Int J Clin Exp Med 2017;10(9):13992-14000
www.ijcem.com /ISSN:1940-5901/IJCEM0053832

Original Article

Synergistic role between Cul1 and PARP1: prognostic and predictive biomarkers in colorectal cancer

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Received February 9, 2017; Accepted September 4, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: Purpose: To study the expression of Cul1 and PARP1 in colorectal cancer (CRC), and evaluate their correlation with clinicopathological parameters and prognosis. Methods: The protein expression of Cul1 and PARP1, were evaluated in CRC samples to determine if their levels are altered in cancer. Immunohistochemical staining was used to determine the association with clinicopathological features and patient outcome on a CRC tissue microarray. Simultaneously, we aimed to investigate Cul1 combined with PARP1 as a prognostic and predictive marker could be even better than alone in CRC. Results: Cul1 and PARP1 were up-regulated expressed in primary CRC compared with adjacent normal tissues. High tumoral Cul1 and PARP1 expression significantly correlated with unfavorable clinicopathologic parameters and decreased overall survival. Multivariate regression analysis showed that high Cul1 and PARP1 expressions, separately and together, were independent negative markers of OS. Conclusions: The results suggest that expression of Cul1 and PARP1 in tumor may be a potential, independent prognostic factor for patients with CRC. A combination of Cul1 and PARP1 expression as efficient prognostic indicators was found for the first time in CRC.

Keywords: Cul1, PARP1, colorectal cancer, prognosis, marker

Introduction

GLOBOCAN 2012 indicated that CRC was the third most frequently diagnosed cancer (1.4 million cases) with 694,000 deaths each year worldwide [1, 2]. Despite the fact that average survival time has been prolonged slightly over the past decades, 5-year survival rate for CRC is still only 50-59% [3]. Most CRC patients died mainly due to distant metastasis [4]. The multimolecular mechanism is involved in the occurrence and development of CRC. Therefore, we could find much more biomarkers to predict the prognosis of CRC patients and take effective treatment measures in order to improve the survival time of CRC patients.

Cul1, a founding member of hydrophobic proteins providing a scaffold for ubiquitin ligases (E3) family, is an essential scaffold of the SKP1-CUL1-F-box protein (SCF) E3 ubiquitin ligase complex [5], which mediates the ubiquitination of proteins involved in cell-cycle progression, signal transduction and transcription [6]. Previous studies have revealed that Cul1 is critical in various diseases, including cancers [7]. Cul1 activity promotes tumor cell proliferation, including breast, prostate, lung and gastric carcinomas [8-11]. Consequently, Cul1 is associated with tumor progression and a poor prognosis for patients with malignant disease [8, 12, 13]. Poly (ADP-ribose) polymerase-1 (PARP1), the main member of the PARP family, is a chromatin associated enzyme which is involved in various biological processes including the regulation of transcription, cell cycle, tumorigenesis, and cellular response to DNA damage [14]. Accumulating evidence shows that the overexpression of PARP1 occurs frequently in a wide range of tumors [15-18] and is correlated with unsatisfactory prognosis [19, 20]. Recently, it has been demonstrated that knock down Cul1 via PARP1 pathway increases apoptosis in human gastric cancer cell lines [21]. Taken together, these findings suggested that

both Cul1 and PARP1 might contribute to cancer progression and poor clinical outcomes. Thus, we are considerably interested if Cul1 would work as a cooperator with PARP1 to improve predictive potency in CRC cancer.

Therefore, the present study aimed to determine expression levels of both Cul1 and PARP1 protein in CRC and to evaluate the association between these values and clinicopathological characteristics, including prognosis. More intriguingly, a hypothesis would be validated on whether Cul1 and PARP1 could be combined as a novel predictor with more accuracy in survival evaluation.

Materials and methods

Patients

A total of 470 patients diagnosed with primary CRC between 2006.01 and 2010.12 from Yixing People's Hospital were enrolled for this retrospective analysis. All the resected tumor tissues were embedded with paraffin. Additional 8-paired fresh samples were frozen in liquid nitrogen immediately after surgical removal and maintained at -80°C until use for Western blot analysis. All patients provided written informed consent, and did not have preoperative chemotherapy and/or radiotherapy. The clinicopathological features are summarized in Table S1. Patient outcome was evaluated as the months of survival from the date of tumor resection up to June 2014 or the date of last follow-up. The criteria of inclusion is to have patients' complete clinicopathologic information and survival follow-up time. In contrast, there is no survival follow-up time, or the patient does not agree, or patients with preoperative radiotherapy and chemotherapy, which were all excluded.

