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Original Article

Silencing PARG decreases invasion in CT26 cells

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Abstract: In this study we investigated the effect of poly (ADP-ribose) glycohydrolase (PARG) deficiency on the ability of invasion in CT26 murine colon carcinoma cell and its possible mechanism. We examined the effects of PARG protein knockdown by RNA interference on invasion, migration, and matrix adhesion of colon carcinoma CT26 cell line *in vitro* and using a murine model of liver metastasis *in vivo* to observe the average survival time. The expression of integrin-β1, matrix metalloproteinases-9 (MMP-9) and matrix metalloproteinases-2 (MMP-2) was detected by western blot. The activities of MMP-9 and MMP-2 in various group supernatants were measured by zymography. We observed that PARG silencing caused a significant decrease in the number of CT26 cells that adhered to fibronectin ($P < 0.5$) and invaded to the lower surface of the membrane ($P < 0.5$). The expression of integrin-β1, MMP-9, and matrix MMP-2 in CT26 cells of the PARG-shRNA group was lower than that of two control groups ($P < 0.5$). Similar results were observed in the activities of MMP-9 and MMP-2 in various group supernatants ($P < 0.5$). The average survival time of BALB/c mice with spleen-transplanted PARG-shRNA CT26 tumors was longer compared with control groups ($P < 0.05$). To conclude, PARG silencing can inhibit the adhesive and invasive abilities of CT26 cells and delay liver metastasis in a mouse model, which might be useful for therapeutic purposes in CRC patients with distant metastasis.

Keywords: PARG, CT26, colorectal carcinoma, invasion, short hairpin RNA, mouse model

Introduction

It is well accepted that colorectal cancer (CRC) is a common type of cancer worldwide, ranking third in terms of incidence but second in terms of mortality [1]. China has experienced increases in both incidence and mortality in the most recent decade [2]. In patients with colorectal cancer, distant metastasis is the leading cause of death. Approximately 50% of patients with colorectal cancer will have liver metastases, the most common distant metastatic site [1, 3]. Thus, new strategies are needed to prevent liver metastasis and reduce its devastating morbidity and mortality in patients with CRC.

Poly (ADP-ribose) glycohydrolase (PARG) is a catabolic enzyme that cleaves ADP-ribose polymers and reactivates the activity of poly (ADP-ribose) polymerase (PARP) [4]. PARG, coordinating with PARP1, is critical for the maintenance of steady-state Poly (ADP-ribose) levels, and has been associated with various cellular processes, including cell proliferation, apoptosis, invasion, and metastasis [5-8]. It has been reported that genetic deletion of the *PARG* gene causes embryonic lethality [5], and decreased PARG activity sensitizes cells to a spectrum of DNA-damaging agents resembling that caused by genetic knockdown of PARP-1 expression or pharmacologic inhibition of PARP activity [9]. Pharmacologic inhibition of the PARG activity inhibits melanoma growth, and decreases the ability of melanoma cells to form lung metastases and to invade the extracellular matrix [10]. More recently, researchers have demonstrated that heightened expression of catalytically active PARG facilitates cell transformation and invasion of normal epithelial cells, associating with a poor prognosis [8]. Therefore, PARG could be a promising target for cancer treatment.

Our previous experiments have provided evidence that RNA interference with the *PARG* gene can significantly inhibit the adhesion of colon cancer cells to platelets [11], and the formation of lymphatic vessels in colon tumors [12], thus preventing the formation of distant metastases to a certain extent. Consistent with this, we demonstrated that *PARG* gene silenc-

ing reduces the abilities of adhesion, migration, and invasion in LoVo cells [13], through down-regulating PARP expression and inhibiting NF-κB transcriptional activity [14]. It is speculated that inhibition of PARG gene expression may play a negative role in colorectal cancer. However, the mechanism whereby PARG promotes invasion and metastasis is just emerging. In the present study, further investigations were implemented to clarify the anti-tumor effect and mechanism of *PARG.*

Materials and methods

Cell lines and animals

The mouse colon adenocarcinoma CT26 cell line was gifted from Professor Yu-Quan Wei (Sichuan University, Chengdu, Sichuan, China). Wei-Qiang Wu had successfully constructed PARG-short hairpin RNA (shRNA) and vector-control CT26 cells [12], cultured in RPMI1640 medium (HyClone, Logan, UT, USA) containing with 10% Fetal Bovine Serum (FBS) (HyClone, Logan, UT, USA) and antibiotic solution (100 μg/ml streptomycin and 100 U/ml penicillin, HyClone) at 37°C in 5% CO_2 incubator. BALB/c mice (6-8 weeks old, 18-22 g) were obtained from the animal experimental center of Chongqing Medical University (Chongqing, China) and placed in the specific pathogen-free feeding room (20-26°C, 12 h: 12 h light/dark cycle) of the animal experimental center at Chongqing Medical University.

