

Original Article

Expression of DNA damage checkpoint 53BP1 is correlated with prognosis, cell proliferation and apoptosis in colorectal cancer

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Abstract: 53BP1, an important mediator of DNA damage checkpoint, plays an essential role in maintaining the cell genome stability, and the aberrant expression of 53BP1 was found to contribute to tumor occurrence and development. In this study, we explored the clinical significance of 53BP1 expression in colorectal cancer and investigated the effects of 53BP1 expression on tumor cell proliferation and apoptosis and its possible mechanisms. Immunohistochemical analysis was performed to detect the expression of 53BP1 in 95 cases of tumor tissues. After establishment of shRNA-mediated knockdown stable HCT-116 cell lines, cell proliferation, apoptosis and cell cycle distribution were detected by MTT and flow cytometry, and expression of up-and down-stream related proteins as γ -H2AX, CHK2 and P53 were tested by Western blot. 53BP1 intensity was found to be associated with tumor location ($P < 0.05$), and the low expression of 53BP1 revealed decreased survival time compared with high expression in subgroups as male, tumor size > 5 cm, tumor located at right side, T stage as T3-T4, NO, clinical stage as I-II ($P < 0.05$). In vitro, shRNA-mediated loss of 53BP1 obviously inhibited HCT-116 tumor cell apoptosis, promoted cell proliferation and increased accumulation of cells in S phase. Meanwhile, the expression of γ -H2AX, CHK2 and P53 was significantly reduced ($P < 0.05$). Our findings suggest 53BP1 may serve as a candidate biomarker for predicting prognosis and disease development in colorectal cancer.

Keywords: Colorectal cancer, 53BP1, prognosis, proliferation, apoptosis

Introduction

Colorectal cancer is still a commonly diagnosed malignant tumor around the whole world, and the incidence is increasing year by year in Asia. About 20%-25% patients developed metastasis at first diagnosis and almost 20%~25% patients would occur metastasis during treatment. Moreover, the mortality rate is as high as 40%~45% [1, 2]. Although many new drugs are now being developed to deal with this disease, the survival time for metastasis colorectal cancer is still about 2 years. Consequently, it is meaningful to study the occurrence and development of colorectal cancer at molecular level for prevention and treatment of colorectal cancer.

Maintaining genome integrity is necessary for cell to function properly and meet basic survival.

DNA damage response pathway (DDR) is essential for the repair of DNA damage and maintaining the genome stability of normal cells [3]. It is currently known that the function abnormalities of key factors in DDR pathway are associated with tumor occurrence, development and treatment resistance [4, 5]. 53BP1 (P53 binding protein 1, 53BP1) is a newly discovered tumor suppressor gene, which is an important member of DNA damage response pathway family. It participates in DNA damage repair, maintaining genomic stability and integrity and regulation of apoptosis [6]. 53BP1 was originally defined as binding protein of tumor suppressor gene p53 that involved in p53-mediated apoptosis [7]. Further studies showed that 53BP1 played a critical role in the signal transduction pathway after DNA double-strand breaks. It is an important early reaction medium. 53BP1 protein includes two tudor domains

Table 1. Correlation between 53BP1 protein expression level and clinic pathologic characteristics

Patient characteristics	53BP1		P value
	Low expression (n)	High expression (n)	
Age, years			
≤ 60	15	9	0.139
> 60	32	39	
Gender			
male	26	21	0.471
female	23	25	
Tumor size			
< 5 cm	24	24	0.756
≥ 5 cm	25	22	
Tumor grade			
I-II	33	25	0.194
III	16	21	
T stage			
T1-T2	8	6	0.651
T3-T4	41	40	
N stage			
N0	29	27	0.961
N1+	20	19	
Clinical stage			
I-IIA	26	29	0.324
IIA-IV	23	17	
Tumor location			
left	22	28	0.026*
right	30	15	

N, number of samples in each group; *P < 0.05, statistically significant.

and a C-terminal BRCT domain [8]. It can gather at the site of DSB by tudor domains [9-11], and interacts with other reaction by C-terminal BRCT domain to take part in DNA damage repair [6]. Moreover, 53BP1 promotes DNA end link and maintains the genome stability during chromosome structure remodeling [12, 13]. The loss of 53BP1 can result in failure of chromosome ends to anchor, chromosome aberrations, increase of the incidence of tumor and promotion of tumor growth [6, 14]. Thus, 53BP1 loss has been regarded as an early event during tumor progression [3].

