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Expression and prognostic significance of cyclin-dependent kinase inhibitor 1A in patients with resected gastric adenocarcinoma

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Abstract. Cyclin-dependent kinase inhibitor 1A (CDKN1A) is an important cell cycleregulator, and has been identified to exhibit aberrant expression in various types of cancer tissues. However, the association between CDKN1A expression level and prognosis in patients with resected gastric adenocarcinoma (RGA) requires additional elucidation. In the present study, the CDKN1A expression profile in RGA tissues obtained from 217 patients were analyzed using immunohistochemistry. Its prognostic significance was evaluated by using the χ^2 test, Kaplan-Meier curves and the log-rank test, and a multivariate Cox model analysis, during a median follow-up time of 51 months. The results demonstrated that CDKN1A expression was significantly correlated with lymph node metastasis (LNM; P=0.001), recurrence (P<0.001) and overall survival (OS; P<0.001). In addition, the recurrence-free survival (RFS) and OS times were significantly shorter in patients with low CDKN1A expression compared with those with high CDKN1A expression (RFS, 20 months vs. 69 months, P<0.001; and OS, 32 months vs. 70 months, P<0.001, respectively). Multivariate analysis additionally confirmed that low CDKN1A expression was significantly correlated with an increased risk of LNM (P=0.001), recurrence (P<0.001) and mortality (P<0.001). Therefore, these data suggest that low expression of CDKN1A has independent prognostic significance indicative of tumor progression and poor survival in patients with RGA. Evaluation of CDKN1A expression may assist in determining prognosis in patients with RGA.

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Introduction

Gastric cancer is the most common digestive tract malignancy that affects ~1 million people per year worldwide (1), and >90% of these cases are gastric adenocarcinoma (GA). At present, surgery remains the primary method for treating patients with GA. Lymph node metastasis (LNM) usually indicates a poor prognosis of resected GA (RGA), as it is closely associated with widespread metastasis that will affect the survival of the patients (2,3). A reduced risk of metastasis and recurrence and improved survival time are the primary goals of cancer therapy. Therefore, it would be useful to identify a biomarker associated with LNM that may assist in predicting prognosis.

Cyclin-dependent kinase inhibitor 1A (CDKN1A; also known as p21), a cell cycle regulator which is encoded by the *CDKN1A* gene at chromosome 6p21.2, is primarily involved in mediating cell growth, proliferation, differentiation, DNA repair and apoptosis (4-6). Data from a number of studies have demonstrated that it assists in regulating tumor development by inducing cell cycle arrest, leading to a reduction in cancer cell growth rate (7,8). Aberrant expression of CDKN1A has been frequently observed in different types of cancer tissues (9-11). In the present study, in order to understand the association between CDKN1A expression and prognosis in patients with RGA, the correlation between the expression of CDKN1A and clinicopathological factors, LNM, recurrence rate, and survival in patients with RGA was evaluated by various statistical analyses.

Materials and methods

Patients. GA tissue samples for immunohistochemistry (IHC) were retrospectively collected from 217 patients who were diagnosed pathologically with primary GA and then underwent surgical resection between January 2003 and November 2008.

Information was obtained from the medical records of each patient and follow-up records subsequent to treatment were monitored until March 2013. Survival data were obtained via telephone contact and the Social Security Death Index. Recurrence was diagnosed according to the criteria described in our previous study (12). Fluoropyrimidine-based regimens (fluoropyrimidine or fluoropyrimidine plus platinum) were used as first-line chemotherapy to treat all of the patients following resection of GA. The standards described in the guidelines of the International Union Against Cancer (UICC; 5th Edition) and the National Comprehensive Cancer Network's (NCCN) Clinical Practice Guidelines in Oncology (v.1.2011) (13,14) were used to determine the tumor-node-metastasis (TNM) stage and histological grade for each of the tissue samples used in the present study.

For comparison of the results of western blotting and IHC, GA tissue samples from 52 cases were collected from patients with primary GA who underwent surgical resection between March 2014 and December 2015.

The study was approved by the Ethics Committee of Fuzhou General Hospital (Fuzhou, China) and all patients provided informed consent.

IHC. IHC was used to determine the levels of CDKN1A in paraffin-embedded RGA (217 cases) and normal gastric tissues (30 cases). The anti-CDKN1A antibody was obtained from ZSGB-BIO (cat. no. TA808128; dilution, 1:150; Beijing, China). IHC was performed as described previously (12,15). The expression intensity of CDKN1A was scored from 0 to 3 according to the standards established by Kawata *et al* (16). Samples with a staining intensity of 0 or 1 were considered as low expression, and with staining intensity of 2 or 3 as high expression.

Hematoxylin and eosin (H&E) staining. H&E staining of paraffin-embedded normal gastric tissues and cancer tissues in patients with RGA were performed by a pathologist using an established methodology. The steps of H&E staining are briefly described as follows: i) Sections of tissue samples were dewaxed with xylene and washed with various concentrations of ethanol (100, 100, 95 and 80%); ii) stained with 0.5% hematoxylin for 5 min, washed with water for 10 min and then distilled water for 5 sec; iii) soaked in hydrochloric acid ethanol for 30 sec and then water for 15 min; iv) 0.5% eosinstaining for 2 min; v) conventional dehydration, transparency and neutral resins mount; vi) the results were observed using light microscopy (magnification, x200). All steps were performed at room temperature (25˚C).

