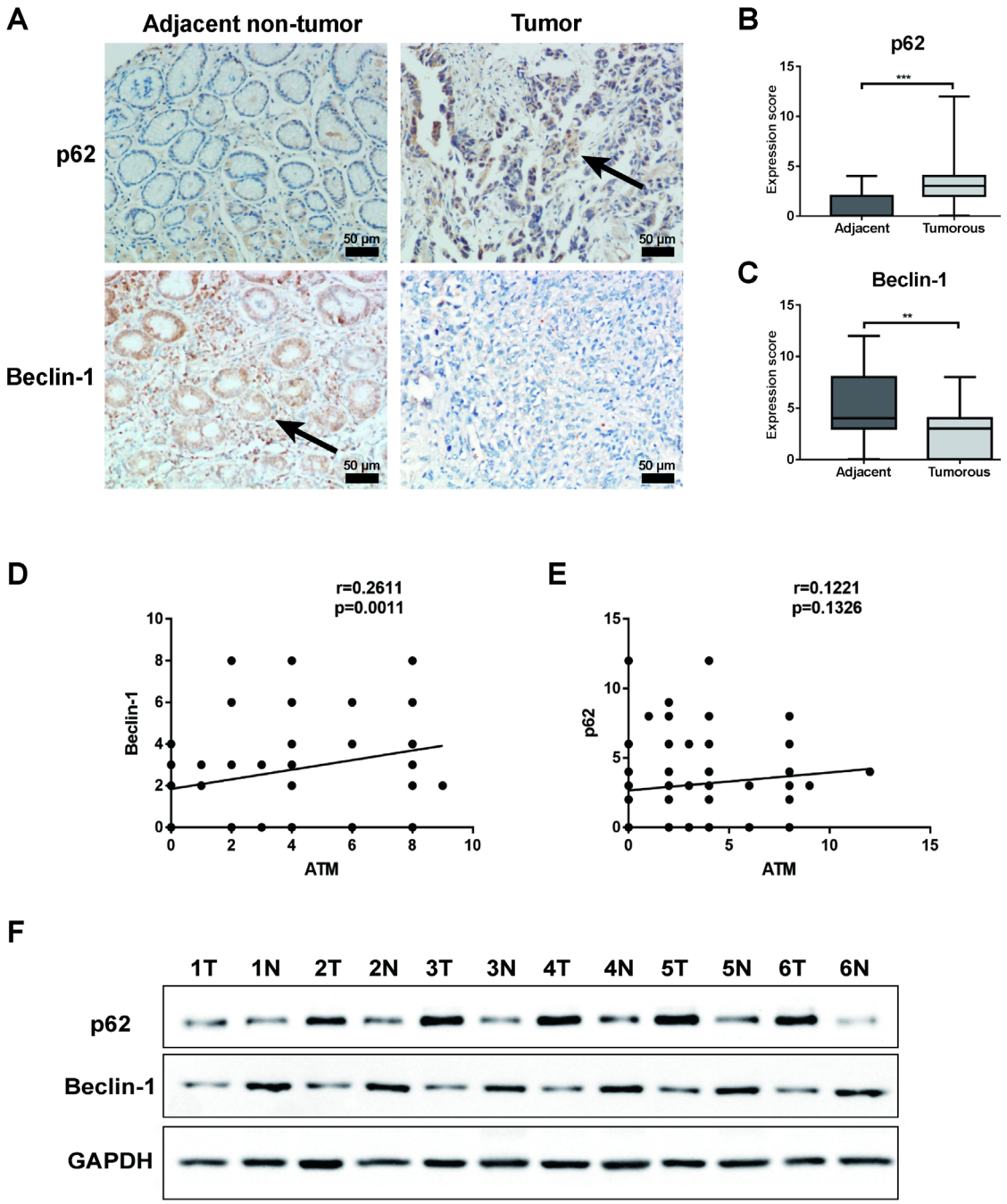

Figure 2. The expression levels of autophagy-related proteins in human GC tissues. A) Representative images of immunohistochemistry staining for p62 and Beclin-1 in human GC tissues and adjacent normal tissues (p62 and Beclin-1 are stained in yellow) B, C). Comparison of the immunohistochemical scores of p62 (B) and Beclin-1 (C) between GC tissues and adjacent non-tumor tissues. D) Pearson correlation analysis between the expression scores of ATM and Beclin-1. E) Pearson correlation analysis between the expression scores of ATM and p62. F) Western Blot for p62 and Beclin-1 in human GC tissues and adjacent normal tissues. Abbreviations: T-Tumor; N-non-tumor

patterns and pathological features including sex, tumor location, histological type, differentiation, invasive depth, and lymph node metastasis. Interestingly, p62 staining was significantly associated with age ($p<0.05$). In summary, these data suggest that the impairment of autophagy may be implicated in gastric tumorigenesis.