Cell invasion assay

The upper surface of 8 μM pore size transwell chambers (Corning, Corning, New York, USA) were coated with 50 μl of Matrigel (Corning, Corning, New York, USA) diluted 1:5 (v/v) with RPMI1640. The matrigel layer was left to air-dry in a 37°C incubator for 4 hours. Thereafter, we rehydrated the dried matrigel layer by adding RPMI1640 without bovine serum at 37°C for 30 minutes. 1×10^5 cells in serum-free RPMI1640 supplemented with 0.2% bovine serum albumin (BSA; BovoStar, Melbourne, Australia) were seeded into the upper chambers. RPMI1640 medium containing 10% fetal bovine serum (FBS) was added into the lower chamber as a chemoattractant. After incubation for 24 hours, matrigel and cells in the upper chamber were wiped with a cotton swab. The cells on the lower surface of the filter fixed with 4% paraformaldehyde (Biological Technology, Shanghai, China) and stained with 0.1% crystal violet (Beyotime Biotechnology, Shanghai, China). Cells were counted in three randomly selected fields and we calculated the mean value.

Cell adhesion assay

Wells of a 96-well tissue culture plate were coated with 50 μl of fibronectin (10 μg/ml, diluted with distilled water; Santa Cruz Biotechnology, Santa Cruz, Northern California, USA) at 4°C overnight. The wells were washed three times with 100 μl of PBS (Boster Biological Technology, Wuhan, China) and then blocked with serum-free RPMI1640 supplemented with 1% BSA for 2 hours at room temperature. Cells were seeded into each well (2×10^4/well) in triplicate and incubated at 37°C in an 5% CO_2 incubator for 1 hour. We removed the non-adhesive cells by washing two times with warm (37°C) PBS. After 4 hours' incubation at 37°C with 50 μl of MTT (2 mg/ml; Beyotime Biotechnology, Shanghai, China), 150 μl of DMSO (Beyotime Biotechnology, Shanghai, China) was added to solubilize crystals with shaking for 10 minutes. Absorbance at 490 nm was measured with a universal microplate reader. The number of attached living CT26 cells was directly proportional to the absorbance of MTT-formazan (490 nm).

Zymography

Cells were seeded into six-well plates at 5×10^6 cells per well and serum-starved for 24 h. The culture supernatants were collected and then centrifuged at low speed (200 × g) to remove cell fragments. Equal volumes of samples were fractionated in 10% polyacrylamide gel containing 0.1% gelatin (Beyotime Biotechnology, Shanghai, China) as substrate. Electrophoresis was carried out at 100 V for 90 minutes at 4°C constant temperature conditions. Gels were washed in 2.5% Triton X-100 (Psiatong, Beijing, China) twice for 30 minutes and then incubated in a buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, 0.02% brij-35) at 37°C for 42 hours. After being stained with 0.1% Coomassie Blue R-250 (Beyotime Biotechnology, Shanghai, China) for 3 hours, the gels were destained in 30% methanol and 10% acetic acid, until bands were clearly visible against the blue background. Gels were scanned with Gel Imaging System (Bio-Rad ChemiDoc XRS) and analyzed with Quantity One software.

Western blot

The extracts of cells were prepared according to each product description. The concentrations of protein were determined by the BCA protein assay (Beyotime Biotechnology, Shanghai, China). Protein extracts were loaded on 8% SDS-PAGE, separated by electrophoresis, and transferred onto polyvinylidene difluoride (PVDF; Millpore, Boston, Massachusetts, USA) membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 1 hour. Then, they were incubated at 4°C overnight with primary antibodies against integrin-β1 (1:500, Santa Cruz), MMP-9 (1:200, Santa Cruz), MMP-2 (1:200, Santa Cruz) and β-actin (1:2000, Bioss Inc). After having been washed thrice with TBST, the membranes were incubated with corresponding secondary antibodies (Proteintech, Wuhan, China) at 37°C for 2 hours. Protein bands were visualized by enhanced chemiluminescence with an ECL kit (Beyotime Biotechnology, Shanghai, China).