Western blotting. As described previously (17), total protein from cancer tissues was extracted using RIPA lysis buffer (cat. no. P0013B) and quantified using the BCA Protein Assay kit (cat. no. P0012S), and western blotting was performed using a SDS-PAGE Gel preparation reagent kit [cat. no. P0012A: P0012A-1, 30% Acr-Bis (29:1); P0012A-2, 1M Tris-HCl, pH 8.8; P0012A-3, 10% SDS; P0012A-4, ammonium persulfate; P0012A-5, TEMED; P0012A-6, 1M Tris-HCl, pH 6.8; SDS-PAGE Sample Loading Buffer (5X), cat. no. P0015], blocking buffer (cat. no. P0023B) and enhanced chemiluminescence reagents (ECL; cat. no. P0018) (all of the above reagents were from Beyotime Institute of Biotechnology, Haimen, China). Briefly, the proteins (30 μg per lane) were subjected to SDS-PAGE gel electrophoresis (concentrated gel, 5%; separation gel, 12%) and then transferred to polyvinylidene membranes. Following incubation with blocking buffer at room temperature for 30 min, the membranes were incubated with primary polyclonal rabbit anti-human CDKN1A antibody (cat. no. SC-397; dilution, 1:300; ZSGB-BIO) or primary polyclonal rabbit anti-human GAPDH antibody (cat. no. 2118S; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. Following three washes with TBST the membranes were incubated with secondary goat anti-rabbit antibody IgG-horseradish peroxidase (cat. no. M21002M; dilution, 1:2,000; abMart Company, Shanghai, China) for 4 h at room temperature. Finally, the bands were visualized using ECL and analyzed with a chemiluminescence imager system (Image Quant LAS4000 mini system; GE Healthcare, Chicago, IL, USA).

Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to conduct data analysis, including the correlation between CDKN1A expression and the clinicopathological characteristics and prognosis of patients. The methods used in this analysis were the χ^2 test, and Kaplan-Meier and log-rank analyses of recurrence-free survival (RFS) and overall survival (OS) times in months. Additionally, univariate and multivariate Cox models were used to assess the prognostic ability of known unfavorable pathological factors, and the correlation between CDKN1A expression profiles and recurrence or survival. The agreement between CDKN1A expression detected by western blot and immunohistochemical analyses was evaluated by Spearman's rank correlation analysis. All tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics of patients. The clinicopathological characteristics of the 217 RGA patients are summarized in Table I. All patients had received fluoropyrimidine or fluoropyrimidine plus platinum agents as first-line chemotherapy for an average of 4 cycles following resection. The patients comprised 153 males and 64 females; 135 patients were <60 years old, and 82 patients were ≥60 years old, the median age was 60 years. At the time of resection, 147 patients (67.7%) presented with LNM. In 145 cases (66.8%), the tumors were moderately or well-differentiated, while the remaining 72 cases (33.2%) exhibited poorly differentiated histology. By the end of follow-up, 126 had developed recurrent disease, and 116 patients had succumbed to the disease. The median times to recurrence and mortality were 41 and 54 months, respectively. The median time of observation was 51 months.

CDKN1A expression and correlation between CDKN1A expression and clinicopathological characteristics. CDKN1A exhibited differential expression patterns in RGA and normal gastric tissues. In normal gastric tissues,

Table I. Patient characteristics (n=217).

Characteristics	Cases	
	n	%
Age, years		
<60	135	62.2
≥60	82	37.8
Sex		
Male	153	70.5
Female	64	29.5
Histological grade		
Well/moderately differentiated	145	66.8
Poorly differentiated	72	33.2
Gross findings		
Apophysis	17	7.8
Invasion	200	92.2
Tumor site		
Proximal	45	20.7
Mid/distal	172	79.3
Lymph node metastasis		
No metastasis	70	32.3
Metastasis	147	67.7
T stage		
T1/T2	101	46.5
T3/T4	116	53.5
Postoperative chemotherapy		
Fluoropyrimidines	62	28.6
Fluoropyrimidines + platinum	155	71.4
CDKN1A expression		
Low	99	45.6
High	118	54.4
Survival		
Alive	101	46.5
Succumbed	116	53.5
Recurrence		
No	91	41.9
Yes	126	58.1

CDKN1A protein was located mainly in the cell nuclei, whereas CDKN1A protein exhibited nuclear and cytoplasmic expression in RGA tissues. Representative IHC results of the different intensities of CDKN1A expression in RGA tissues and in normal gastric tissues are presented in Fig. 1, along with their corresponding H&E staining results. Representative western blot results are shown in Fig. 2. Resected tumor tissues from 118 cases (54.4%) of RGA exhibited high CDKN1A expression, whereas those from 99 patients (45.6%) demonstrated low CDKN1A expression (Table I). As summarized in Table II, CDKN1A expression was significantly associated with LNM (P=0.001), recurrence (P<0.001), and survival (P<0.001), as determined by χ^2 test. No significant associations between CDKN1A expression