Establishment of CRC tissue microarray to examine the expression of Cul1 and PARP1 proteins

The colon cancer tissue microarray was established by State Engineering Research Center of Shanghai. Tumor tissues of 1.0 mm were obtained from the center of paraffin embedded tumor mass and the respective para-carcinoma tissues, which were used for tissue control. The biopsy samples of normal colonic mucosal epithelial cells were inserted into the four corners and center of each microarray. The expression of Cul1 and PARP1 were determined by standard procedures. The colon cancer tissue microarray was treated at 55°C for 20 min and then washed with xylene for 3 times (5 min each time) to remove paraffin. Afterwards, the chip was washed with absolute ethyl alcohol, 950 mL/L ethyl alcohol, 800 mL/L ethyl alcohol and distilled water respectively for 5 min. Antigen retrieval was then performed with samples in 10 mmol/L sodium citrate (pH 6.0) at 95°C for 30 min. The samples were incubated with hydrogen peroxide to block the activity of endogenous peroxidase. Serum blocking was carried out for 30 minutes and monoclonal rabbit anti-Cul1 (1:200, Epitomics, California, USA), anti-PARP1 (1:200, Cell Signaling Technology, MA, USA) were incubated with the sample sections at 4°C overnight. The respective second antibody was incubated for 30 min followed by hematoxylin staining by 3,3'-diamido-plate. Dehydration was then performed and sample sections were sealed by cover glasses. Cul1 and PARP1 antibody incubation was not included in the negative control group. For the sample sections in each chip, the quality standard of staining followed the staining of normal colonic mucosa epithelial tissue.

Evaluation of immunostaining

Staining of Cul1 or PARP1 in the tissue was scored independently by two pathologists blinded to the clinical data, by applying asemiquantitative immunoreactivity score (IRS) in the training cohort. The intensity of immunostaining was shown in Figures S1, S2. Interpretation of tissue staining results: Cul1 or PARP1 staining level was evaluated by immune staining score (IRS), which was calculated through multiplying staining intensity with the percentage of positive cells in panaramic scan instrument. According to IRS, Cul1 or PARP1 staining was divided into different levels: negative (IRS: 0), weak (IRS: 1-2), moderate (IRS: 3-6) and strong (IRS: 8-12) [22]. The optimum value of cutoff points of the Cul1 or PARP1 IRS was shown to be 4 or 3 since it had the best predictive value for survival (Figures S3, S4).

Western blot

Tissue proteins were extracted using the RIPA strong lysis buffer and PMSF. The concentra-