ATM expression correlated with autophagy-related marker Beclin-1 in GC. The DNA damage sensor ATM is

well known to promote autophagy by activating the AMPK/mTOR signaling pathway [18]. We next evaluated the correlations between ATM and autophagy-related markers in GC tissues. Pearson correlation analysis predicted that the levels of ATM expression were positively correlated with the levels of Beclin-1 (r=0.2611, p=0.0011; Figure 2D). However, there was no significant correlation between ATM and p62 expression (r=0.1221, p=0.1326; Figure 2E).

Prognostic analysis. The 153 patients with GC were divided into the positive or negative expression group according to their staining score. An immunoreactive score ≤2 was defined as negative, whereas a score >2 was defined as positive. The 5-year Kaplan-Meier overall survival analysis for the cohort of patients with GC is shown in Figure 3. Total ATM and CHK2 were not significantly associated with GC-specific survival (p>0.05; Figures 3A, 3B). However, tumors with a positive expression of phosphorylated ATM at S1981 were associated with an adverse clinical outcome with poor GC-specific survival (p<0.01) compared to tumors with negative p-ATM (Figure 3C). Regarding phosphorylated CHK2, there was no significant difference between the patients with positive and negative expression (Figure 3D). Furthermore, we found that the negative expression of the DDR downstream protein p-p53 (S15)

