

PARP1 Bound to XRCC2 Promotes Tumor Progression in Colorectal Cancer

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Research

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Abstract

Background: By complexing poly (ADP-ribose) (PAR) in reaction to broken strand, PAR polymerase1 (PARP1) acts as the key enzyme participated in DNA repair. However, recent studies suggest that unrepaired DNA breaks results in persistent PARP1 activation, which leads to a progressively reduce in hexokinase1 (HK1) activity and cell death. So the molecular mechanism of PARP1 remains elusive.

Methods: 212 colorectal cancer (CRC) patients who had the operation at our hospital were recruited. Used immunohistochemistry to evaluate PARP1 expression. Survival analysis was calculated based on PARP1 expression.

Results: Compared with matching adjacent noncancerous tissue, in CRC tissue, PARP1 expression was remarkably higher, which was correlated with the degree of differentiation, TNM stage, depth of invasion, distant metastasis, and 5-year survival. Furthermore, after constructing CRC cell lines stably expressing low or high PARP1, we found that PARP1 overexpression promoted proliferation, and proved PARP1 interacted with XRCC2 in CRC cells by immunoprecipitation (IP) analysis.

Conclusions: PARP1 was upregulated in CRC cells and promoted its proliferation of colorectal cancer cells. Furthermore, PARP1 expression status was significantly related to some clinicopathological features and 5-year survival.

Introduction

Colorectal cancer (CRC), which ranks fourth (6.1%) in the light of incidence but second (9.2%) in accordance with mortality in diagnosed cancer, takes a place of the most usual gastrointestinal cancers worldwide. Currently, in 2018, around 1.8 million people were confirmed CRC worldwide, and over 881,000 patients died of the disease, occupying about 10% cancer cases and deaths.¹ More importantly, in recent decades, the CRC incidence and mortality have increased in China.² Scholars have made progress in targeted therapy of CRC; nevertheless, superior targeting drugs are desired since the current treatment cannot generate satisfactory results.

Changes in PARP1 levels play a vital role in CRC.³ PARP1 plays a fundamental role in preserving genome stability and regulating chromatin structure. PARP1 takes part in several DNA repair pathways, which include DNA single-strand break (SSB) repair, HR repair (HRR) pathway of DNA double-stranded breaks (DSBs), and base excision repair (BER). HRR-deficient neoplasms are extremely susceptible to PARP1 inhibitors based on the synthetic lethality theory.⁴⁻⁶ PARP1 inhibitors chiefly prevent the PARP1 catalytic activity and raise the single-strand breaks levels persistently, resulting in DNA DSBs upon replication.^{5,7,8} On the other hand, unrepaired DNA breaks, arising from DNA repair deficiency and/or overexposure of genotoxin, results in persistently PARP1 activated and cell death.⁹ Uncontrolled or excessive activation of PARP1 leads to numerous pathological results, including the onset of diabetes, streptozotocin-induced pancreatic beta-cell death, myocardial ischemia, and tissue injury from cerebral.¹⁰⁻¹³ What's more, the

study of Fouquerel¹⁴ has demonstrated that by inhibiting HK1 independent of NAD⁺ depletion, PARP1 regulates glycolysis negatively. The result showed that activated PARP1 inhibited the activity of HK1, which resulted in the inhibition of ATP synthesis and cell death.

Therefore, PARP1 as an important member of DNA damage repair and energy metabolism pathway of tumor cells, its specific role and mechanism have not been clarified. In this research, we intend to study the effect of PARP1 in CRC cells and provide an important basis for PARP1 to become a new therapeutic target for CRC.

Methods

Patients

From October 2010 to December 2012, 212 primary CRC tissues and 47 matching adjacent noncancerous tissues were obtained from patients who had the operation at the Hunan Provincial People's Hospital. Patients with preoperative chemotherapy and/or radiotherapy, and palliative surgery were excluded. Survival analysis excluded patients who died within 30 days after surgery, since their death could be attributed to surgical complications. All patients have signed an informed consent. The Ethical Review Board of Hunan Provincial People's Hospital have approved the study.

Immunohistochemistry (IHC)

According to previously described methods, we performed the following procedures by the classic biotin-streptavidin-peroxidase IHC staining protocols.¹⁵ We obtained sections from the Hunan Provincial People's Hospital Pathology Department, and incubated overnight at 48°C with polyclonal primary antibody against PARP1 (1:100; Abcam, Cambridge, UK). Following incubated with diaminobenzidine and horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody (Beyotime; Guangzhou, China), used Mayer's hematoxylin to counterstain the slides. Positive control was primary CRC tissue slides. In negative control staining, used phosphate-buffered saline (PBS) buffer to replace the primary antibody. The immunostaining results were scored according to the methods previously described below.¹⁶

Culture and treatment of Cell

We obtained human CRC cell lines FHC, SW480, SW620, LoVo, SW403, HT-29, COLO205 and COLO320DM from the American Type Culture Collection (Manassas, VA, USA). Incubated cell lines in DMEM/RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) complemented with penicillin (100U/mL), streptomycin (100µg/ml), and 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO₂.