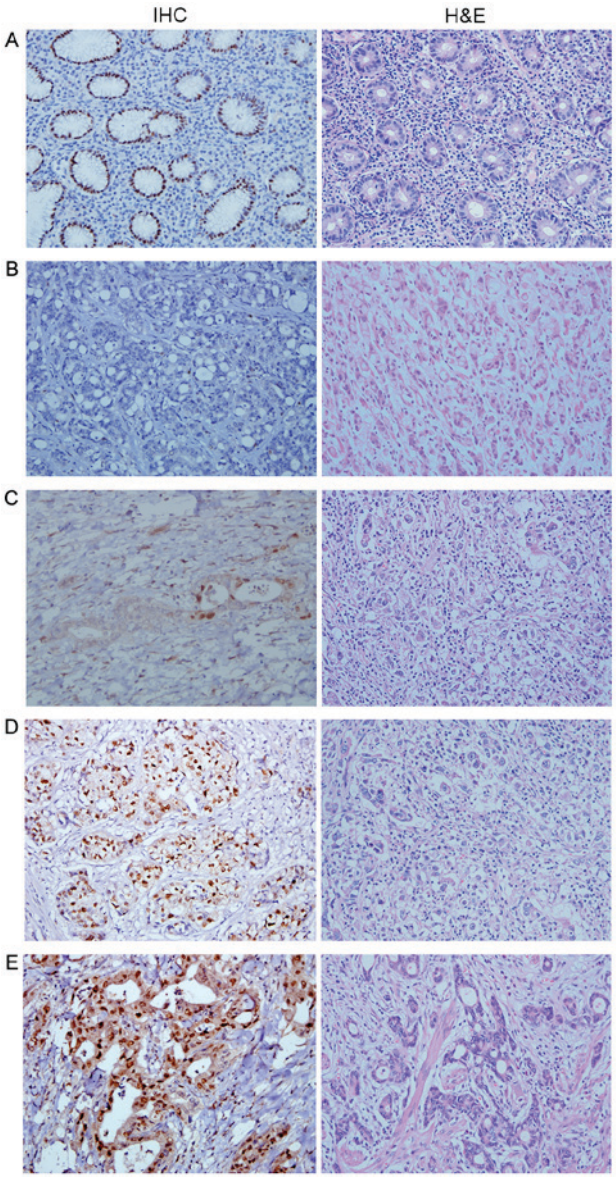


Figure 1. Representative CDKN1A IHC and corresponding H&E staining results in normal gastric and RGA tissues. (A) Normal gastric tissue; (B) score '0' for CDKN1A staining in RGA tissues; (C) score '1' for CDKN1A staining in RGA tissues; (D) score '2' for CDKN1A staining; (E) score '3' for CDKN1A staining. Magnification, x200. IHC, immunohistochemistry; H&E, hematoxylin and eosin staining; CDKN1A, cyclin-dependent kinase inhibitor 1A; RGA, resected gastric adenocarcinoma.

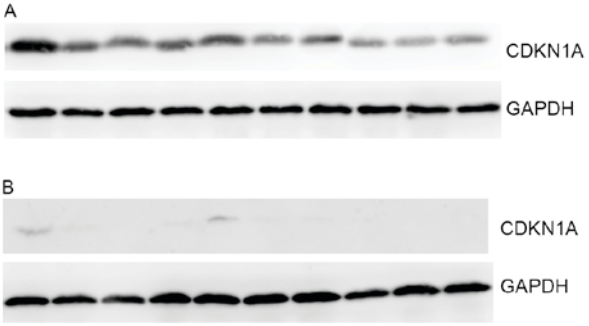


Figure 2. Representative western blotting results. (A) High expression of CDKN1A. From left to right, the lanes represent the tissue sample number 3, 8, 12, 17, 18, 32, 43, 50, 55 and 131. (B) Low expression of CDKN1A. From left to right, the lanes represent the tissue sample number 13, 41, 45, 51, 52, 69, 71, 80, 161 and 182. CDKN1A, cyclin-dependent kinase inhibitor 1A.

Table II. Association between CDKN1A expression and characteristics of patients with resected gastric adenocarcinoma (n=217).

Characteristics	CDKN1A expression, n (%)		P-value
	Low	High	
Age, years			0.072
<60	68 (50.4)	67 (49.6)	
≥60	31 (37.8)	51 (62.2)	
Sex			0.720
Male	71 (46.4)	82 (53.6)	
Female	28 (43.8)	36 (56.2)	
Histological grade			0.593
Well/moderately differentiated	68 (46.9)	77 (53.1)	
Poorly differentiated	31 (43.1)	41 (56.9)	
Gross findings			0.701
Apophysis	7 (41.2)	10 (58.8)	
Invasion	92 (46.0)	108 (54.0)	
Tumor site			0.621
Proximal	22 (48.9)	23 (51.1)	
Mid/distal	77 (44.8)	95 (55.2)	
Lymph node metastasis			0.001[a]
No metastasis	20 (28.6)	50 (71.4)	
Metastasis	79 (53.7)	68 (46.3)	
T stage			0.053
T1/T2	39 (38.6)	62 (61.4)	
T3/T4	60 (51.7)	56 (48.3)	
Postoperative chemotherapy			0.085
Fluoropyrimidines	34 (54.8)	28 (45.2)	
Fluoropyrimidines + platinum	65 (41.9)	90 (58.1)	
Survival			<0.001[a]
Alive	25 (24.8)	76 (75.2)	
Succumbed	74 (63.8)	42 (36.2)	
Recurrence			<0.001[a]
No	20 (22.0)	71 (78.0)	
Yes	79 (62.7)	47 (37.3)	

[a]Statistically significant (P<0.05). CDKN1A, cyclin-dependent kinase inhibitor 1A.

and other clinicopathological characteristics, including age, gender, histological grade, gross pathology, tumor site, and postoperative chemotherapy, were observed (Table II).

Identification of prognostic factors associated with OS. The 5-year OS rate of the entire patient group was 46.5% (Table I). Data from the χ^2 and log-rank tests indicated that patients with low CDKN1A expression exhibited a significantly poorer survival rate (25.3%; χ^2 P<0.001) and lower median OS time (log-rank P<0.001; Tables II and III) compared with patients with high CDKN1A expression. As demonstrated in Table III, other factors identified by χ^2 and log-rank tests to be associated with poor survival included LNM (P<0.001 and P<0.001, respectively), advanced T stage (P<0.001 and P<0.001, respectively), and postoperative chemotherapy with fluoropyrimidine only (P=0.008 and P=0.002, respectively). The results of the Kaplan-Meier analysis demonstrated that patients with low CDKN1A expression exhibited a significantly shorter OS time compared with those with high CDKN1A expression (median, 32 months vs. 70 months, P<0.001; Fig. 3A).