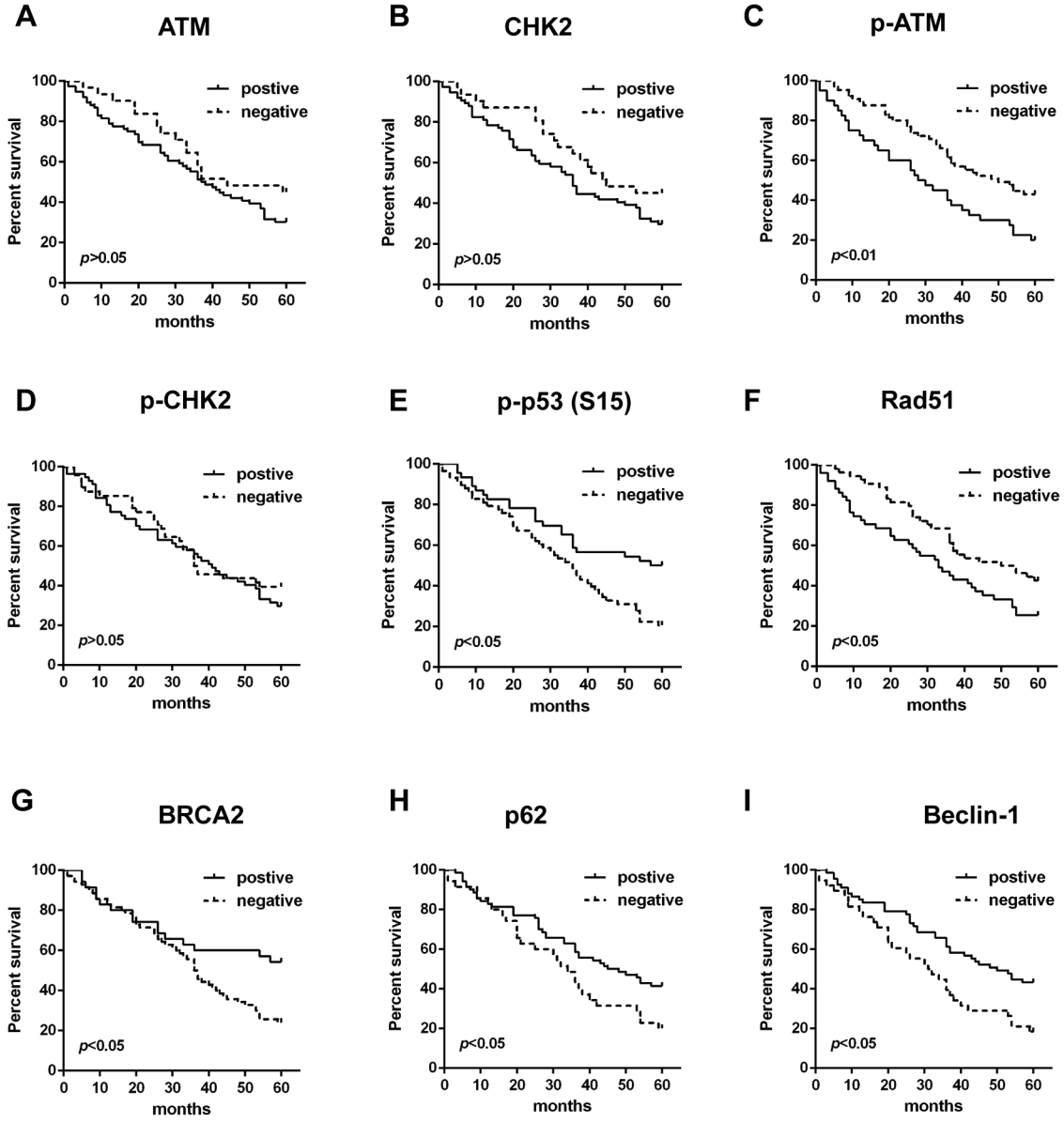

Figure 3. Correlation between the expression levels of DDR and autophagy-related proteins and the prognosis of GC by Kaplan-Meier analyses of overall survival. A) ATM; B) CHK2; C) p-ATM; D) p-CHK2; E) p-P53 (S15); F) Rad51; G) BRCA2; H) p62; I) Beclin-1

was associated with decreased 5-year overall survival in GC patients (Figure 3E).

Next, we examined the prognostic effect of the expression levels of DNA repair and autophagy-related proteins on GC patients. Patients with higher BRCA, p62, and Beclin-1 expression levels were statistically correlated with a better 5-year survival outcome than patients with lower expression levels of the respective markers ($p<0.05$; Figures 3G–3I). Nevertheless, Rad51-positive tumors showed a poor clinical prognosis compared with Rad51-negative tumors (Figure 3F).

Discussion

The maintenance of genomic integrity depends on the coordinated manipulation of a network of cellular biological processes, including cell cycle checkpoints, DNA replication, DNA repair, and so on. Generally, the cellular response to DNA damage initiates the DDR signaling pathway, which is orchestrated by the ATM and CHK protein kinases. A large number of genes are involved, directly or indirectly, in the regulation of the DDR signaling pathway, including the upstream DDR kinases ATM and CHK, the cell cycle-associated protein p53, and the DNA repair markers DNA-PKcs, Rad51, and BRCA1/2 [19, 20]. Autophagy, a highly conserved process for intracellular degradation and recycling, has been recently linked to the DDR pathway [21]. Growing evidence indicates that upon DNA damage autophagy can be induced, which in turn regulates the DNA damage repair process, cell cycle progression, and other processes [21]. In this study, we reported the expression patterns of the DDR-related proteins ATM, CHK2, p53, Rad51, and BRCA2 in human GC tissues. Additionally, we examined the effect of the autophagy-related markers p62 and Beclin-1 in human gastric samples. The data suggested that their aberrant expression may be involved in gastric tumorigenesis and tumor progression.