Most previous studies have focused on the role of 53BP1 in DNA damage repair, and only few studies involved in the relationship between the expression of 53BP1 and the development of tumor. It was also revealed that 53BP1 loss

in tumor tissue was related with tumor progression and poor prognosis [15, 16]. However, the relevance between expression of 53BP1 and colorectal cancer progression and prognosis is still unclear until now. Thus, in this present study the expression of 53BP1 was detected by immunohistochemistry in colorectal cancer tissues and the correlation with clinicopathological parameters was evaluated. Moreover, the potential influence of 53BP1 on tumor cell proliferation and apoptosis was investigated *in vitro*.

Materials and methods

Patients' specimens and data collection

The tumor tissue from colorectal cancer patients (n = 95) were collected from the Union Hospital of Tongji Medical College of Huazhong University of Science and Technology (Hubei, China) from July 2006 to May 2007. The study was approved by the Human Ethics Review Board and informed consent was obtained from all of the patients. The diagnosis of specimens was confirmed by immunohistochemistry, and all of the patients were staged according to the 7th AJCC staging system. Clinicopathological information obtained included patient demographics, tumor stage, tumor grade and tumor location et al. Patients were followed up annually until 2013. None of the patients received radiotherapy or chemotherapy before surgery. The patients' data are shown in **Table 1**.

Cell culture and transfection

HCT116 cells were obtained from the laboratory of the General Surgical Department, Union Hospital, Tongji Medical College, Huazhong University of Science & Technology and maintained in RPMI-1640 Medium supplemented with 10% foetal bovine serum. All cells were cultured in a humidified incubator with 5% CO₂ at 37°C. The plasmids were constructed and the cells were transfected as previously described [17]. The sense small hairpin RNA (shRNA) target sequences for 53BP1 were as follows: GCACAAGAACTTATGGAAAGT, the non-specific random shRNA as the negative control and the sequence is GCTTGAGTTCTCACA-GAATTG. The Stable transfected cells were enriched by G418 (400 µg/ml) selection, and the transfection efficiency was confirmed by western blot and quantitative reverse-transcription PCR analysis.

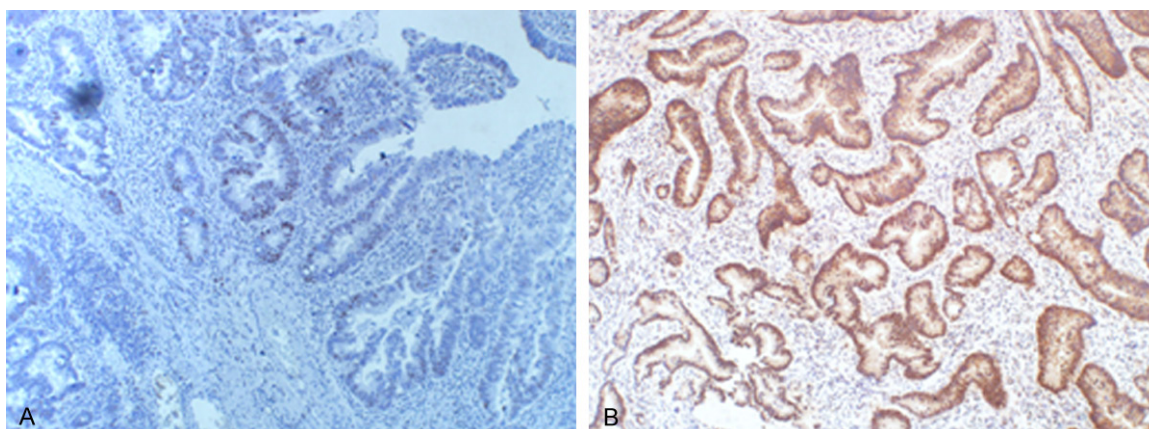


Figure 1. Representative immunohistochemistry staining for 53BP1 expression in colorectal cancer tissue. (A) is low expression of 53BP1 and (B) is expression of 53BP1.