Vectors and retroviral infection

Through subcloning the PCR-amplified human PARP1 coding sequence into a pBABE-puro vector, we generated pBABE/PARP1-overexpressing human PARP1. Cloned two RNA interference (RNAi)

oligonucleotides into pSuper-retro-puro vectors to produce the pSuper-retro-PARP1-RNAi respectively, thereby silencing endogenous PARP1. As previously described, the generation and infection of retroviruses were carried out.¹⁷ After infection 48 hours, cell line stably expressing PARP1 (pBABE-puro-PARP1 or SW480/SW620-PARP1; control, pBABE-puro or SW480/SW620-Vector, respectively) or PARP1 RNAi (pSuper-retro-puro-si PARP1 or SW480/SW620-PARP1/RNAi; control, pSuperretro-puro or SW480/SW620-Scramble, respectively) were selected using puromycin (0.5mg/ml) over 10 days. SDS-PAGE was used to segregate SW480 and SW620 cell lysates to detect PARP1 protein levels.

Extraction and reverse transcription of RNA, real-time quantitative PCR

In line with manufacturer's illustrations, applied Trizol reagent (Invitrogen, Carlsbad, CA, USA) to accomplish total RNA extraction from cultured cells or tissues. Equipped with the ABI PRISM 7500 system (Applied Biosystems, Foster City, CA, USA), SYBR Green I (Invitrogen) was applied for real-time quantitative PCR. Selected housekeeping gene GAPDH as an internal control. Used the primers below:

PARP1 forward, 5'-ACAGTGTGCAGGCCAAGGTG -3', and reverse 5'-CTCGGC TTCTTCAGAATCTCTGTC-3';
XRCC2 forward: 5'-TCACCTGTGCATGGTG ATATT-3', and reverse: 5'-TTCCAGGCCACCTTCTGATT-3';
GAPDH forward: 5'-GACTCATGACCACAGTCCATGC-3', and reverse: 5'-AGAGGCAGGGATGATG

TTCTG-3'; p21 forward: 5'-CGATGCCAACCTCCTCAACGA-3', and reverse: 5'-TCGCAGACCTCCAGCATCCA-3'; cyclin D1 forward: 5'-AACTACCTGGA CCGCTTCCT-3', and reverse: 5'-CCACTTGAGCTTGTTACCA-3'.

Western blotting

According to manufacturer's instructions proteins were prepared from cell lysates, isolated on SDS-PAGE, and transferred to PVDF membranes. To detect specific proteins, primary antibodies that were used included α -Tubulin mouse monoclonal antibody (1:1000; Sigma-Aldrich, St. Louis, MO, USA), anti-human XRCC2 mouse monoclonal antibody (1:1500; Abcam), anti-human PARP1 mouse monoclonal antibody (1:1500; Abcam), anti-human cyclin D1 rabbit monoclonal antibody (1:1500; Abcam), and anti-human P21 rabbit monoclonal antibody (1:1500; Abcam). The secondary antibody was goat anti-mouse antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the membrane exposed to electrochemiluminescence reagent (GE Healthcare, Buckinghamshire, UK), storm imaging system (Amersham Biosciences, Piscataway, NJ, USA) was used for visualization to achieve signal amplification and detection.

Cell proliferation detection

In accordance with the manufacturer's illustrations, used the Cell Counting Kit-8 (CCK-8) cell proliferation kit (Dojindo Laboratories, Kumamoto, Japan) to assess cell proliferation. Concisely, seeded the cells into 96-well plates (2×10^3 cells/well), and cultured under regular circumstances with 100 μ L complete medium. At the specified time, incubated cells with RPMI-1640 medium (100 μ L) plus CCK8 reagent

(10 μ L) for 2 hours at 37°C. After that, measured the absorbance at 450nm wavelength on a microplate reader (Bio-Rad, La Jolla, CA, USA). Conducted three repetition experiments independently.

Colony formation assay

In brief, plated exponential growth cells into 6-well plates at 1000 cells/well and cultured for 10-14 days at 37°C with 5% CO₂. For visualization and counting, used 75% ethanol to fix the colonies for 30mins and stained with 0.5% crystal violet (Beyotime, Nanjing, China) afterwards. When colonies with more than 50 cells would be manually calculated. Every group of cells comprised three wells, and three independent repeat experiments were conducted.

Statistical analysis

SPSS 20.0 (SPSS Inc, Chicago, IL, USA) was used for statistic analysis. Employed the Chi-square test to evaluate the association between PARP1 expression and clinicopathological characteristics. The significant differences between two groups of data were analyzed with the Student's t test. The log-rank test and the Kaplan-Meier method were employed for survival curves analysis. The time from surgery to last follow-up date or patient's death was 5-year overall survival (OS). The time from radical operation to recurrence, last follow-up date, or death was defined as Relapse-free survival (RFS). Recurrences were defined as local and distant relapses. Statistically significant was set at $p < 0.05$.

Results

In CRC cell lines and clinical tissues PARP1 was upregulated.