It is well known that DSBs are one of the most cytotoxic DNA lesions. The ATM-CHK2 dependent DDR pathway is activated primarily to address DSBs. ATM can be recruited to the broken DNA ends and autophosphorylates serine 1981 for activation, which results in the phosphorylation of a number of downstream substrates such as CHK2 and p53 [22]. Our study indicated that the protein levels of ATM, CHK2, and p53 were significantly decreased in human GC tissues compared to normal tissues. This finding appears to be supported by a previous study, which showed that loss of ATM and CHK2 was observed in GC [23]. Human malignant tumors result from the accumulation of oncogenic mutations triggered by DNA damage, which leads to uncontrolled cell growth and hyperproliferation. Based on our observations, DDR signaling pathways are supposed to function as a barrier to activation in the early evolution of gastric tumorigenesis, but the loss of DDR-related proteins may result in irreparable damage to DNA and in cancerous cells, ultimately promoting the occurrence of cancer [24]. Increasing evidence has confirmed the involvement of DDR-associated factors in various human diseases, particularly in a broad range of human cancers [25]. Aljarbou et al. indicated that DNA sensors ATR, CHK1, and CHK2 were overexpressed in Saudi patients with colon cancer compared with the adjacent mucosa [26]. In addition, we observed the significant upregulation of phosphorylated ATM and CHK2 in advanced malignant gastric tumor tissues. The high expression of ATM phosphorylation was associated with poor prognosis in GC. Recently, several selective molecule inhibitors of ATM have been developed in preclinical and clinical experiments. These ATM inhibitors could also be utilized to sensitize cancer cells to the synthetic lethal effects of genotoxic modalities, therefore improving the efficiency of chemotherapy and radiotherapy [27]. At the same time, it has been reported that the combination of DDR targeting drugs with immune checkpoint inhibitors (ICIs) could open up a new approach to cancer treatment [28]. Currently, the exact mechanism involved in the regulation of the DDR pathways in GC remains unclear. Further studies are needed to understand the interplay between these DDR-related proteins in tumorigenesis and tumor progression.

DNA repair activated by DDR signaling is essential for genomic integrity. We observed the dysregulation of HR repair via the upregulation of Rad51 and downregulation of BRCA2 in human GC samples. There is growing evidence that their aberrant expression is implicated in the progression of human malignancies [29, 30]. Elevated Rad51 levels have been identified to confer resistance to chemotherapy or radiotherapy with DNA damage agents in cancer cells [31]. However, BRCA2 as a core mediator of HR repair is a well-known tumor suppressor. A high frequency of BRCA2 gene mutations was recently reported to increase the risk of multiple tumors due to increased levels of genomic instability [32].

Autophagy, an intracellular self-degradative process that delivers cytoplasmic components to the lysosome, plays an important role in the maintenance of energy homeostasis. Autophagy has been demonstrated to play a multifaceted role in tumor initiation and progression [33]. In this study, we observed the loss of the autophagy-related gene Beclin-1 and the accumulation of autophagy selective-substrate p62 in gastric carcinoma tissues. Our research suggests that the inhibition of autophagy may be implicated in gastric neoplasia. These findings were supported by previous data showing that the depletion of Beclin-1 was found in various tumors, including hepatocellular carcinoma, ovarian cancer [34, 35]. The loss of Beclin-1 can lead to the inhibition of autophagosome and cell hyperproliferation, suggesting that Beclin-1 functions as a tumor suppressor. The induction of autophagy in response to DNA lesions is required to maintain cellular genomic integrity and to protect against disease [13]. It has been reported that DNA sensor ATM promotes autophagy through activation of AMPK. In this study, we showed that ATM was positively correlated with Beclin-1 in

GC, further suggesting the induction of autophagy response to DNA damage in gastric cancer progression.

Given the importance of DDR signaling pathways and autophagy in tumor initiation and progression, we systematically detected the expression patterns of their related proteins in human gastric carcinoma tissues and further analyzed their association with the prognosis of neoplasms. In summary, this study suggested that the impairment of DDR pathways and inhibition of autophagy might be implicated in gastric tumorigenesis. Additionally, autophagy defects may result in the accumulation of DNA damage. Further investigations involving the crosstalk among DDR pathways, autophagy, and GC could potentially lead to the development of a therapeutic strategy that selectively targets autophagy and DNA damage proteins.

Supplementary information is available in the online version of the paper.

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Expression and prognostic significance of the DNA damage response pathway and autophagy markers in gastric cancer

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Supplementary Information

Supplementary Table S1. Clinicopathological association of ATM and CHK2 expression in patients with GC.