Experimental metastasis model

48 BALB/c mice were divided at random into three groups: Non-transfected group, Empty Vector transfected group, and PARG-shRNA group. The cells in logarithmic growth phase were diluted in PBS to adjust the cell density to 1×10^7/mL. The mice were anesthetized by intraperitoneal injection of 2% chloral hydrate (0.015 mL/g; Esite Biotechnology, Chengdu, China). A small longitudinal incision was made at the junction of the left subcostal margin and axillary posterior line. we slowly injected 50 μl of the above cell suspension into the exteriorized spleen. The abdominal wound was sutured on different tissue strata. Mouse model received no treatment, up until its natural death, and we recorded the survival time after inoculation. In rectangular coordinates, we took survival time as the horizontal axis and survival ratio as the vertical axis. Kaplan-Meier survival curves were obtained.

Statistical analysis

All statistical analyses were performed by SPSS 17.0. One-way ANOVA was used to compare quantitative data among three groups. Log rank test was used for survival curves. A *P* value less than 0.05 ($P < 0.05$) was considered significant.

Results

PARG-shRNA decreased cell invasion

The average number of cells in the non-transfected CT26 group was 120 versus 53 for PARG-shRNA treatment group ($P < 0.05$). Similar results were measured for Empty Vector control group and PARG-shRNA treatment group (135 versus 53; $P < 0.05$). However, there was no statistical significance between non-transfected CT26 group and Empty Vector transfected group (120 versus 135; $P > 0.05$; **Figure 1A** and **1B**).

PARG-shRNA also decreased cell adhesion

No significant difference in cell adhesion was observed between the Empty Vector transfected group and non-transfected CT26 group, whereas the adherence capacity of PARG shRNA CT26 group significantly weakened ($P < 0.05$; **Figure 1C**).

PARG positively regulated the activities of MMP-9 and MMP-2

The degradation of gelatin, which reflected the gelatinolytic activities of MMP-9 and MMP-2, was evaluated by Quantity One Soft. Analysis of independent samples revealed that both MMP-9 and MMP-2 were present in all samples. The levels of MMP-9 and MMP-2 were significantly decreased in PARG-shRNA CT26 cells ($P < 0.05$), compared with CT26 cells non-transfected and transfected with Empty Vector (**Figure 2A** and **2B**).

Lower expressions of integrin-β1, MMP-9, and MMP-2 in PARG-shRNA CT26 cells

The expression levels of integrin-β1, MMP-9, and MMP-2 in PARG-shRNA CT26 cells were all lower than those in the control groups ($P < 0.05$). No significant differences were identified between the Empty Vector transfected and non-transfected groups ($P > 0.05$; **Figure 3A** and **3B**).

Longer survival time of BALB/c mice with spleen transplanted PARG-shRNA CT26 tumor

The average survival time of BALB/c mice with spleen-transplanted PARG-shRNA CT26 tumors was longer compared with Empty Vector transfected and non-transfected groups ($P < 0.05$). For average survival, no significant differences