PARP1 expression in primary CRC tissue and matching adjacent noncancerous tissue was described in Fig. 1. In 157 of 212 (74.1%) primary CRC tissues, positive PARP1 staining was discovered. By contrast, compared with primary CRC tissue, in the matching adjacent noncancerous tissues, PARP1 staining positive rate was only 53.2% (25/47 samples, $p = 0.005$; Fig. 1A-D; Table 1). Furthermore, applied western blotting and real-time PCR to detect eight CRC patients PARP1 expression in tumors and the matching adjacent noncancerous tissues. Different from normal tissues, PARP1 was upregulated remarkably in tumors ($p < 0.05$; Fig. 2A, B). Moreover, we also used western blotting and real-time PCR to examine PARP1 expression in normal colonic epithelial cell (FHC) and eight CRC cell lines. Compared with colonic epithelial cell, PARP1 was upregulated remarkably in CRC cell lines ($p < 0.05$; Fig. 2C, D).

Table 1
PARP1 expression in primary CRC tissue and adjacent noncancerous tissue.

Tissue sample	n	Expression of PARP1		<i>p</i>
		Positive (%)	Negative (%)	
Primary colorectal cancer	212	157(74.1)	55(25.9)	0.005
Adjacent normal colorectal mucosa tissues	47	25(53.2)	22(46.8)	

Correlation between PARP1 expression and CRC patients clinicopathological characteristics.

Table 2 described the relevance between PARP1 expression and clinicopathological features, including age, gender, differentiation, distant metastasis, tumor-nodes-metastasis (TNM) stage, tumor size, lymph node metastasis, tumor site, and depth of invasion. The expression status of PARP1 in primary CRC was remarkably associated with the degree of differentiation, TNM stage, distant metastasis, and depth of invasion ($p = 0.015$; $p = 0.002$; $p = 0.001$; $p = 0.001$, respectively).

Table 2
Clinicopathological characteristics and PARP1 expression status of patients with colorectal cancer.

Clinical characteristics	n	Positive (%)	Negative (%)	<i>p</i>
Gender				
male	102	73(71.6)	29(28.4)	0.426
female	110	84(76.4)	26(23.6)	
Age				
< 50y	55	44(80.0)	11(20.0)	0.503
50-70y	122	88(72.1)	34(27.9)	
> 70y	35	25(71.4)	10(28.6)	
Tumor site				
rectum	83	60(72.3)	23(27.7)	0.830
left	85	63(74.1)	22(25.9)	
right	44	34(77.3)	10(22.7)	
Tumor size				
< 3cm	24	17(70.8)	7(29.2)	0.149
3-5cm	111	77(69.4)	34(30.6)	
> 5cm	77	63(81.8)	14(18.2)	
Lymph node metastasis				
Yes	144	112(77.8)	32(22.2)	0.072
No	68	45(66.2)	23(33.8)	
Distant metastasis				
Yes	54	49(90.7)	5(9.3)	* 0.001
no	158	108(68.4)	50(31.6)	
Depth of invasion				
T1	8	3(37.5)	5(62.5)	* 0.001
T2	20	9(45.0)	11(55.0)	
T3	45	33(73.3)	12(26.7)	

* *p* < 0.05.

Clinical characteristics	n	Positive (%)	Negative (%)	<i>p</i>
T4	139	112(80.6)	27(19.4)	
Degree of differentiation				
high	4	1(25.0)	3(75.0)	* 0.015
moderately	161	116(72.0)	45(28.0)	
low	47	40(85.1)	7(14.9)	
TNM				
I/II	37	20(54.1)	17(45.9)	* 0.002
III/IV	175	137(78.3)	38(21.7)	
* <i>p</i> < 0.05.				

Survival analysis and prognostic significance of PARP1 expression.

Figure 3A, B showed the Kaplan-Meier estimates for the group with positive PARP1 and group with negative PARP1. The median time to total OS for 212 patients was 43.9 months. And the median OS time for two groups was 40.7 months and 53.1 months separately. The two survival curves were remarkably different (Fig. 3A; $\chi^2 = 12.095$; $p = 0.001$). 212 patients total median RFS time was 38.0 months. While the median RFS time for two groups was 34.7 months and 48.1 months respectively. The two survival curves were also significantly different (Fig. 3B; $\chi^2 = 10.848$; $p = 0.001$). These results showed that, compared with negative PARP1 expression, patients with positive PARP1 expression had shorter OS and RFS.

Stable cell lines expressing high or low PARP1.

Western blotting and quantitative PCR (QPCR) was used to detect PARP1 expression after cell lines expressing high or low PARP1 were constructed. Western blotting revealed that PARP1 expression in SW480/SW620- PARP1/RNAi was lower than that in SW480/SW620-Scramble cells. What's more, PARP1 expression in SW480/SW620-PARP1 cells was higher than that in SW480/SW620-Vector cells (Fig. 4B; $p < 0.05$). Moreover, the results obtained by QPCR were similar (Fig. 4A; $p < 0.05$). The above results demonstrated that cell lines stably expressed low or high PARP1 had been constructed successfully.

PARP1 interacted with XRCC2 during SW620 cell proliferation.

In previous studies, we found that the polymorphism or low expression of XRCC2 affected the sensitivity of CRC cells to PARP1 inhibitors^{15,18}. In the light of previous theory, we assumed that PARP1 binds to XRCC2 in SW620 cells. To define the combination between PARP1 and XRCC2, IP was performed. Using PARP1 and XRCC2 primary antibodies to perform two-way verification, both of which showed that, during SW620 cell differentiation, PARP1 interacted with XRCC2 (Fig. 4C).