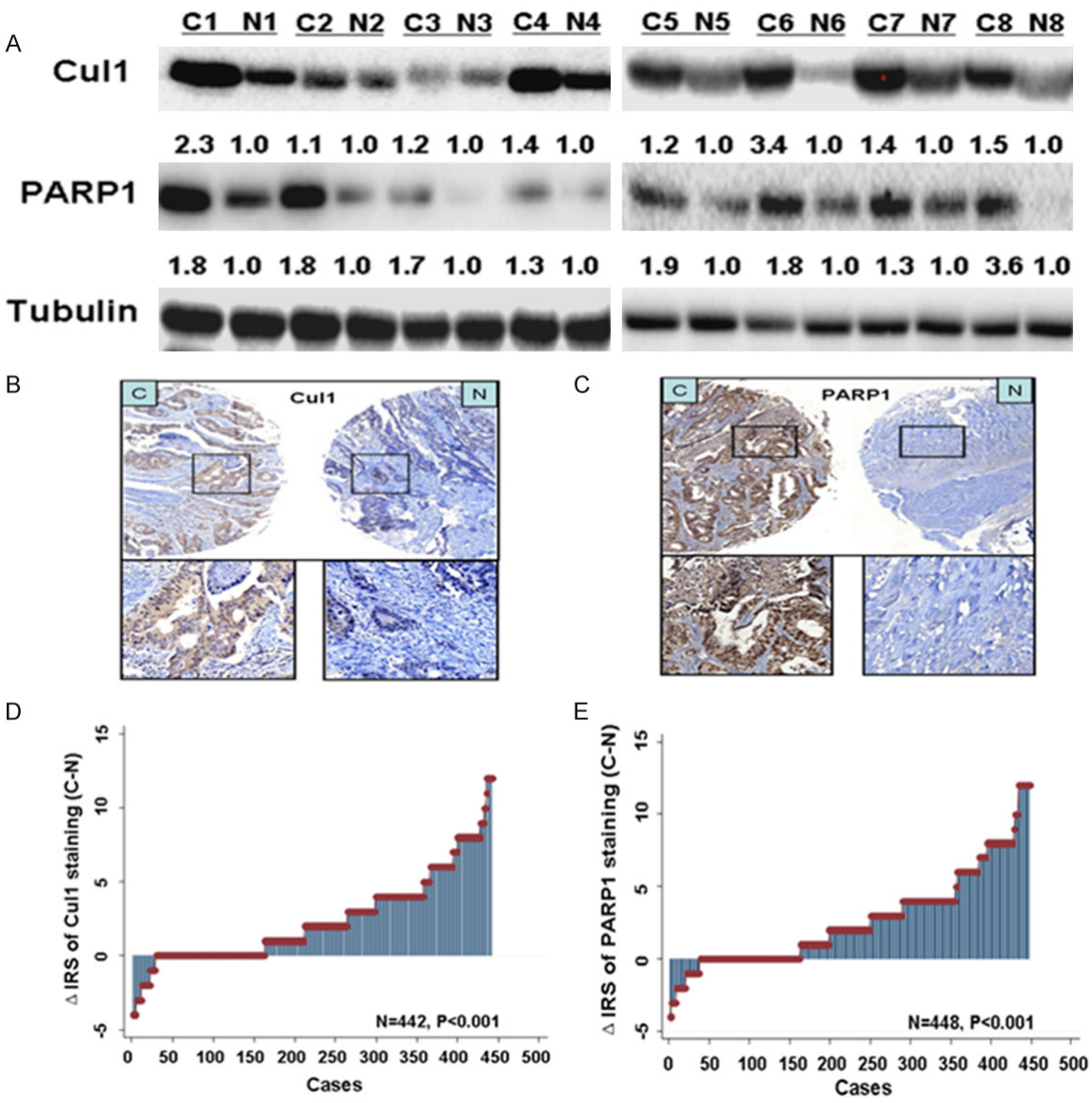

Figure 1. A: Cul1 or PARP1 was elevated in CRC and associated with poor prognosis of CRC patients. Expression of Cul1 or PARP1 was increased in cancer tissues (C) compared with paired normal colonic tissues (N) by Western blot. B, D: Representative images of Cul1 or PARP1 immunohistochemical staining in TMA are showed, respectively. Note: Top panel: original magnification, 40×; bottom panel: 200×. C, E: The distribution of the difference of Cul1, PARP1 staining in CRC compared with paired normal tissues, respectively.

tion of protein was measured according to the instructions of the BCA kit. The protein samples were analyzed by 10% SDS PAGE. After transferring the samples to a polyvinylidene fluoride membrane (Beyotime Institute of Biotechnology), 5% skim milk powder was applied for blocking and samples were incubated at room temperature for 2 hours. After washing the membrane with Tris Buffered saline with Tween 20, the protein samples were incubated with the rabbit anti-Cul1 (1:1,000, Epitomics, California, USA), the rabbit anti-PARP1 (1:1,000, Cell Signaling Technology, MA, USA) and monoclonal mouse anti-β-actin antibody (1:2,000 dilution; Boster Biotechnology) at 4°C overnight, immunoreactive bands were detected with a Phototope-horseradish peroxide Western blot detection kit (Cell Signaling Technology Inc.). The intensity of the Cul1 and PARP1 protein bands were analyzed by densitometry, after normalization to the corresponding β-actin level.

Table 1. Relationship between expression levels of Cul1 and clinicopathological features in CRC patients

Variables	n=464 cases		
	Low (%)	High (%)	P[a]
All patients	266 (57.3)	198 (42.7)	
Age (years)			0.139
≤65	157 (59.7)	106 (40.3)	
>65	109 (54.2)	92 (45.8)	
Gender			0.175
Males	154 (55.4)	124 (44.6)	
Females	112 (60.2)	74 (39.8)	
Pathological classification[b]			0.302
I	4 (80.0)	1 (20.0)	
II	242 (57.9)	176 (42.1)	
III	17 (47.2)	19 (52.8)	
Depth of invasion[b]			0.005
T1/T2	71 (68.9)	32 (31.1)	
T3/T4	193 (54.1)	164 (45.9)	
Lymph node metastasis[b]			0.001
N0	173 (63.4)	100 (36.6)	
N1/N2	91 (48.4)	97 (51.6)	
TNM stage[b]			0.015
I	60 (68.2)	28 (31.8)	
II	107 (60.8)	69 (39.2)	
III	88 (49.4)	90 (50.6)	
IV	89 (47.1)	9 (52.9)	
Tumor diameter[b]			0.543
≤5 cm	214 (57.2)	160 (42.8)	
>5 cm	51 (57.3)	38 (42.7)	
Distant metastasis			0.423
M0	256 (57.5)	189 (42.5)	
M1	10 (52.6)	9 (47.4)	

a Two-sided Fisher's exact tests; b Some patients missing these clinical pathological parameters.

Statistical analysis

Statistical analysis of IHC scores for the CRC TMAs was performed using SPSS 20.0 software (SPSS Inc, Chicago, IL, USA). The association between Cul1 or PARP1 expression and clinicopathological parameters was determined using the Fisher's exact test. IRS of Cul1 or PARP1 was assessed by the paired Wilcoxon test (raw scores). We used the Kaplan-Meier method to assess the survival time (OS). Univariate or multivariate Cox regression analysis was conducted to estimate the crude HRs, adjusted HRs and their 95% CIs, with adjustment for potential confounders. We evaluated the performances of different scores by plotting (t, AUC [t]) for different values of follow-up time (t). All statistical analyses were managed using the STATA statistical software (version 10.1; Stata Corp, College Station, TX). Differences were considered significant when the *P* value was less than 0.05 (two-side).