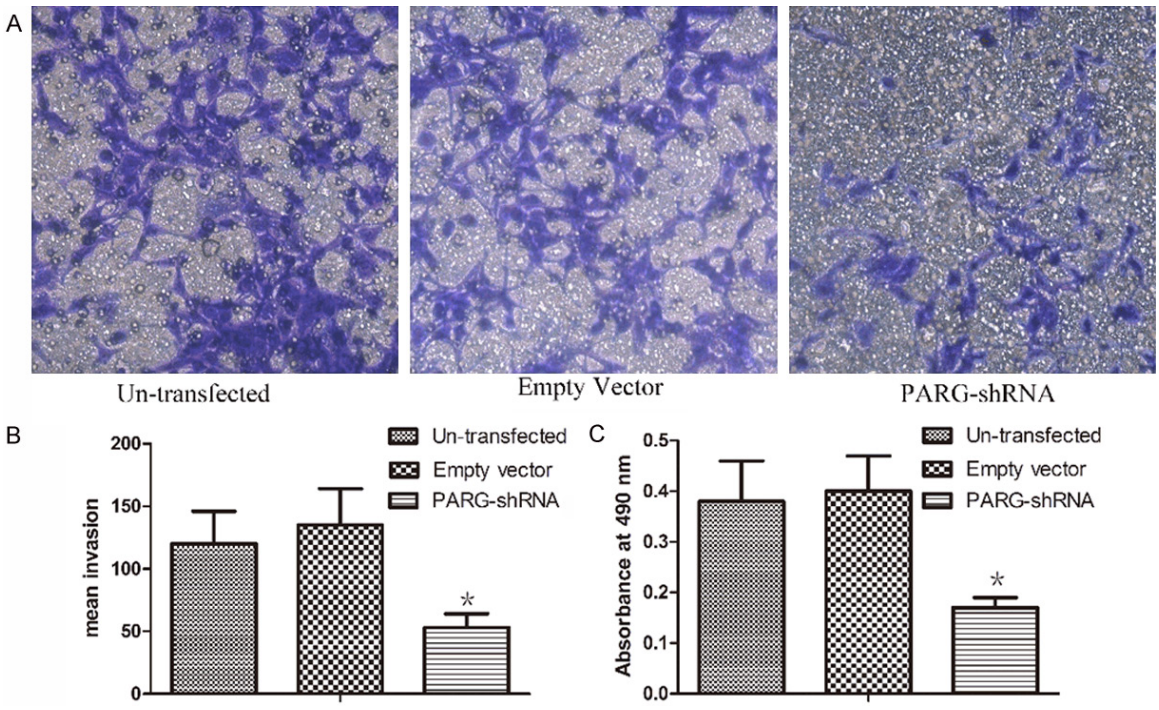

Figure 1. A. Photographic images of CT26 cells crossing the matrigel-coated layer and that adhered to the lower surface are shown. B. A significant decrease was seen in PARG-shRNA treatment group ($P < 0.05$; compared with non-transfected CT26 group and Empty Vector group). C. The absorbance of non-transfected CT26 and Empty Vector CT26 groups were 0.38 ± 0.08 and 0.40 ± 0.07 respectively, and the absorbance of PARG-shRNA was 0.17 ± 0.02, less than the two control groups ($P < 0.05$).

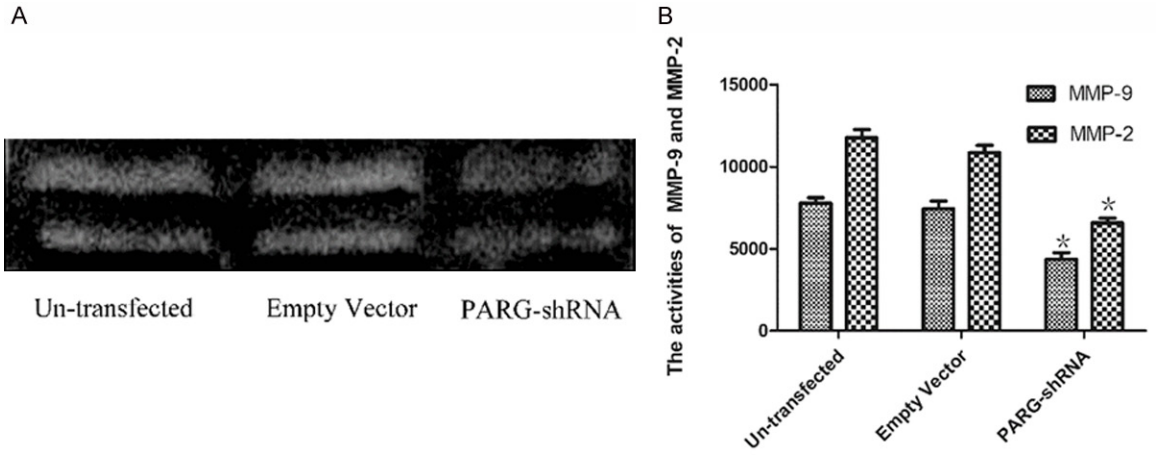

Figure 2. The gelatinolytic activities of MMP-9 and MMP-2. A. A representative Gelatin zymography showing the culture supernatants levels of MMP-9 and MMP-2 in each group. B. Quantitative analysis revealed that the levels of MMP-9 and MMP-2 in PARG-shRNA CT26 cells were decreased compared with other two control groups ($P < 0.05$).

were identified between the Empty Vector transfected and non-transfected groups ($P > 0.05$; **Table 1**; **Figure 4**).