PARP1 overexpression promoted proliferation in CRC cells.

Through CCK-8 assay and colony formation assay, we assessed PARP1 overexpression effect on CRC cell proliferation to study whether PARP1 affects CRC development and progression. Compared with PARP1/RNAi, PARP1 upregulation remarkably increased SW480/SW620 cells growth rate 48 hours after PARP1 transduction (Fig. 5A, B; $p < 0.05$). As expected, PARP1 overexpression increased the expression of cyclin D1, while significantly reducing p21 expression in CRC cells (Fig. 5C, D; $p < 0.05$). The results demonstrate that PARP1 may make a significant impact in CRC cells proliferation.

Discussion

These traditional views believe that PARP1 had biological functions. For instance, DNA repair, apoptosis, synthetic lethality, histone binding, necrosis, and so on.¹⁹ After activated by DNA damage, PARP1 startup the DNA damage repair process as a tumor suppressor gene, binds to target proteins, DNA signal- and double- strand breaks, and other proteins of DNA repair.²⁰ However, the latest research showed that PARP1 participated nucleus to mitochondria communication, which involved in cell energy metabolism. Activation of PARP1 sparks off cell death and energy crumble, which indicate novel understanding on the significance of PARP1 activation. Therefore, the role of PARP1 in cells is undefined. Current researches have pointed out that PARP1 may be related to the CRC occurrence. Studies have proved that PARP1 (Ala762Val, rs1136410) was associated with the susceptibility to colorectal carcinoma of adult and children.^{21, 22} Moreover, the study of Sakthianandeswaren has found that MACROD2 deletions or haploinsufficient caused chromosome instability (CIN) and impaired PARP1 activity in CRC. In turn, it drove the evolution of cancer.^{23, 24} Therefore, we focus on the significance of PARP1 in colorectal cancer.

By Immunohistochemistry, real-time PCR, and western blotting, compared with normal tissues, we discovered that PARP1 was upregulated significantly in tumors. These lead us to further study the significance of PARP1 in the development of CRC. Furthermore, after analyzing the correlation between PARP1 expression and the CRC clinicopathological features, we surprised to find that PARP1 expression status was remarkably correlated with the degree of differentiation, TNM stage, distant metastasis, and depth of invasion in primary CRC. Increasing PARP1 expression may promote invasive behavior and metastatic process of CRC. However, the mechanism of how PARP1 affects the progression of CRC remains unknown. Former researchers hold the assumption that PARP1 plays a vital part in facilitating the growth and proliferation of tumor cell. Santos JC²⁵ revealed that the expression of PARP1 is related to tumor location (tumor of colon or rectum) and tumor stage (III/IV or I/II grade). Similarly, Li²¹ proved that PARP1 (Ala762Val) was associated with the susceptibility to CRC. Thus, it may not be surprising that PARP1 is involved in the metastasis and invasion of CRC. It worth further studies to clarify the mechanism involved.

What's more, in this research we found that the positive PARP1 expression and CRC patients' poor survival after surgery was correlated significantly. Patients with positive PARP1 expression had shorter OS and RFS compared with those patients with negative PARP1 expression. The two survival curves were

remarkably different. The finding indicated that the PARP1 protein may affect the prognosis of CRC patients. However, the study of Li ²¹ found that the mutation of PARP1 (Ala762Val) may have nothing to do with the prognosis of CRC patients intrinsically. Therefore, to define the relevance between PARP1 and CRC patients prognosis, expand the sample and enroll more advanced CRC patients is necessary.

To further figure out the effect of PARP1 in CRC progression and development, cell lines stably expressing high (SW480-PARP1, SW620-PARP1) or low PARP1 (SW480-PARP1/RNAi, SW620-PARP1/RNAi) were constructed. Firstly, we investigated the affection of high or low PARP1 expression on the growth of SW480/SW620 cells. Since one of the vital characteristics of the cancer cell phenotype is unconstrained growth. The current result revealed that suppressing PARP1 effectively decrease proliferation, whereas upregulating it significantly promote proliferation, indicating that PARP1 could be a vital regulator of CRC cell proliferation.

Schaaf ²⁶ found that PARP1 plays a vital part in the chemosensitization mechanism of hyperthermia of CRC. Previous study has pointed out that two genes simultaneously deficiencies lead biological systems lethality, otherwise the absence of one gene will be abided.²⁵ Based on this concept, restraining PARP1 could be a latent therapeutic schedule for the therapy of cancers with defects in precise DNA repair genes, such as XRCC2, BRCA1/2, and MRE11.^{26,27} A number of clinical trials have been launched with PARP inhibitors apply to CRC patients.²⁷ Furthermore, our study reveals that CRC cell proliferation restrained by olaparib in a dosage and time dependent way.¹⁵ These studies show that PARP1 expression can promote the proliferation of CRC cells. Moreover, PARP1 inhibitors have been shown to play an anti-tumor role in many tumors.^{15,28} Both PARP1 and XRCC2 participate in the double-stranded DNA HRR pathway. In the previous study, we found that CRC cell proliferation restrained by PARP1 inhibitor (olaparib) in a dosage and time dependent way. These findings indicated that there is a closed relationship between XRCC2 and PARP1. The effect of PARP1 inhibitor may require the presence of XRCC2. In the present study, we found that PARP1 interacted with XRCC2 in SW480/SW620 cells. However, up to now, there is no convinced proof to define the relevance between XRCC2 and PARP1. In future experiments, we will further explore the close relationship between PARP1 and XRCC2.