Results

Cul1 and PARP1 were differentially expressed in primary CRC and adjacent normal tissues

Western blot and immunohistochemical staining in TMA were used to detect Cul1 and PARP1 expression in primary CRC and adjacent normal tissues. Western blot analysis indicated that Cul1 and PARP1 expressions were up-regulated in all eight CRC tissues compared with matched adjacent tissues (**Figure 1A**). Meanwhile, Immunohistochemistry staining was utilized in TMA slides to further investigate Cul1 and PARP1 expressions in CRC tissues and paired adjacent non-cancerous tissues (**Figure 1B**, **1C**) in the training cohort. Because some samples were lost during antigen retrieval or with no relevant cells present in the core, Cul1 and PARP1 expressions were examined in 442 and 448 CRC patients respectively, which have both of CRC tissues and adjacent normal tissues. We observed that Cul1 or PARP1 expression was up-regulated in tumor tissues compared with paired adjacent non-tumor tissues (P<0.001, **Figure 1D**, **1E**).

Increased Cul1 and PARP1 expression correlated with unfavorable CRC clinicopathological features

The patients with CRC were classified into four groups according to Cul1 and PARP1 expression status, including Cul1 (low/high) and PARP1 (low/high). The Fisher's exact tests were then carried out and the results showed that high expression of Cul1 and PARP1 significantly correlated with an aggressive CRC phenotype, including depth of invasion (P=0.005 and P=0.002, respectively), lymph node metastasis (P=0.001 and P=0.029, respectively), and TNM stage (P=0.015 and P=0.013, respectively). But no significant association with other clinical factors (**Tables 1**, **2**).

Table 2. Relationship between expression levels of PARP1 and clinicopathological features in CRC patients

Variables	n=458		
	Low (%)	High (%)	P[a]
All patients	222 (48.5)	236 (51.5)	
Age (years)			0.391
≤65	128 (49.2)	132 (50.8)	
>65	94 (47.5)	104 (52.5)	
Gender			0.236
Males	130 (46.9)	147 (53.1)	
Females	92 (50.8)	89 (49.2)	
Pathological classification[b]			1.000
I	2 (40.0)	3 (60.0)	
II	200 (48.5)	212 (51.5)	
III	17 (47.2)	19 (52.8)	
Depth of invasion[b]			0.002
T1/T2	63 (61.8)	39 (38.2)	
T3/T4	158 (44.9)	194 (55.1)	
Lymph node metastasis[b]			0.029
N0	143 (52.4)	130 (47.6)	
N1/N2	78 (42.9)	104 (57.1)	
TNM stage[b]			0.013
I	53 (60.9)	34 (39.1)	
II	86 (48.6)	91 (51.4)	
III	71 (41.3)	101 (58.7)	
IV	11 (64.7)	6 (35.3)	
Tumor diameter[b]			0.242
≤5 cm	174 (47.4)	193 (52.6)	
>5 cm	47 (52.2)	43 (47.8)	
Distant metastasis			0.141
M0	210 (47.8)	229 (52.2)	
M1	12 (63.2)	7 (36.8)	

a Two-sided Fisher's exact tests; b Some patients missing these clinical pathological parameters.

Increased Cul1 and PARP1 expression correlated with poor CRC prognosis

Kaplan-Meier analysis showed that a high Cul1 or PARP1 expression was correlated with a significantly shorter survival time compared with a low Cul1 or PARP1 expression in patients with CRC (P<0.05 for both; **Figure 2A**, **2B**). Meanwhile, when Cul1 and PARP1 expression were considered as covariables, the results showed that patients with both low had the most favorable survival, followed by those with one low, while patients with both high had the poorest survival (P<0.001; **Figure 2C**). Univariate analysis indicated a number of variables were associated with a poor prognosis, including high-level Cul1 expression, high-level PARP1 expression, pathological classification, depth of invasion, lymph node metastasis, TNM stage, distant metastasis (**Table 3**). In the multivariate Cox regression model, the results showed that high Cul1 expression, high PARP1 expression, high Cul1 and PARP1 co-expression, age, distant metastasis, TNM stage were independent prognostic markers of overall survival of patients (**Table 4**).

Synergetic effect of Cul1 with PARP1 expression on OS in CRC patients

In order to further evaluate the prognostic efficacy of Cul1 and PARP1 expressions, we conducted a time-dependent ROC analysis for the censored data, which indicated that the combination of the clinical risk score (pathological classification, depth of invasion, lymph node metastasis, TNM stage, distant metastasis, tumor diameter) and Cul1 or PARP1 or Cul1 plus PARP1 contributed much more than either one alone in the whole cohort (**Figure 3**). In the cohort, the AUC at year 5 was 0.715 (95% CI: 0.662-0.769) for clinical risk score, whereas it was significantly increased to 0.912 (95% CI: 0.881-0.942) when combination of the clinical risk score with Cul1 plus PARP1 risk score.