Results from the univariate and multivariate Cox analyses additionally confirmed that positive LNM (P<0.001 and P=0.006, respectively), advanced T stage (T3/T4) (P<0.001 and P=0.001, respectively), postoperative chemotherapy with fluoropyrimidine only (P=0.003 and P=0.003, respectively) and low CDKN1A expression (P<0.001 and P<0.001, respectively) were significantly associated with increased risk of mortality (Table IV). Taken together, these data suggest that CDKN1A was a statistically significant independent prognostic significance for OS in patients with RGA.

Table III. Association between OS and characteristics of patients with resected gastric adenocarcinoma (n=217).

Characteristics	Survival		P-value[a]	Median OS time (months)	P-value[b]
	Alive (%)	Deceased (%)			
Age, years			0.743		0.783
<60	64 (47.4)	71 (52.6)		57.0	
≥60	37 (45.1)	45 (54.9)		48.0	
Sex			0.718		0.611
Male	70 (45.8)	83 (54.2)		51.0	
Female	31 (48.4)	33 (51.6)		53.0	
Histological grade			0.468		0.417
Well/moderately differentiated	70 (48.3)	75 (51.7)		54.0	
Poorly differentiated	31 (43.1)	41 (56.9)		41.0	
Gross findings			0.290		0.229
Apophysis	10 (58.8)	7 (41.2)		63.0	
Invasion	91 (45.5)	109 (54.5)		47.0	
Tumor site			0.751		0.733
Proximal	20 (44.4)	25 (55.6)		51.0	
Mid/distal	81 (47.1)	91 (52.9)		53.0	
Lymph node metastasis			<0.001[c]		<0.001[c]
No metastasis	46 (65.7)	24 (34.3)		76.0	
Metastasis	55 (37.4)	92 (62.6)		39.0	
T stage			<0.001[c]		<0.001[c]
T1/T2	63 (62.4)	38 (37.6)		69.0	
T3/T4	38 (32.8)	78 (67.2)		35.0	
Postoperative chemotherapy			0.008[c]		0.002[c]
Fluoropyrimidines	20 (32.3)	42 (67.7)		33.0	
Fluoropyrimidines + platinum	81 (52.3)	74 (47.7)		63.0	
CDKN1A expression			<0.001[c]		<0.001[c]
Low	25 (25.3)	74 (74.7)		32.0	
High	76 (64.4)	42 (35.6)		70.0	

[a]P-value was obtained from the χ^2 test; [b]P-value was obtained from the log-rank test; [c]statistically significant (P<0.05). OS, overall survival; CDKN1A, cyclin-dependent kinase inhibitor 1A.

Identification of prognostic factors associated with recurrence risk. The overall recurrence rate of the whole patient cohort was 58.1% (Table I). Data from the χ^2 and log-rank tests indicated that patients with low CDKN1A expression exhibited a significantly higher recurrence rate (79.8%; χ^2 P<0.001) and lower median recurrence-free survival (log-rank P<0.001) compared with those with high CDKN1A expression (Tables II and V). As demonstrated in Table V, other factors identified by χ^2 and log-rank tests to be associated with a higher recurrence rate included LNM (P<0.001 and P<0.001, respectively), and T3/T4 stage (P<0.001 and P<0.001, respectively). The results of the Kaplan-Meier analysis indicated that patients with low CDKN1A expression exhibited a significantly shorter RFS time compared with those with high CDKN1A expression (median, 20 vs. 69 months, P<0.001; Fig. 3B).

Results from the univariate and multivariate Cox analyses demonstrated that LNM (P<0.001 and P=0.001, respectively), advanced T stage (P<0.001 and P=0.003, respectively), postoperative chemotherapy with fluoropyrimidine only (P=0.038 and P=0.007) and low CDKN1A expression (P<0.001 and P<0.001) were significantly associated with increased risk of recurrence (Table VI). Taken together, these data suggest that CDKN1A was a statistically significant independent prognostic significance for recurrence risk in patients with RGA.

Identification of risk factors associated with LNM. At the time of surgical resection, 79 of 99 patients (79.8%) with low CDKN1A expression presented with LNM, compared with 68 of 118 patients (57.6%) with high CDKN1A expression, a difference that was identified to be significant (P=0.001 by χ^2 test; Tables II and VII). Univariate and multivariate logistic regression analyses additionally demonstrated that low CDKN1A expression was significantly associated with increased risk of LNM (P=0.001 and P=0.001, respectively).

Table IV. Univariate and multivariate Cox regression analyses of overall survival.

	Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years		0.785		0.366
<60	1.00		1.00	
≥60	1.05 (0.73-1.53)		1.20 (0.81-1.78)	
Sex		0.614		0.188
Male	1.00		1.00	
Female	0.90 (0.60-1.35)		0.75 (0.49-1.15)	
Histological grade		0.422		0.252
Well/moderately differentiated	1.00		1.00	
Poorly differentiated	1.17 (0.80-1.71)		1.26 (0.85-1.89)	
Gross findings		0.237		0.441
Apophysis	1.00		1.00	
Invasion	1.59 (0.74-3.41)		1.36 (0.62-2.98)	
Tumor site		0.735		0.462
Proximal	1.00		1.00	
Mid/distal	0.93 (0.60-1.44)		0.84 (0.53-1.34)	
Lymph node metastasis		<0.001[a]		0.006[a]
No metastasis	1.00		1.00	
Metastasis	2.40 (1.53-3.76)		1.94 (1.21-3.14)	
T stage		<0.001[a]		0.001[a]
T1/T2	1.00		1.00	
T3/T4	2.38 (1.61-3.51)		2.03 (1.36-3.03)	
Postoperative chemotherapy		0.003[a]		0.003[a]
Fluoropyrimidines	1.00		1.00	
Fluoropyrimidines + platinum	0.56 (0.38-0.81)		0.55 (0.37-0.82)	
CDKN1A expression		<0.001[a]		<0.001[a]
Low	1.00		1.00	
High	0.33 (0.22-0.48)		0.39 (0.26-0.58)	

[a]Statistically significant (P<0.05). HR, hazard ratio; CI, confidence interval; CDKN1A, cyclin-dependent kinase inhibitor 1A.