Immunostaining for 53BP1 and evaluation

Paraffin-embedded sections were deparaffinised in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 min. Antigen retrieval was performed by autoclave sterilisation in sodium citrate buffer for 3 min, and slides were then incubated with 10% normal goat serum solution for 20 min. The rabbit polyclonal antibody directed against 53BP1 (IHC-00001, 1:200, Bethyl Laboratories, INC, USA) was added to sections and incubated at 4°C overnight. HRP-conjugated secondary antibody was used according to the manufacturer's instructions. The slides were then incubated with DAB to visualize 53BP1 expression, followed by hematoxylin counterstaining. PBS was used rather than the primary antibody as a negative control. The images were captured using a RGB JVC solid-state camera connected to an Olympus BH2 microscope. Staining results were assessed independently and blindly by two pathologists. Nucleus staining was considered positive.

The score for 53BP1 staining was graded as follows: The percentage of positively stained tumor cells was scored in 4 grades: 1 (0%-25%), 2 (25%-50%), 3 (50%-75%) and 4 (75%-100%). To achieve objectivity, the intensity of positive staining was also used in a four scoring system: 0 (negative staining), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), and 3 (strong staining exhibited as brown). The intensity and percentage of positively stained tumor cells were scored after

counting at least 5 high-power fields at 200×. 53BP1 expression index = (intensity score) (positive score). The value for high and low levels of expression was identified as scores ≥ 8 for high expression and scores < 8 for low expression of 53BP1.

Cell viability assays

HCT-116 (2×10^5 cells/ml) was cultured in 96-well plates with 10% FBS RPMI-1640. Microculture tetrazolium assay (MTT) was performed to measure cell proliferation activity by adding 3-(4,5-diethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (5 mg/ml) (Sigma, St Louis, MO, USA) at 24, 48, 72 and 96 hours, respectively.

Assessment of cell apoptosis

Twenty-four hours after seeding, the trypsinised cells were re-suspended in $1 \times$ binding buffer at a concentration of 2×10^5 cells/ml, and annexin V-FITC and propidium iodide (PI) (Sigma, St Louis, MO, USA) were then added. The cells were incubated for an additional 15 minutes at room temperature in the dark and then subjected to analysis with flow cytometry (BD Biosciences, San Jose, CA, USA). A minimum of ten thousand cells in each sample were analysed using the Cell Quest software (BD Biosciences).

Cell cycle analysis

As previous study described [18], the cells were washed and centrifugated after harvested 24 hours, the cell pellets were fixed in 70% cold

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Table 2. Univariate and multivariate Cox regression analysis of potential prognostic factors for colorectal cancer patients

	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age	1.300	0.633-2.541	0.503			
Gender	0.863	0.507-1.557	0.679			
Tumor size	2.001	1.095-3.657	0.024*	1.766	0.937-3.325	0.078
Tumor grade	2.155	1.189-3.905	0.011*	1.074	0.552-2.093	0.883
N stage	3.621	1.964-6.292	0.000**	0.075	0.006-0.720	0.033*
Clinical stage	4.308	2.124-6.890	0.000**	49.250	4.419-584.558	0.002**
Tumor location	2.220	1.314-3.750	0.003**	2.107	1.176-3.776	0.012*
53BP1 expression	0.496	0.288-0.912	0.023*	0.619	0.325-1.181	0.145

HR, hazard ratio; CI, confidence interval; *P < 0.05, **P < 0.01 statistically significant.

ethanol and stained with a DNA staining solution (1 mg/ml of propidium iodide and 5 mg/ml of RNase A) for 30 minutes. The stained cells were then suspended and immediately subjected to analysis with a flow cytometer and the Cell Quest software (BD Biosciences) was used to analyse the cell cycle distribution.