Discussion

CRC is one of the largest threats to human health. Currently the only prognostic system routinely employed for CRC management is based on the AJCC TNM stage classification system, according to the results of pathological analysis [23]. However, this staging system is not sufficiently reliable, because the outcome is not only to the clinicopathological features, but also to the biologic aggressiveness of the individual disease, which is characterized by high potential for metastasis and resistance to anticancer therapy. The discovery of molecular biological prognostic factors may aid in a more accurate prediction of clinical outcome and may also reveal novel predictive factors and therapeutic targets [24].

A previous study indicated that deregulation of cell proliferation was a prerequisite for carcinogenesis and cancer progression [25]. The asso-

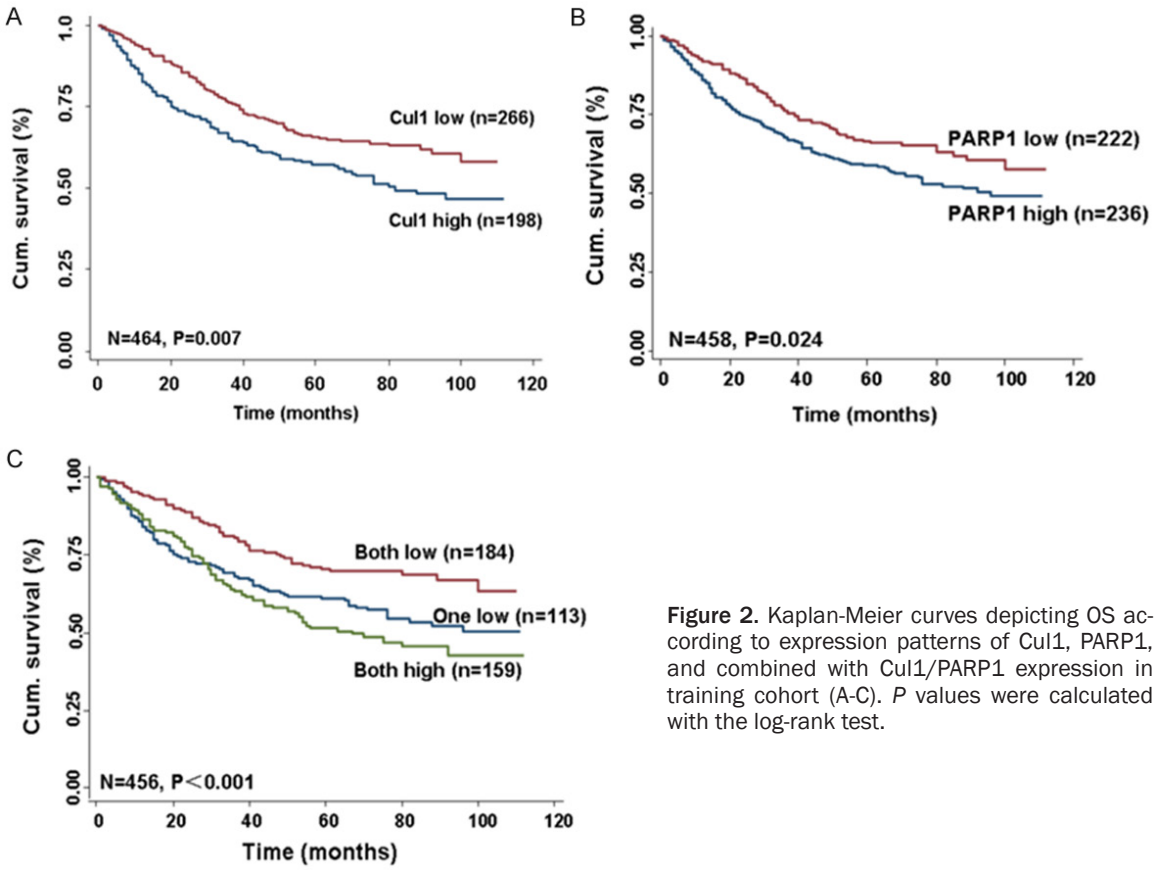

Figure 2. Kaplan-Meier curves depicting OS according to expression patterns of Cul1, PARP1, and combined with Cul1/PARP1 expression in training cohort (A-C). *P* values were calculated with the log-rank test.

Table 3. Univariate Cox regression analysis of Cul1 or PARP1 expression and clinicopathological variables predicting survival in patients with CRC patients