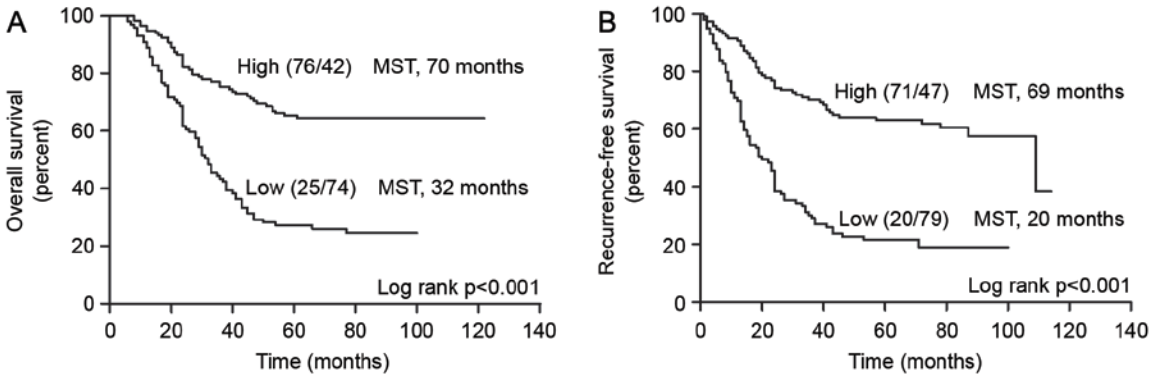


Figure 3. Kaplan-Meier curves according to high (n=118) and low (n=99) CDKN1A expression. (A) Overall survival; values in brackets represent 'number of patients alive/number of patients deceased'. (B) Recurrence-free survival; values in brackets represent 'number without recurrence/number of patients with recurrence'. P-values were calculated by log-rank test. MST, median survival time; CDKN1A, cyclin-dependent kinase inhibitor 1A.

Other factors, including gross pathology (P=0.02 and P=0.035 from univariate and multivariate analyses, respectively) and advanced T stage (T3/T4; P=0.001 and P=0.005, from univariate and multivariate analyses, respectively) were

Table V. Association between recurrence and characteristics of patients with resected gastric carcinoma (n=217).

Characteristics	Recurrence, n (%)		P-value[a]	Median RFS time (months)	P-value[b]
	No	Yes			
Age, years			0.647		0.461
<60	55 (40.7)	80 (59.3)		35.0	
≥60	36 (43.9)	46 (56.1)		42.0	
Sex			0.961		0.876
Male	64 (41.8)	89 (58.2)		39.0	
Female	27 (42.2)	37 (57.8)		41.0	
Histological grade			0.727		0.684
Well/moderately differentiated	62 (42.8)	83 (57.2)		41.0	
Poorly differentiated	29 (40.3)	43 (59.7)		36.0	
Gross findings			0.338		0.249
Apophysis	9 (52.9)	8 (47.1)		63.0	
Invasion	82 (41.0)	118 (59.0)		36.0	
Tumor site			0.330		0.428
Proximal	16 (35.6)	29 (64.4)		24.0	
Mid/distal	75 (43.6)	97 (56.4)		40.0	
Lymph node metastasis			<0.001[c]		<0.001[c]
No metastasis	43 (61.4)	27 (38.6)		70.5	
Metastasis	48 (32.7)	99 (67.3)		24.0	
T stage			<0.001[c]		<0.001[c]
T1/T2	56 (55.4)	45 (44.6)		68.0	
T3/T4	35 (30.2)	81 (69.8)		24.0	
Postoperative chemotherapy			0.068		0.035[c]
Fluoropyrimidines	20 (32.3)	42 (67.7)		24.0	
Fluoropyrimidines + platinum	71 (45.8)	84 (54.2)		53.0	
CDKN1A expression			<0.001[c]		<0.001[c]
Low	20 (20.2)	79 (79.8)		20.0	
High	71 (60.2)	47 (39.8)		69.0	

[a]P-value was obtained from the χ^2 test; [b]P-value was obtained from the log-rank test; [c]statistically significant (P<0.05). RFS, recurrence-free survival; CDKN1A, cyclin-dependent kinase inhibitor 1A.

also significantly associated with LNM (Table VII). Taken together, these data suggest that CDKN1A was a statistically significant independent risk factor for LNM in patients with RGA.

Correlation between western blot and IHC analyses for detection of CDKN1A. As presented in the representative results of Fig. 2, CDKN1A protein levels in tissues from 52 patients with RGA detected by western blotting and IHC exhibited a good correlation (r=0.872, P<0.001), which was obtained by scanning the western-blot bands with the gel analysis system to obtain the gray value, and then performing Spearman's rank correlation analysis to analyze the correlation between the gray values and their corresponding IHC scores so as to plot the scatter plot (Fig. 4). These data suggest that CDKN1A expression in RGA tissues may be determined by either western blotting or immunohistochemical analysis.

Discussion

The results of the present study demonstrated that low CDKN1A expression in GA tissues was associated with LNM and poor prognoses, as indicated by the shorter survival times and increased recurrence risks in these patients. Thus, CDKN1A appears to have independent prognostic significance in patients with RGA.