Quantitative reverse-transcription PCR analysis (qRT-PCR)

Total RNA was extracted with TRIZOL reagents (Thermo, USA). RNA was reverse transcribed to cDNA by Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo, K1622). qRT-PCR was performed with Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems. All the experiments were repeated at least three times.

Western blot analyses

As previous study described [18], cells were lysed with RAPA for 30 minutes and centrifuged with 12000 g for 15 minutes, the supernatant liquid was collected. Then, the samples were mixed with SDS loading buffer and heated for 5 minutes at 100°C, 10% SDS-PAGE and PVDF membrane was used to separate the protein. the membranes was incubated with primary antibody against 53BP1 (Abcam, ab175933, 1:2000), CHK2 (Abgent, AP4999a, 1:1000), p-P53 (CST, 9286, 1:1000), γ-H2AX (Epitomics, EPR895, 1:5000) or β-actin antibody (Santa Cruz, 1:1000) overnight at 4°C and rinsed with TBST three times, followed by incubation with secondary antibodies labelled with horseradish peroxidase (HRP). The bands were then visualised using enhanced chemiluminescence (ECL). Band intensities were analyzed by

enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA).

Statistical analyses

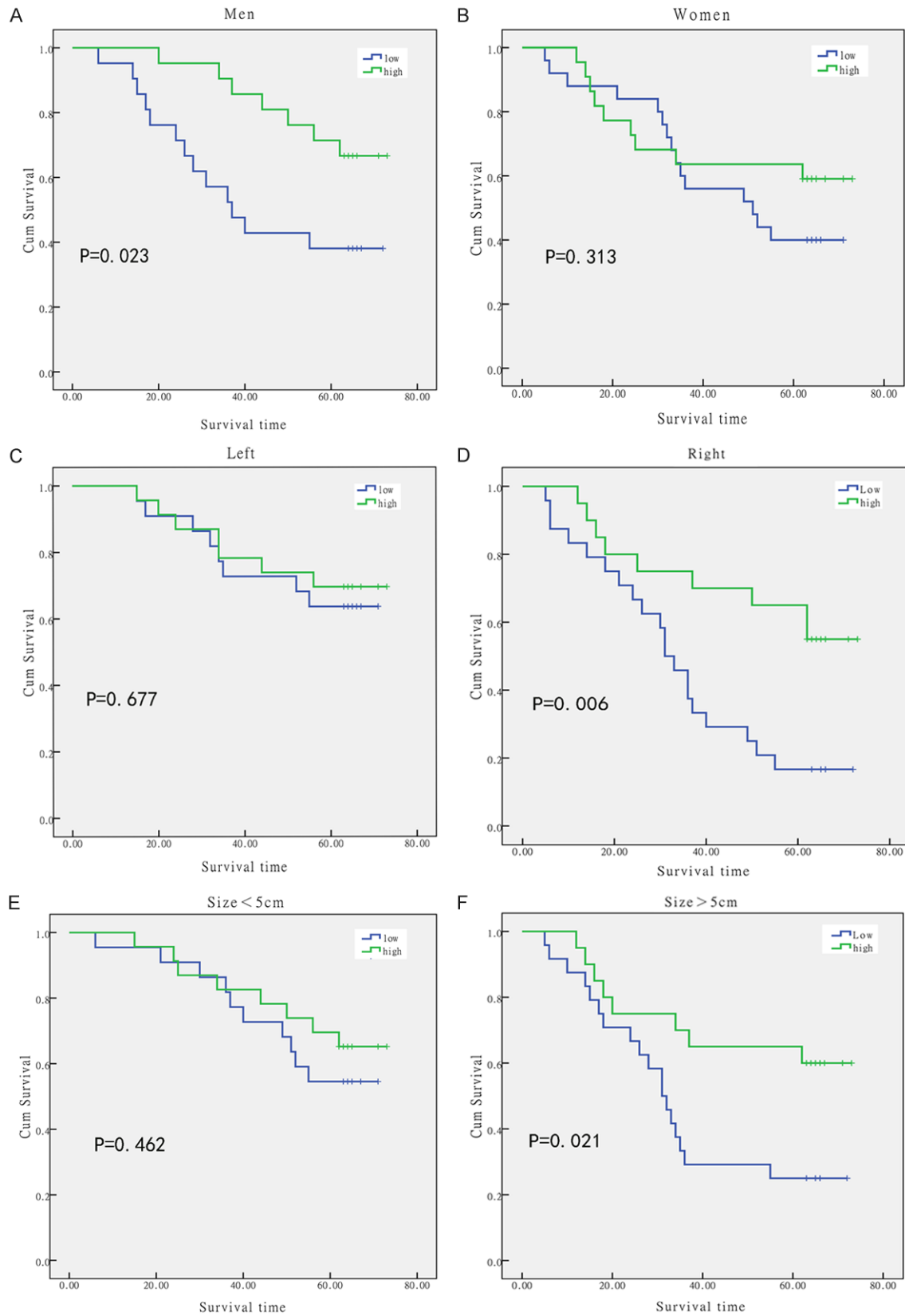
All statistics data were analyzed by SPSS16.0 software. Chi-square test and Fisher's exact tests were employed to analysis the correlation between 53BP1 expression and clinical pathological characteristics. The primary endpoint was defined as the time from diagnosis to the date of all-cause death (overall survival, OS). Relative risks (RR) of death, and relationship with 53BP1 expression and other variables were evaluated by unvaried and multivariate Cox proportional hazards model. For vitro study, the results were shown as mean ± SD and significance between two groups was determined by T test all experiments. A P < 0.05 was considered statistically significant.