Discussion

Poly-ADP-ribosylation (PARylation) is a reversible and dynamic process. The main catalytic enzymes involved in this progress are PARP1, a member of PARPs, and PARG, which have been described to covalently add and remove ADP-ribose (ADPr) respectively. Although the constitutive level of PARylation in un-stimulated cells is very low, its function is most important, implicated in a wide range of physiological and pathophysiological processes, such as DNA

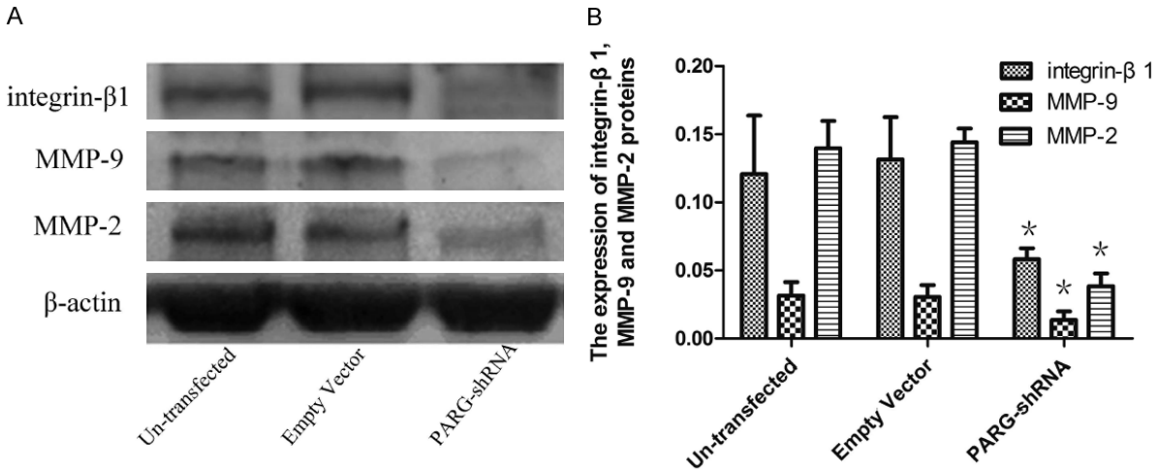

Figure 3. A. Western blot showing the expression levels of integrin-β1, MMP-9 and MMP-2 in CT26 cells. B. Quantitative analysis revealed that the expression levels of integrin-β1, MMP-9 and MMP-2 in PARG-shRNA CT26 cells were decreased compared with other two control groups ($P < 0.05$).

Table 1. Survival time of mice in different groups (days)

Group	Survival time (d)					
Non-transfected	20	21	23	23	23	26
Empty Vector	19	22	22	23	24	24
PARG-shRNA	31	33	35	37	38	38*

*$P < 0.05$, vs Non-transfected group and Empty Vector group.

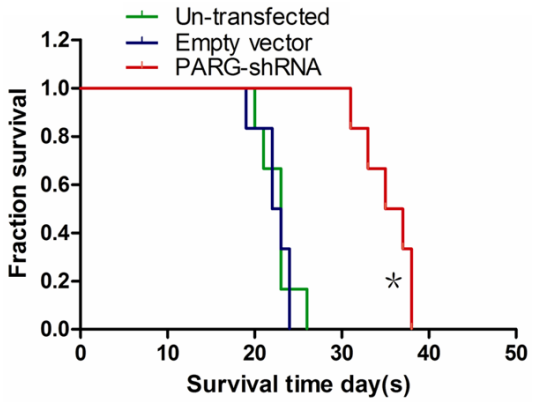

Figure 4. Kaplan-Meier survival curves of BALB/c mice with spleen-transplanted CT26 tumors ($P < 0.05$).

damage repair, transcriptional and cell death regulation [15, 16]. Therefore, the intracellular content of PAR polymer is under strict control, achieved through rapid degradation by PARG. Also, it has been reported that inhibition of PARG can downregulate PARP1 gene expression and its promoter activity, subsequently influencing downstream effectors [17]. There is growing research showing that PARP1 is critical for many tumors [18-20]. However, little is known about the role of PARG in anti-tumor effect.