Previously published data have demonstrated that CDKN1A is a tumor suppressor due to its ability to induce cell cycle arrest (18-21). Confirming this role, downregulation of CDKN1A has been identified in different cancer tissues, and has been associated with poor prognoses in various types of human cancer, such as pancreatic and colon cancer, primary hepatocellular carcinoma, early-stage human papillomavirus-associated lung cancer and ovarian cancer (4,22-27). Khalili *et al* (28) revealed that low CDKN1A expression level may be associated with

Table VI. Univariate and multivariate Cox regression analysis of recurrence.

	Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years		0.466		0.733
<60	1.00		1.00	
≥60	0.87 (0.61-1.26)		0.94 (0.64-1.37)	
Sex		0.877		0.165
Male	1.00		1.00	
Female	0.97 (0.66-1.42)		0.75 (0.50-1.13)	
Histological grade		0.687		0.507
Well/moderately differentiated	1.00		1.00	
Poorly differentiated	1.08 (0.75-1.56)		1.14 (0.77-1.68)	
Gross findings		0.257		0.470
Apophysis	1.00		1.00	
Invasion	1.51 (0.74-3.10)		1.31 (0.63-2.75)	
Tumor site		0.434		0.315
Proximal	1.00		1.00	
Mid/distal	0.85 (0.56-1.28)		0.80 (0.51-1.24)	
Lymph node metastasis		<0.001[a]		0.001[a]
No metastasis	1.00		1.00	
Metastasis	2.54 (1.66-3.90)		2.09 (1.33-3.27)	
T stage		<0.001[a]		0.003[a]
T1/T2	1.00		1.00	
T3/T4	2.16 (1.50-3.13)		1.77 (1.21-2.58)	
Postoperative chemotherapy		0.038[a]		0.007[a]
Fluoropyrimidines	1.00		1.00	
Fluoropyrimidines + platinum	0.67 (0.46-0.98)		0.58 (0.39-0.87)	
CDKN1A expression		<0.001[a]		<0.001[a]
Low	1.00		1.00	
High	0.31 (0.22-0.45)		0.38 (0.26-0.56)	

[a]Statistically significant (P<0.05). HR, hazard ratio; CI, confidence interval; CDKN1A, cyclin-dependent kinase inhibitor 1A.

gastric cancer initiation and progression. However, there have been conflicting results in terms of the CDKN1A expression profiles in different cancer tissues. Certain studies have identified that CDKN1A overexpression in cancer tissues is associated with tumor aggressiveness and poor survival: Dai *et al* (29) suggested that CDKN1A expression in breast cancer tissues was correlated with LNM and poor survival of patients with breast cancer, and increased MDA-MB231 breast cancer cell migration and invasion via transforming growth factor β-mediated mothers against decapentaplegic homolog 3 acetylation. In addition to the aforementioned studies (4,22-27), there have been other investigations indicating that low expression of CDKN1A in various cancer tissues is associated with poor prognosis (30,31). The molecular mechanism by which different CDKN1A expression levels affect prognoses in patients with different types of cancer remains unclear and requires additional investigation. The present study identified that low expression of CDKN1A in RGA

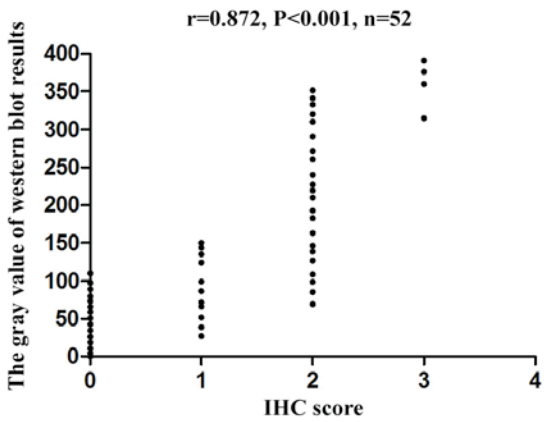


Figure 4. Spearman's correlation scatter plot of western blotting and analyses for detection of CDKN1A expression. The Spearman's correlation analysis between the gray value of the western blotting band and corresponding immunohistochemistry score of CDKN1A expression demonstrated a high-positive correlation (r=0.872, P<0.001, n=52). IHC, immunohistochemistry; CDKN1A, cyclin-dependent kinase inhibitor 1A.

Table VII. Univariate and multivariate analyses of variables correlated with LNM.

	LNM, n (%)		Univariate analysis		Multivariate analysis	
Characteristics	No	Yes	OR (95% CI)	P-value	OR (95% CI)	P-value
Age, years				0.643		0.206
<60	42 (31.1)	93 (68.9)	1.00		1.00	
≥60	28 (34.1)	54 (65.9)	0.87 (0.49-1.56)		0.99 (0.52-1.91)	
Sex				0.247		0.991
Male	53 (34.6)	100 (65.4)	1.00		1.00	
Female	17 (26.6)	47 (73.4)	1.47 (0.77-2.80)		1.58 (0.78-3.19)	
Histological grade				0.584		0.363
Well/moderately differentiated	45 (31.0)	100 (69.0)	1.00		1.00	
Poorly differentiated	25 (34.7)	47 (65.3)	0.85 (0.47-1.54)		0.73 (0.37-1.43)	
Gross findings				0.020[a]		0.035[a]
Apophysis	10 (58.8)	7 (41.2)	1.00		1.00	
Invasion	60 (30.0)	140 (70.0)	3.33 (1.21-9.17)		3.31 (1.09-10.02)	
Tumor site				0.587		0.736
Proximal	13 (28.9)	32 (71.1)	1.00		1.00	
Mid/distal	57 (33.1)	115 (66.9)	0.82 (0.40-1.68)		0.87 (0.40-1.92)	
T stage				0.001[a]		0.005[a]
T1/T2	44 (43.6)	57 (56.4)	1.00		1.00	
T3/T4	26 (22.4)	90 (77.6)	2.67 (1.49-4.81)		2.44 (1.31-4.53)	
Postoperative chemotherapy				0.200		0.102
Fluoropyrimidines	24 (38.7)	38 (61.3)	1.00		1.00	
Fluoropyrimidines + platinum	46 (29.7)	109 (70.3)	1.50 (0.81-2.77)		1.78 (0.89-3.55)	
CDKN1A expression				0.001[a]		0.001[a]
Low	20 (20.2)	79 (79.8)	1.00		1.00	
High	50 (42.4)	68 (57.6)	0.34 (0.19-0.64)		0.33 (0.17-0.64)	