Variables	n=470 cases	
	HR (95% CI)	P
Age (≤65 vs. >65)	1.607 (1.215-2.126)	0.001
Gender (male vs. female)	1.013 (0.762-1.347)	0.927
Pathological classification (I/II vs. III)	2.475 (1.587-3.860)	<0.001
Depth of invasion (T1/T2 vs. T3/T4)	3.687 (2.270-5.990)	<0.001
Lymph node metastasis (N0 vs. N1/N2)	2.807 (2.112-3.731)	<0.001
TNM stage (I/II vs. III/IV)	3.214 (2.407-4.291)	<0.001
Distant metastasis (M0 vs. M1)	8.150 (4.849-13.699)	<0.001
Tumor diameter (≤5 cm vs. >5 cm)	1.196 (0.848-1.688)	0.307
PARP1 expression (low vs. high)	0.721 (0.541-0.961)	0.026
Cul1 expression (low vs. high)	0.683 (0.515-0.905)	0.008

ciation between proliferation and poor prognosis has been proved [26]. Cul1 has been proposed to be involved in tumor cell proliferation, such as breast, prostate, lung and gastric carcinomas [8-11]. In this study, a significant increase in Cul1 levels were observed in CRC tissues compared with matched adjacent tissues. Clinical analysis found that high expression of Cul1 was significantly correlated with depth of invasion, lymph node metastasis and TNM stage in CRC. Further analysis showed that high expression of Cul1 is a novel independent factor for poor prognosis in CRC. These results are in agreement with previous findings in which high levels of Cul1 were shown to be an indicator of poor prognosis in gastric cancer [27], melanoma [28] and breast cancer [29] patients.

PARP1 is important in the repair of DNA damage as it immediately binds to DNA breaks to induce recruitment and activation of other DNA repair proteins [30, 31]. However, the major role of PARP1 in the repair of DNA single-stand breaks could induce progression of human malignant tumors [32]. The aberrant DNA repairing activity from the overexpression of PARP1 in tumor

Table 4. Multivariate Cox regression analysis of Cul1, PARP1, Cul1/PARP1 expression and clinicopathological variables predicting survival in patients with CRC

Variables	HR (95% CI)	P[a]
Cul1		
Age (5% CI) ate Cox	1.833 (1.376-2.442)	<0.001
Gender (male vs. female)	0.912 (0.682-1.219)	0.533
Pathological classification (I/II vs. III)	1.724 (1.063-2.798)	0.027
Distant metastasis (M0 vs. M1)	4.503 (2.588-7.834)	<0.001
TNM stage (I/II vs. III/IV)	3.170 (2.337-4.299)	<0.001
Tumor diameter (v5 cm vs. >5 cm)	1.123 (0.776-1.625)	0.539
Cul1 expression (low vs. high)	0.749 (0.563-0.996)	0.040
PARP1		
Age (0.996 ssion)	1.859 (1.392-2.483)	<0.001
Gender (male vs. female)	0.887 (0.661-1.189)	0.422
Pathological classification (I/II vs. III)	1.767 (1.084-2.879)	0.022
Distant metastasis (M0 vs. M1)	4.926 (2.807-8.645)	<0.001
TNM stage (I/II vs. III/IV)	3.095 (2.282-4.198)	<0.001
Tumor diameter (v5 cm vs. >5 cm)	1.106 (0.763-1.601)	0.595
PARP1 expression (low vs. high)	0.685 (0.512-0.917)	0.011
Cul1/PARP1		
Age (10.917 ssion)	1.855 (1.388-2.478)	<0.001
Gender (male vs. female)	0.894 (0.666-1.199)	0.666
Pathological classification (I/II vs. III)	1.700 (1.049-2.756)	0.031
Distant metastasis (M0 vs. M1)	4.497 (2.567-7.878)	<0.001
TNM stage (I/II vs. III/IV)	3.206 (2.360-4.357)	<0.001
Tumor diameter (v5 cm vs. >5 cm)	1.133 (0.782-1.641)	0.509
Cul1/PARP1 expression		
Both low vs. one low	1.689 (1.162-2.456)	0.006
Both low vs. both high	0.619 (0.434-0.883)	0.008

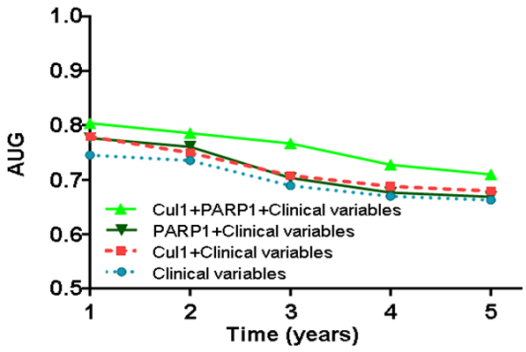

Figure 3. Time-dependent ROC analyses for clinical risk score (TNM stage, histologic type, and tumor diameter), or the combination of Cul1, PARP1, or Cul1 plus PARP1. AUC = area under the curve.