In conclusion, we found that PARP1 was upregulated in CRC and promotes colorectal cancer cells proliferation. Furthermore, PARP1 expression status in primary CRC was remarkably correlated with the degree of differentiation, TNM stage, distant metastasis, depth of invasion, and 5-year survival. Lastly, the results of IP show that XRCC2 protein interacting with PARP1, but need further explore.

Declarations

Ethics approval and consent to participate

All patients have signed an informed consent. The Ethical Review Board of Hunan Provincial People's Hospital have approved the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Kaiwu Xu: project development, data analysis and collection, manuscript writing and editing. Zhige Yu, Tailiang Lu, Wei Peng, Yongqiang Gong: data collection. Chaowu Chen: Project design and quality control, manuscript editing.

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Figures

Figure 1

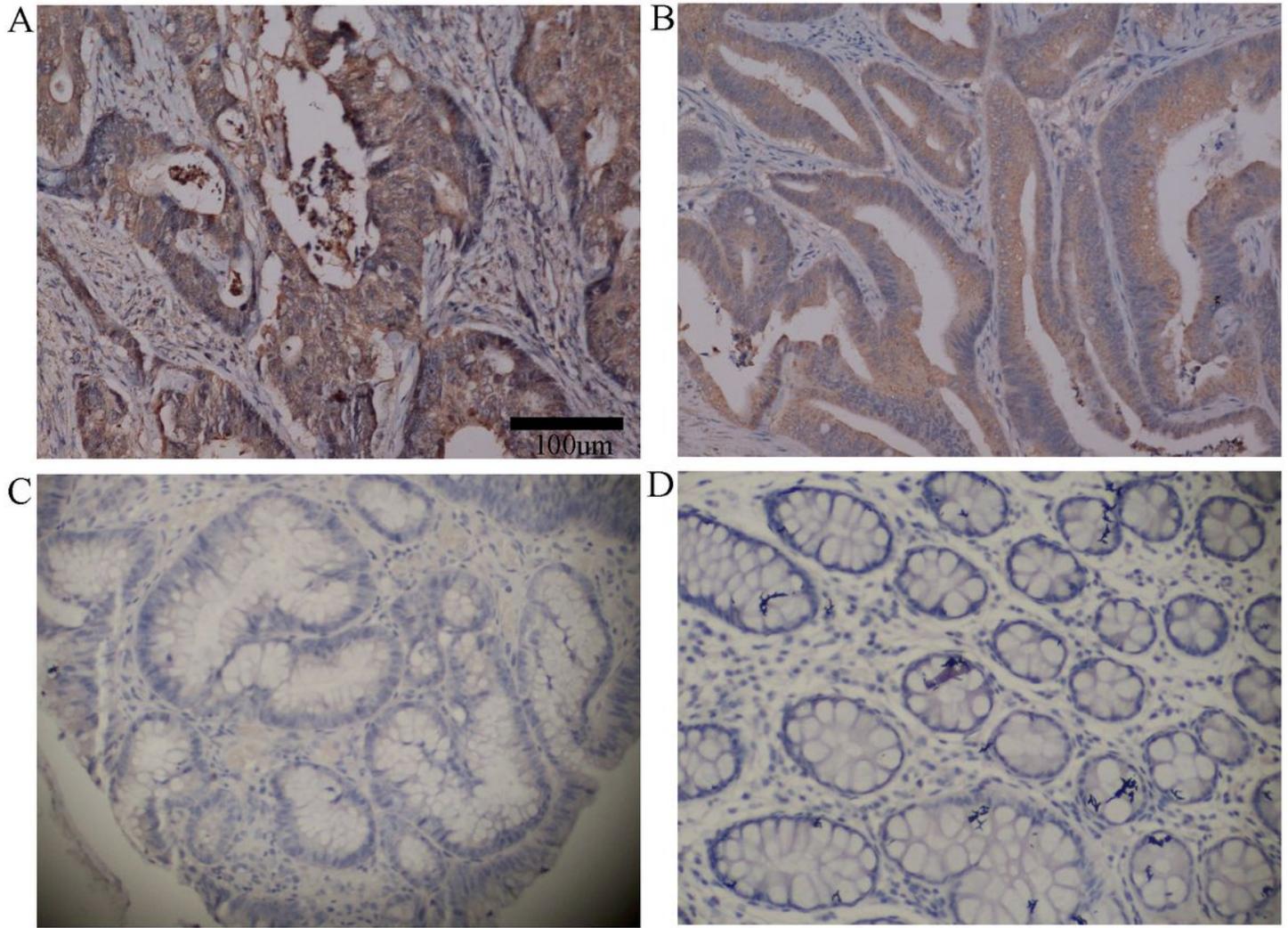


Figure 1

Expression of PARP1 in CRC and adjacent normal mucosal tissues. Primary CRC (A-C): (A) positive; (B) weakly positive (X200 magnification); (C) negative. (D) normal colorectal mucosa tissue. The scale bar represents 100µm.