[a]Statistically significant (P<0.05). LNM, lymph node metastasis; OR, odds ratio; CDKN1A, cyclin-dependent kinase 1A.

tissues was associated with poor prognosis in patients with gastric cancer.

As CDKN1A is a tumor suppressor, we hypothesize that epigenetic changes, such as promoter methylation, may be one of the mechanisms underlying the low CDKN1A expression in RGA tissues; promoter methylation is capable of inhibiting gene transcript and protein expression, as identified by Watanabe *et al* (32), who suggested that abnormal genomic methylation may be involved in the reduced expression of CDKN1A in adult T-cell leukemia/lymphoma (32). A study conducted by Nie *et al* (33) indicated that long noncoding RNAs (lncRNA) may also serve a role in regulation of CDKN1A transcription; the authors demonstrated that the lncRNA antisense noncoding RNA in the INK4 locus was upregulated in non-small cell lung cancer (NSCLC) tissues, and that this lncRNA promoted NSCLC cell proliferation and inhibited NSCLC cell apoptosis through the inhibition of Krüppel-like factor 2 and CDKN1A transcription (33). These studies additionally demonstrate that CDKN1A is a key regulator of the cell cycle, and may be a target for cancer treatment.

In conclusion, the present study indicated, via multiple statistical analyses, that low expression level of CDKN1A in RGA tissues was significantly associated with LNM, a shorter survival time and a high recurrence rate and risk of mortality. CDKN1A demonstrated important prognostic significance, and may be used as an independent prognostic factor for patients with RGA.