cells could enhance the anti-apoptotic property of tumor cells [32]. Therefore, it is indicated that PARP1 could affect tumor development, and the overexpression of PARP1 is associated with advanced clinicopathological features and poor survival of human malignant tumors, including breast carcinoma [19, 33], melanoma [34], and glioblastoma [16]. However, the potential effect of PARP1 on the survival of patients with CRC remains unknown. In the present study, our research results found that the expression level of PARP1 was increased in CRC tissues as compared with matched adjacent tissues. Furthermore, high expression of PARP1 was significantly correlated with advanced depth of invasion, lymph node metastasis and TNM stage in CRC. High expression of PARP1 was correlated with the reduced overall survival of CRC patients. Previous studies have reported that the combined biomarkers may be more efficient than the single one in the prognosis of various human carcinomas. Our previous study has showed that Cul1 could regulate PARP1 in the regulation of tumor apoptosis [21]. We hypothesized that the prognostic significance of the combination of Cul1 and PARP1 might be better than Cul1 or PARP1 alone. To certify this assumption, we analyzed the correlations of Cul1 and PARP1 combined expression, Cul1 expression, and PARP1 expression with overall survival of CRC patients, respectively. Our data showed that Cul1 and PARP1 combined expression could be more powerfully in predicting the prognosis of CRC patients, indicating that the detection of co-expression of Cul1 and PARP1 could be used to design appropriate, individualized treatment and be helpful to characterize patients who may benefit from close following up after surgery.

In summary, the present findings indicated that Cul1 and PARP1 are all high expression in CRC. Importantly, the multivariate analyses revealed the significant role of Cul1 and PARP1 as an independent prognostic factor for patients with CRC. Combination of Cul1 and PARP1 expres-

sion may function as a promising biomarker for prognostication of the CRC.

However, this study had several limitations. First, this data was from a single-center. Second, this study was retrospective and only patients who underwent curative resection were included. These questions would be solved in our next study.

Compliance with ethical standards

All procedures involved human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Acknowledgements

Research was supported by malignant tumor biomarkers in Jiangsu Province Key Laboratory (#11ZLKF09).

Disclosure of conflict of interest

None.

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Table S1. The patients' clinicopathologic information in CRC

Variables	n	
All patients	470	(%)
Age (years)		
≤65	267	56.8
>65	203	43.2
Gender		
Males	281	59.8
Females	189	40.2
Pathological classification[b]		
I	5	1.1
II	423	91.2
III	36	7.7
Depth of invasion[b]		
T1	9	2.0
T2	94	20.2
T3	347	74.6
T4	15	3.2
Lymph node metastasis[b]		
N0	276	59.2
N1	126	27.0
N2	64	13.8
TNM stage[b]		
I	88	18.9
II	179	38.6
III	180	38.8
IV	17	3.7
Tumor diameter[b]		
≤5 cm	378	80.6
>5 cm	91	19.4
Distant metastasis		
M0	451	95.9
M1	19	4.1

[b]Some patients missing these clinical pathological parameters.

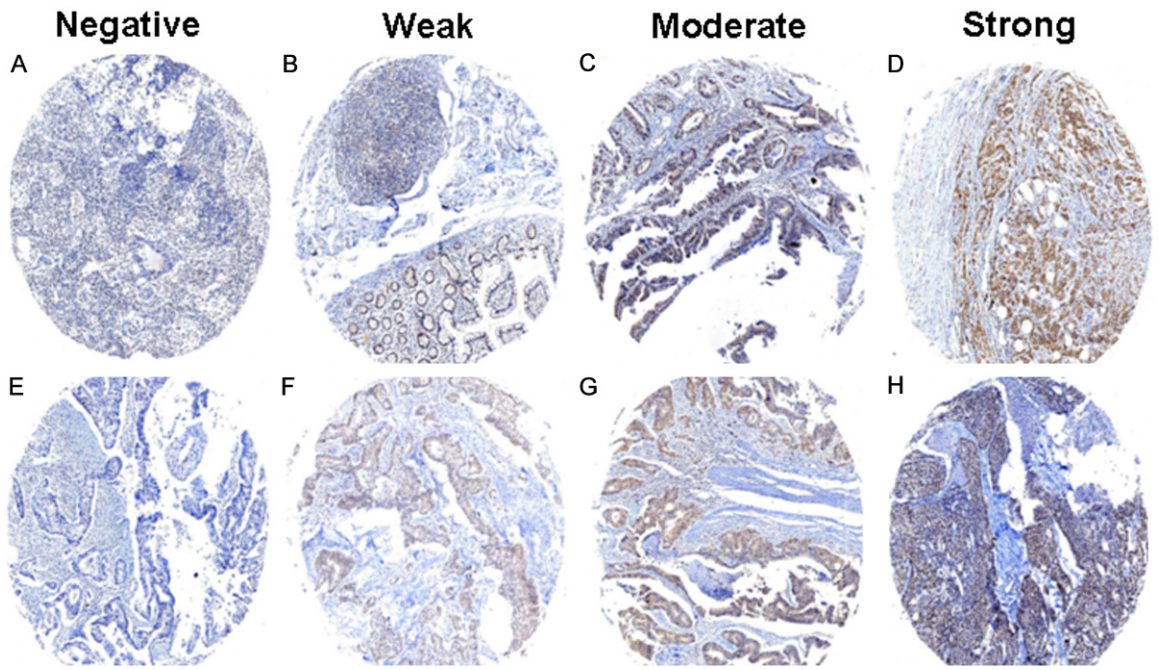

Figure S1. Representative images of Cul1 immunohistochemical staining in normal gastric tissue and gastric cancers. (A-D) is adjacent normal tissue; (E-H) is cancer tissue. (A, E) Negative staining. (B, F) Weak staining. (C, G) Moderate staining. (D, H) Strong staining. All panels: original magnification, 40×.