Figure 2

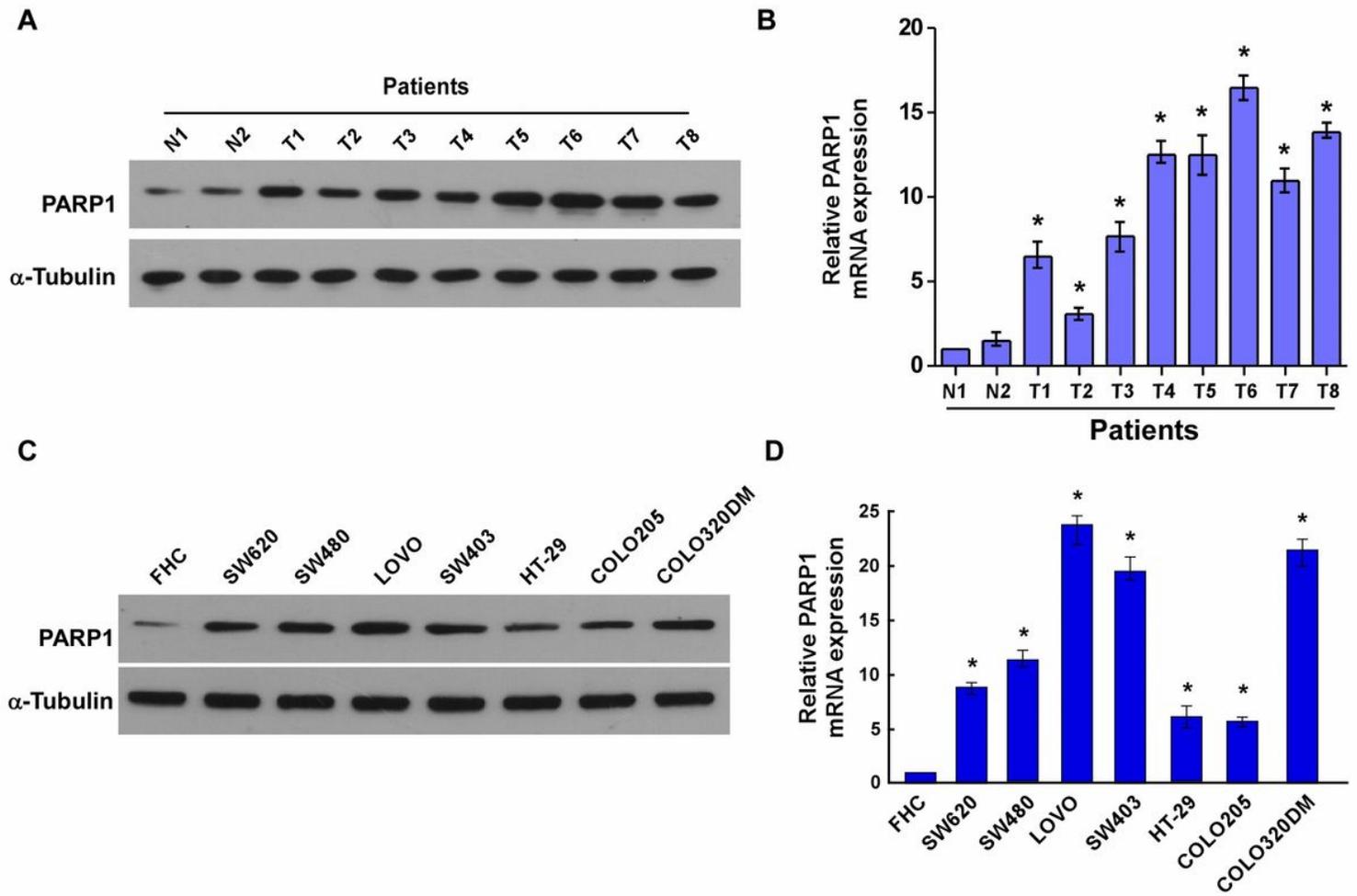


Figure 2

PARP1 expression in eight CRC tumors and matched adjacent noncancerous tissues (A-B): (A) Western blotting; (B) real-time PCR. PARP1 expression in eight CRC cell lines and normal colonic epithelial cell (FHC) (C-D): (C) Western blotting; (D) real-time PCR ($p < 0.05$; Figure 2 C, D). * $p < 0.05$, (Student's t test).