Characteristics	n	Overall score of ATM expression						Overall score of CHK2 expression					
		–, n	+, n	++, n	+++, n	%[a]	p-value	–, n	+, n	++, n	+++, n	%[a]	p-value
Gender													
Male	100	30	50	19	1	70.0	>0.05	31	62	7	0	69.0	>0.05
Female	53	16	32	4	1	69.8		19	32	2	0	64.2	
Age (years)													
≥55	91	28	47	16	0	69.2	>0.05	31	56	5	0	67.0	>0.05
<55	62	18	34	8	2	71.0		19	38	5	0	69.4	
Location													
Antrum	78	27	42	8	1	65.4	>0.05	28	46	4	0	64.1	>0.05
Body and cardia	75	19	40	15	1	74.7		22	48	5	0	70.7	
Gross type (Borrmann)													
I+II	76	20	45	10	1	73.7	>0.05	28	45	3	0	63.2	>0.05
III+IV	77	26	38	12	1	66.2		22	49	6	0	71.4	
Differentiation													
Well and moderately	85	31	46	7	1	63.5	<0.05	36	45	4	0	57.6	<0.01
Poorly and Undifferentiated	68	15	37	15	1	77.9		14	49	5	0	79.4	
Invasive depth													
Submucosa and Muscularis	38	12	23	3	0	68.4	>0.05	14	23	1	0	63.2	>0.05
Below subserosa	115	34	59	20	2	70.4		36	71	8	0	68.7	
TNM													
I+II	62	22	37	3	0	64.5	<0.05	26	33	3	0	58.1	>0.05
III+IV	91	24	45	20	2	73.6		24	61	6	0	73.6	
Lymph node metastasis													
With	119	40	57	20	2	66.4	>0.05	37	74	8	0	68.9	>0.05
Without	34	6	25	3	0	82.4		13	19	2	0	61.8	

Note: [a]percentage of immunostaining

Supplementary Table S2. Clinicopathological association of p-ATM (S1981), p-CHK2 (T68) and p53 (S15) proteins expression in patients with GC.

Characteristics	n	Overall score of p-ATM(S1981) expression						Overall score of p-CHK2(T68) expression						Overall score of p53(S15) expression					
		−, n	+, n	++, n	+++, n	%[a]	p-value	−, n	+, n	++, n	+++, n	%[a]	p-value	−, n	+, n	++, n	+++, n	%[a]	p-value
Gender																			
Male	100	63	28	9	0	37.0	>0.05	45	41	11	3	55.0	>0.05	56	37	5	2	44.0	>0.05
Female	53	27	25	1	0	49.1		25	18	5	5	52.8		36	14	2	1	32.1	
Age (years)																			
≥55	91	60	24	7	0	34.1	<0.05	45	31	9	6	50.5	>0.05	50	34	5	2	45.1	>0.05
<55	62	30	30	2	0	51.6		25	27	8	2	59.7		42	16	3	1	32.3	
Location																			
Antrum	78	53	20	5	0	32.1	<0.05	38	31	6	3	51.3	>0.05	50	22	6	0	35.9	>0.05
Body and cardia	75	37	33	5	0	50.7		32	27	10	6	57.3		42	28	2	3	44.0	
Grosstype (Borrmann)																			
I+II	76	48	25	3	0	36.8	>0.05	35	31	8	2	53.9	>0.05	47	23	4	2	38.2	>0.05
III+IV	77	42	29	6	0	45.5		35	27	9	6	54.5		45	28	3	1	41.6	
Differentiation																			
Well and moderately	85	60	20	5	0	29.4	<0.01	45	35	4	1	47.1	<0.01	51	28	4	2	40.0	>0.05
Poorly and Undifferentiated	68	30	32	6	0	55.9		25	22	14	7	63.2		41	22	4	1	39.7	
Invasive depth																			
Submucosa and muscularis	38	23	15	0	0	39.5	>0.05	19	14	3	2	50.0	>0.05	24	11	2	1	36.8	>0.05
Below subserosa	115	67	39	9	0	41.7		51	43	15	6	55.7		68	40	5	2	40.9	
TNM																			
I+II	62	44	18	0	0	29.0	<0.01	34	23	3	2	45.2	>0.05	41	16	4	1	33.9	>0.05
III+IV	91	46	36	9	0	49.5		36	35	14	6	60.4		51	34	4	2	44.0	
Lymph node metastasis																			
With	119	64	45	10	0	46.2	<0.05	53	44	14	8	55.5	>0.05	68	44	5	2	42.9	>0.05
Without	34	26	8	0	0	23.5		17	15	2	0	50.0		24	7	2	1	29.4	

Note: [a]percentage of immunostaining