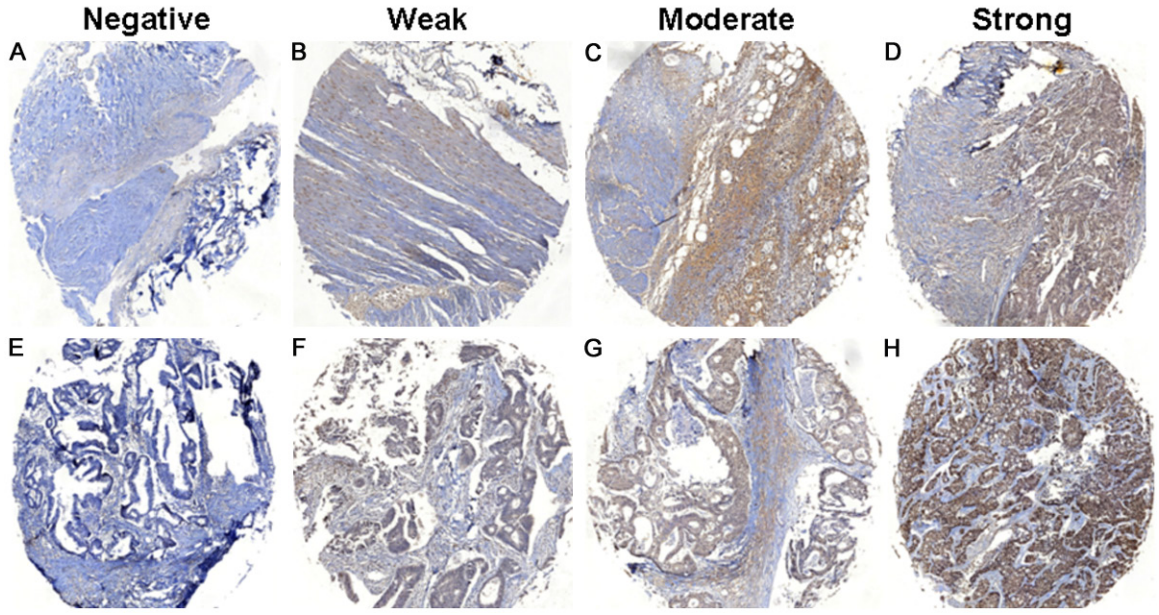

Figure S2. Representative images of PARP1 immunohistochemical staining in normal gastric tissue and gastric cancers. (A-D) is adjacent normal tissue; (E-H) is cancer tissue. (A, E) Negative staining. (B, F) Weak staining. (C, G) Moderate staining. (D, H) Strong staining. All panels: original magnification, 40×.

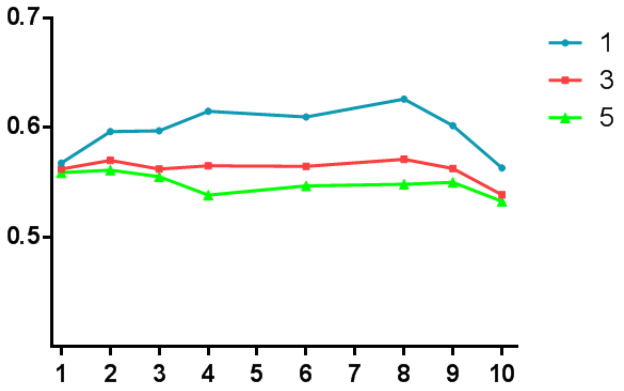

Figure S3. Receiver operating characteristic (ROC) curves were obtained to show the relation between area under the curve (AUC) at different cutoff values of Cul1 immunoreactivity score (IRS) for 1, 3 and 5 years of overall survival time.

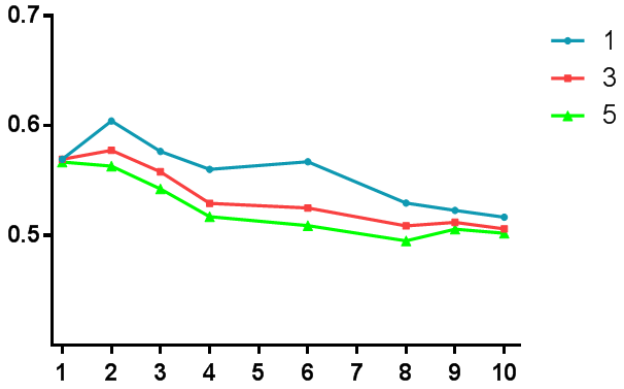

Figure S4. Receiver operating characteristic (ROC) curves were obtained to show the relation between area under the curve (AUC) at different cutoff values of PARP1 immunoreactivity score (IRS) for 1, 3 and 5 years of overall survival time.