Figure 3

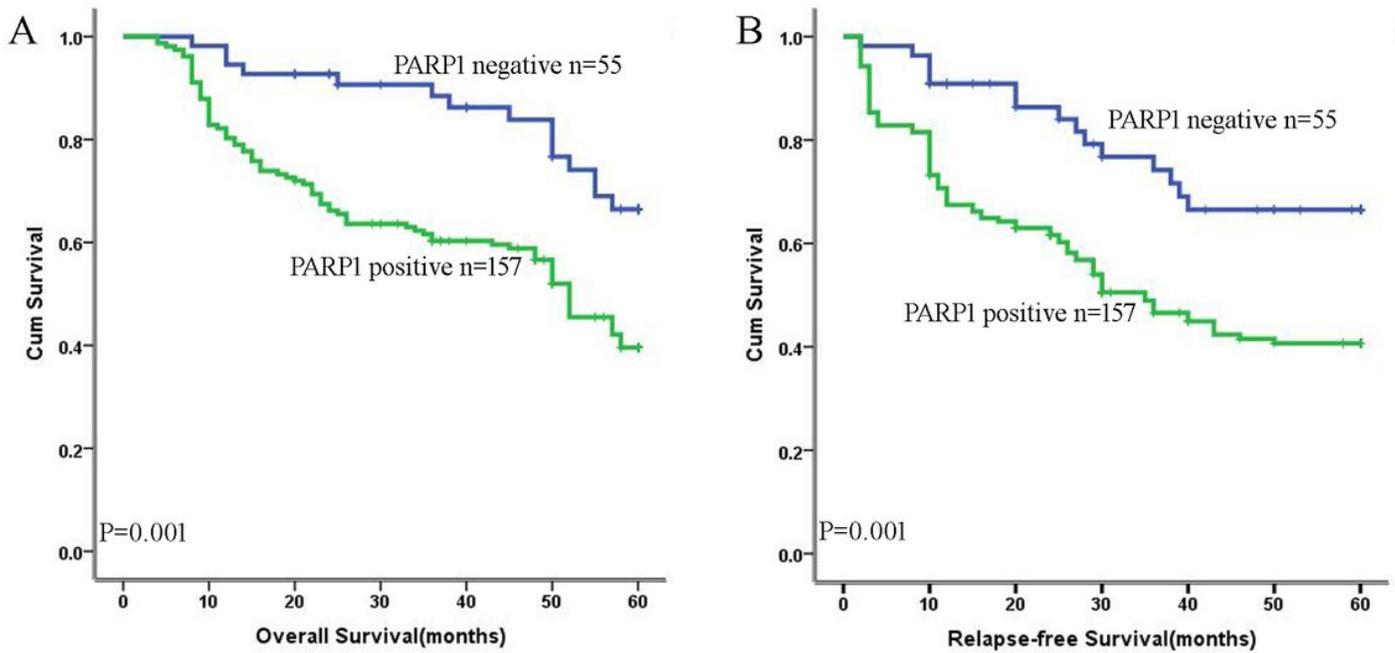


Figure 3

Survival curve in regards to PARP1 expression and the PARP1 expression in SW480/SW620 cells. Positive PARP1 expression patients have shorter OS (A) and RFS (B) than negative PARP1 expression patients ($p = 0.001$; $p = 0.001$, respectively).

Figure 4

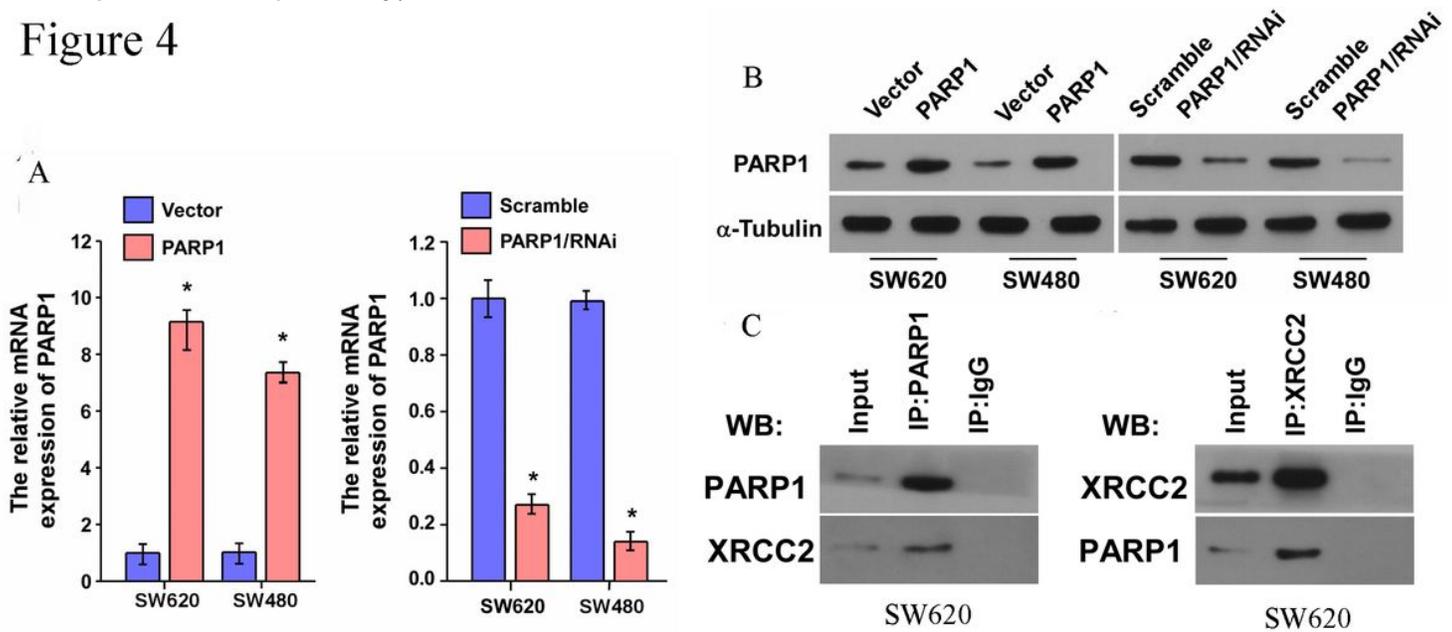


Figure 4

(A) QPCR analysis of PARP1 mRNA expression in SW480 and SW620 cells. $*p < 0.05$, (Student's t test).
 (B) Western blotting analysis of PARP1 expression in SW480/SW620-PARP1, SW480/SW620-Vector,