Results

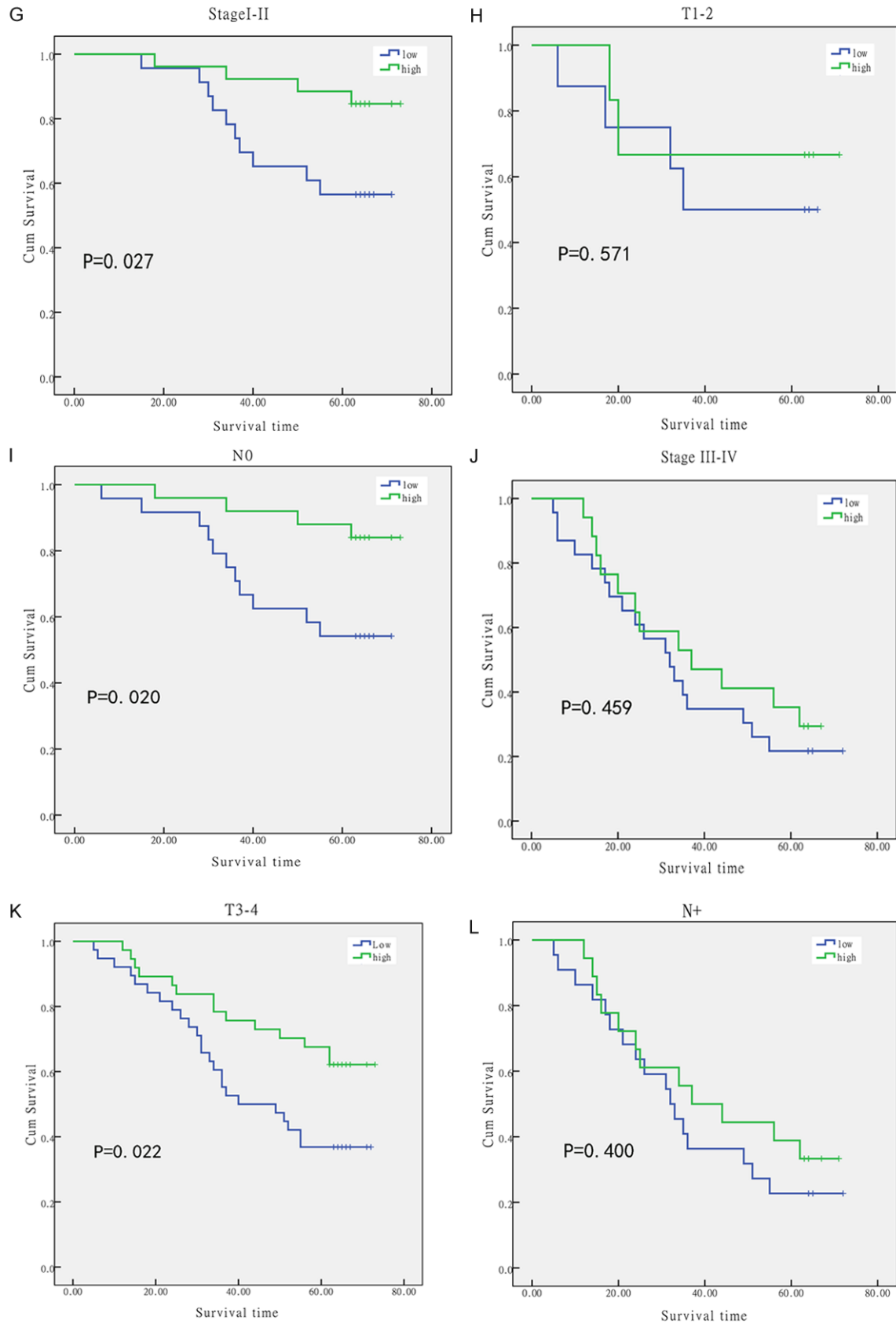
Correlation between 53BP1 expression and pathologic characteristics of colorectal cancer

We examined 53BP1 expression in tumor tissues from 95 patients with colorectal cancer by immunohistochemistry, and the representative staining was presented in **Figure 1**. Baseline characteristics of 95 patients with colorectal cancer included age, gender, clinical stage, histological grade, and tumor size and tumor location. The correlation between 53BP1 protein expression and clinic pathological factors are shown in **Table 1** and **Figure 1**. 53BP1 intensity was found to be associated with tumor location (P < 0.05), but not associated with patients' age, gender, tumor stage, tumor grade, tumor size and clinical stage (P > 0.05).

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Figure 2. Association of 53BP1 protein levels and overall survival time. A-F. The difference between survival and 53BP1 expression were observed according to gender, tumor location and tumor size. No difference was found in female, tumor located at left side or tumor size < 5 cm ($P > 0.05$), but in male, tumor located at right side or tumor size > 5 cm low expression of 53BP1 leads to a decrease in survival time ($P < 0.05$). G-L. The association between survival and 53BP1 expression were investigated according to clinical stage. Patients with low 53BP1 expression revealed poor survival time in I-IIA stage, T3-T4, N0 ($P < 0.05$). No difference was found in clinical stage as IIB-IV, T1-T2, N+ ($P > 0.05$).