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, *et al*: Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. N Engl J Med 355: 11-20, 2006.
3. Liu C, Lu P, Lu Y, Xu H, Wang S and Chen J: Clinical implications of metastatic lymph node ratio in gastric cancer. BMC Cancer 7: 200, 2007.
4. Ohta K, Hoshino H, Wang J, Ono S, Iida Y, Hata K, Huang SK, Colquhoun S and Hoon DS: MicroRNA-93 activates c-Met/PI3K/Akt pathway activity in hepatocellular carcinoma by directly inhibiting PTEN and CDKN1A. Oncotarget 6: 3211-3324, 2015.
5. Cazzalini O, Scovassi AI, Savio M, Stivala LA and Prosperi E: Multiple roles of the cell cycle inhibitor p21 (CDKN1A) in the DNA damage response. Mutat Res 704: 12-20, 2010.
6. Prives C and Gottifredi V: The p21 and PCNA partnership: A new twist for an old plot. Cell Cycle 7: 3840-3846, 2008.
7. Andries V, Vandepoele K, Staes K, Berx G, Bogaert P, Van Isterdael G, Ginneberge D, Parthoens E, Vandenbussche J, Gevaert K, *et al*: NBPF1, a tumor suppressor candidate in neuroblastoma, exerts growth inhibitory effects by inducing a G1 cell cycle arrest. BMC Cancer 15: 391, 2015.
8. Park C, Jeong NY, Kim GY, Han MH, Chung IM, Kim WJ, Yoo YH and Choi YH: Momilactone B induces apoptosis and G1 arrest of the cell cycle in human monocytic leukemia U937 cells through downregulation of pRB phosphorylation and induction of the cyclin-dependent kinase inhibitor p21Waf1/Cip1. Oncol Rep 31: 1653-1660, 2014.
9. Wei CY, Tan QX, Zhu X, Qin QH, Zhu FB, Mo QG and Yang WP: Expression of CDKN1A/p21 and TGFBR2 in breast cancer and their prognostic significance. Int J Clin Exp Pathol 8: 14619-14629, 2015.
10. Li S, Wang C, Yu X, Wu H, Hu J, Wang S and Ye Z: miR-3619-5p inhibits prostate cancer cell growth by activating CDKN1A expression. Oncol Rep 37: 241-248, 2017.
11. Tang H, Wu Y, Liu M, Qin Y, Wang H, Wang L, Li S, Zhu H, He Z, Luo J, *et al*: SEMA3B improves the survival of patients with esophageal squamous cell carcinoma by upregulating p53 and p21. Oncol Rep 36: 900-908, 2016.
12. Wang X, Lin Y, Lan F, Yu Y, Ouyang X, Wang X, Huang Q, Wang L, Tan J and Zheng F: A GG allele of 3'-side AKT1 SNP is associated with decreased AKT1 activation and better prognosis of gastric cancer. J Cancer Res Clin Oncol 140: 1399-1411, 2014.
13. Yoo CH, Noh SH, Kim YI and Min JS: Comparison of prognostic significance of nodal staging between old (4th edition) and new (5th edition) UICC TNM classification for gastric carcinoma. International union against cancer. World J Surg 23: 492-497, 1999.
14. Ward JH: NCCN Guidelines and the International community. J Natl Compr Canc Netw 9: 133-134, 2011.
15. Wang X, Lin Y, Lan F, Yu Y, Ouyang X, Liu W, Xie F, Wang X and Huang Q: BAX and CDKN1A polymorphisms correlated with clinical outcomes of gastric cancer patients treated with postoperative chemotherapy. Med Oncol 31: 249, 2014.
16. Kawata N, Tsuchiya N, Horikawa Y, Inoue T, Tsuruta H, Maita S, Satoh S, Mitobe Y, Narita S and Habuchi T: Two surviving polymorphisms are cooperatively associated with bladder cancer susceptibility. Int J Cancer 129: 1872-1880, 2011.
17. Huang Q, Huang Q, Chen W, Wang L, Lin W, Lin J and Lin X: Identification of transgelin as a potential novel biomarker for gastric adenocarcinoma based on proteomics technology. J Cancer Res Clin Oncol 134: 1219-1227, 2008
18. Galeano F, Rossetti C, Tomaselli S, Cifaldi L, Lezzerini M, Pezzullo M, Boldrini R, Massimi L, Di Rocco CM, Locatelli F, *et al*: ADAR2-editing activity inhibits glioblastoma growth through the modulation of the CDC14B/Skp2/p21/p27 axis. Oncogene 32: 998-1009, 2013.
19. Morton JP, Jamieson NB, Karim SA, Athineos D, Ridgway RA, Nixon C, McKay CJ, Carter R, Brunton VG, Frame MC, *et al*: LKB1 haploinsufficiency cooperates with Kras to promote pancreatic cancer through suppression of p21-dependent growth arrest. Gastroenterology 139: 586-597, 2010.
20. Brown SG, Knowell AE, Hunt A, Patel D, Bhosle S and Chaudhary J: Interferon inducible antiviral MxA is inversely associated with prostate cancer and regulates cell cycle, invasion and Docetaxel induced apoptosis. Prostate 75: 266-279, 2015.
21. Koh DI, Han D, Ryu H, Choi WI, Jeon BN, Kim MK, Kim Y, Kim JY, Parry L, Clarke AR, *et al*: KAISO, a critical regulator of p53-mediated transcription of CDKN1A and apoptotic genes. Proc Natl Acad Sci USA 111: 15078-15083, 2014.
22. Sun Y, Yang S, Sun N and Chen J: Differential expression of STAT1 and p21 proteins predicts pancreatic cancer progression and prognosis. Pancreas 43: 619-623, 2014.
23. Belt EJ, Brosens RP, Delis-van Diemen PM, Bril H, Tijssen M, van Essen DF, Heymans MW, Beliën JA, Stockmann HB, Meijer S, *et al*: Cell cycle proteins predict recurrence in stage II and III colon cancer. Ann Surg Oncol 19 (Suppl 3): S682-S692, 2012.
24. Mitomi H, Ohkura Y, Fukui N, Kanazawa H, Kishimoto I, Nakamura T, Yokoyama K, Sada M, Kobayashi K, Tanabe S, *et al*: P21WAF1/CIP1 expression in colorectal carcinomas is related to Kras mutations and prognosis. Eur J Gastroenterol Hepatol 19: 883-889, 2007.
25. Wu DW, Liu WS, Wang J, Chen CY, Cheng YW and Lee H: Reduced p21 (WAF1/CIP1) via alteration of p53-DDX3 pathway is associated with poor relapse-free survival in early-stage human papillomavirus-associated lung cancer. Clin Cancer Res 17: 1895-1905, 2011.
26. Young TW, Rosen DG, Mei FC, Li N, Liu J, Wang XF and Cheng X: Up-regulation of tumor susceptibility gene 101 conveys poor prognosis through suppression of p21 expression in ovarian cancer. Clin Cancer Res 13: 3848-3854, 2007.
27. Buchynska LG, Nesina IP, Yurchenko NP, Bilyk OO, Grinkevych VN and Svintitsky VS: Expression of p53, p21WAF1/CIP1, p16INK4A and Ki-67 proteins in serous ovarian tumors. Exp Oncol 29: 49-53, 2007.
28. Khalili M, Vasei M, Khalili D, Alimoghaddam K, Sadeghizadeh M and Mowla SJ: Downregulation of the genes involved in reprogramming (SOX2, c-MYC, miR-302, miR-145, and P21) in gastric adenocarcinoma. J Gastrointest Cancer 46: 251-258, 2015.
29. Dai M, Al-Odaini AA, Arakelian A, Rabbani SA, Ali S and Lebrun JJ: A novel function for p21Cip1 and acetyltransferase p/CAF as critical transcriptional regulators of TGF β-mediated breast cancer cell migration and invasion. Breast Cancer Res 14: R127, 2012.
30. Zhang H, Zhang X, Ji S, Hao C, Mu Y, Sun J and Hao J: Sohlh2 inhibits ovarian cancer cell proliferation by upregulation of p21 and downregulation of cyclin D1. Carcinogenesis 35: 1863-1871, 2014.
31. Liu J, Hu Y, Hu W, Xie X, Ela Bella A, Fu J and Rao D: Expression and prognostic relevance of p21WAF1 in stage III esophageal squamous cell carcinoma. Dis Esophagus 25: 67-71, 2012.
32. Watanabe M, Nakahata S, Hamasaki M, Saito Y, Kawano Y, Hidaka T, Yamashita K, Umeki K, Taki T, Taniwaki M, *et al*: Downregulation of CDKN1A in adult T-cell leukemia/lymphoma despite overexpression of CDKN1A in human T-lymphotropic virus 1-infected cell lines. J Virol 84: 6966-6977, 2010.
33. Nie FQ, Sun M, Yang JS, Xie M, Xu TP, Xia R, Liu YW, Liu XH, Zhang EB, Lu KH, *et al*: Long noncoding RNA ANRIL promotes non-small cell lung cancer cell proliferation and inhibits apoptosis by silencing KLF2 and P21 expression. Mol Cancer Ther 14: 268-277, 2015.