SW480/SW620-PARP1/RNAi and SW480/SW620-Scramble cells. (C) The IP results of XRCC2 protein interacting with PARP1 protein. Input represents the positive control group, IgG represents the negative control group, IP represents the target experimental group.

Figure 5

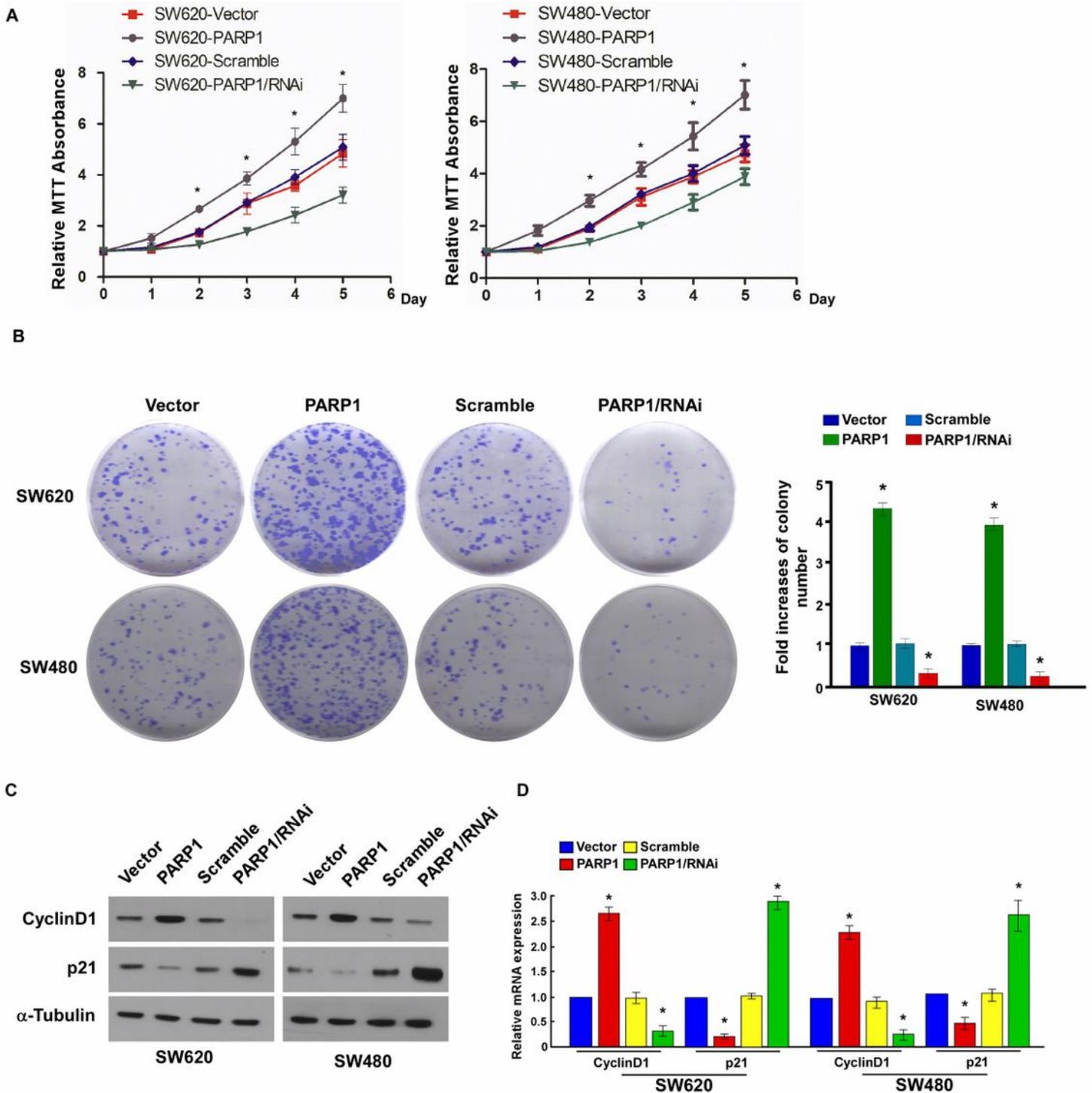


Figure 5

Cell proliferation analysis. CCK-8 assay (A) and Colony formation assay (B) of SW480/SW620 cells transfected with PARP1, PARP1/RNAi, and Vector/Scramble. Cyclin D1 and p21 expression (C-D): (C)

Western blot analysis; (D) QPCR analysis. * $p < 0.05$, (Student's t test).