We are actively engaged in an effort to understand the effect of PARG on colorectal carcinoma. Our previous research has detected that PARG, PARP, and NF-κB were highly expressed in colorectal carcinoma. Colon cancer cells treated with PARG inhibitor or transfected with PARG-shRNA could decrease expressions of PARP, NF-κB and NF-κB-dependent genes, suggesting that PARG may have effects on colon tumor angiogenesis, migration, and invasion [11, 13, 21]. For further verification, in our current study, we have provided the insights into the probable mechanism by which PARG-deficient CT26 cells displayed a decrease in invasion.

Tumor metastasis is a complicated process involving multiple genes and follows many stages and steps [22, 23]. Metastatic tumor cells must detach from the site of the original tumor, and adhere to the extracellular matrix (ECM) mediated by some specific receptors on the surface of cells. When it is suitable for the tumor cells, they will secrete several kinds of protease to breakdown the basement membrane (BM) and degrade the ECM, thus beginning the process of invasion [24]. Many researchers have found that integrins and matrix metalloproteinases (MMPs) play a crucial role during the above process [25-27].

The integrin family contains 24 αβ heterodimeric members that mediate cell attachment to ECM and cell-cell interactions [28, 29]. They can be grouped into 8 subgroups based on β subunits [29]. Integrin-β1 is a large subgroup that can partner with 11 distinct chains [30], widely expresses in multiple cells [29], and binds to a variety of matrix substrates including collagen I and IV, laminins, fibronectin, and vitronectin [30, 31]. In this study, we observed longer survival times of BALB/c Mice with spleen transplanted with PARG-shRNA CT26 tumor. We also found that the cell invasion, adhesion, and integrin-β1 expression in CT26 cells decreased with PARG silencing. The decrease of integrin-β1 expression may be responsible for weakness in attachment of CT26 cells on fibronectin, preventing tumor cells adhering to the extracellular matrix, which is the first step of invasion. In addition, integrin-β1 can positively regulate expression of MMPs and activation of MMPs. In the tumor cells in which we eliminated the integrin-β1 receptor, both the secretion and activation of MMPs were significantly inhibited. The high expression and activities of MMPs can directly destroy and degrade ECM, preparing for the metastasis of cancer cells [32, 33].

MMPs are a family of calcium and zinc-dependent endopeptidases, which degrade almost all ECM components, preparing the path for tumor cells to metastasize [34, 35]. Two of these endopeptidases, MMP-9 and MMP-2, are closely related to the colonic carcinoma, especially a significant increase in MMP-2 [36, 37]. They can degrade the ECM components of type-IV collagen, which plays a critical role in maintaining the integrity of the ECM, enhance the abilities of tumor cells to invade adjacent tissues and migrate to distant organs [35]. In this study, western blot analysis of the expressions of MMP-9 and MMP-2 both in CT26 cells indicates significant decreases of them occurred at PARG-shRNA treatment group compared to controls. In addition, the activities of MMP-9 and MMP-2 in PARG-shRNA treatment group supernatant were weaker than those in controls. Combined with the results of cell invasion, it is speculated that after knocking down PARG, the reduced invasive ability of CT26 cells could be related to the low expressions and activities of MMP-9 and MMP-2.

The expression levels of MMP-9 and MMP-2 are all related to NF-κB activity [38-40]. Moreover, NF-κB signaling directly regulates integrin-β1 expression [41]. In our previous study we have found that inhibiting PARP1 in colon carcinoma could reduce the formation of PARP1-NF-κB complex, and simultaneously reduce NF-κB activity and NF-kB-dependent gene expressions [42]. We have also determined that PARP expression was significantly suppressed in PARG-shRNA cells [21]. Taken together, we speculate that by knocking down PARG, the expression of PARP1 and activity of NF-κB can be down-regulated, and finally decrease invasion and distant metastasis.

In conclusion, we silenced PARG gene expression by short hairpin RNA (shRNA) and then observed the suppressive effects on the capacity of cell adhesion and invasion. Silencing PARG can reduce NF-κB-related gene expression (integrin-β1, MMP-9 and MMP-2) by inhibiting PARP1 expression and NF-κB activity, thus leading to the suppression of adhesive and invasive abilities of CT26 cells, and prolonged survival time in amouse model. According to our study, the strategy of decreasing PARG expression in tumor cells might be useful for therapeutic purposes in CRC patients with distant metastasis.

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Disclosure of conflict of interest

None.

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