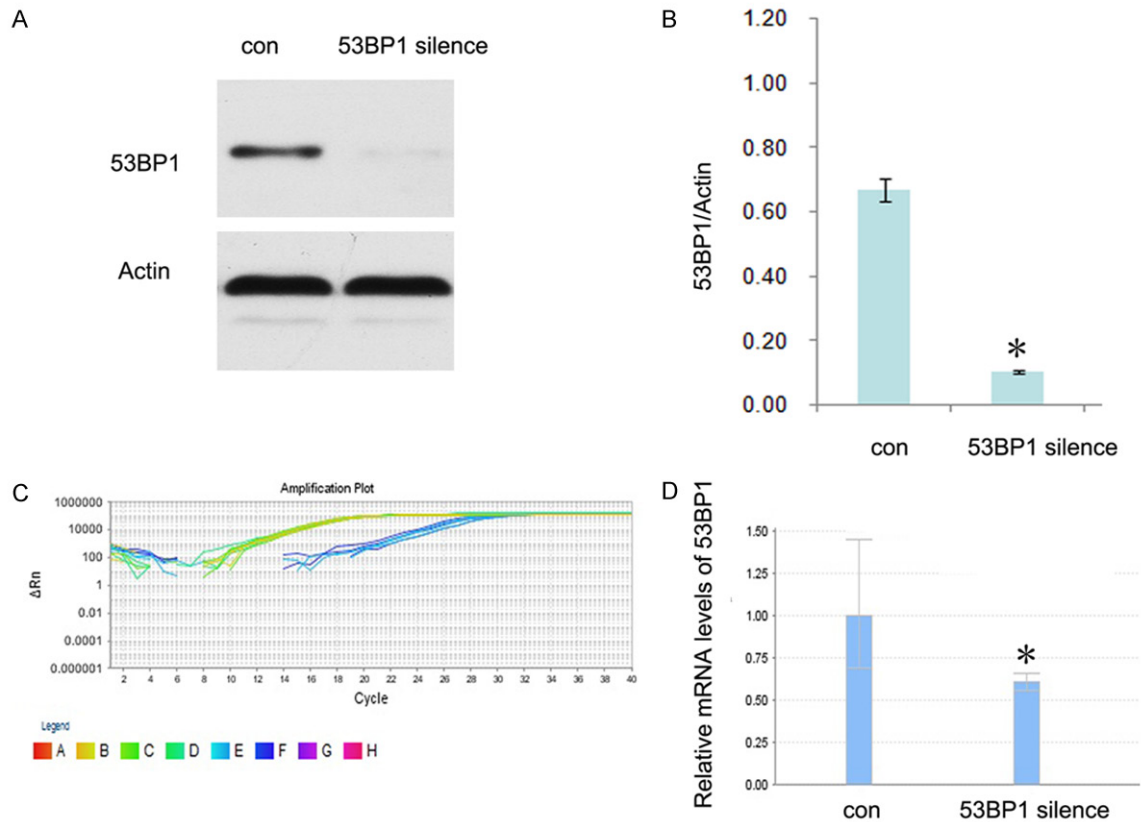


Figure 3. Transfection efficiency was measured by western blot analysis and RT-PCR. A, B. The transfection efficiency was measured by western blot analysis, results are shown from three independent experiments, Columns, means; Error bars, SEM, * $P < 0.05$. C, D. Quantitative detection of 53BP1 by RT-PCR, results are shown from three independent experiments, Columns, means; Error bars, SEM, * $P < 0.05$.

Kaplan-Meier survival analysis, univariate analysis and multivariate analysis

We next performed Kaplan-Meier survival analysis to test the influence of different 53BP1 expression on OS in patient subgroups. It was found that the low expression of 53BP1 revealed decreased survival time compared with high expression in subgroups as male, tumor size > 5 cm, tumor located at right side, T stage as T3-T4, N0, clinical stage as I-II ($P < 0.05$). In addition, some factors such as histological grade, clinical stage, N stage, tumor location and 53BP1 intensity were found to be

associated with OS through univariate analysis, ($P < 0.05$). However, only the factors of clinical stage and tumor location were found to be associated with OS in multivariable analysis. Moreover, an interaction variable for tumor size, tumor location and 53BP1 were analyzed by multivariable model, and we observed low expression of 53BP1 revealed poor survival compared with high expression of 53BP1 when tumor size > 5 cm ($P = 0.022$, HR = 2.033, 95% CI = 1.109-3.728), and tumor located at right ($P = 0.002$, HR = 0.374, 95% CI = 0.200-0.701). Results are summarized in **Table 2**; **Figure 2**. Our results suggest 53BP1 loss maybe the

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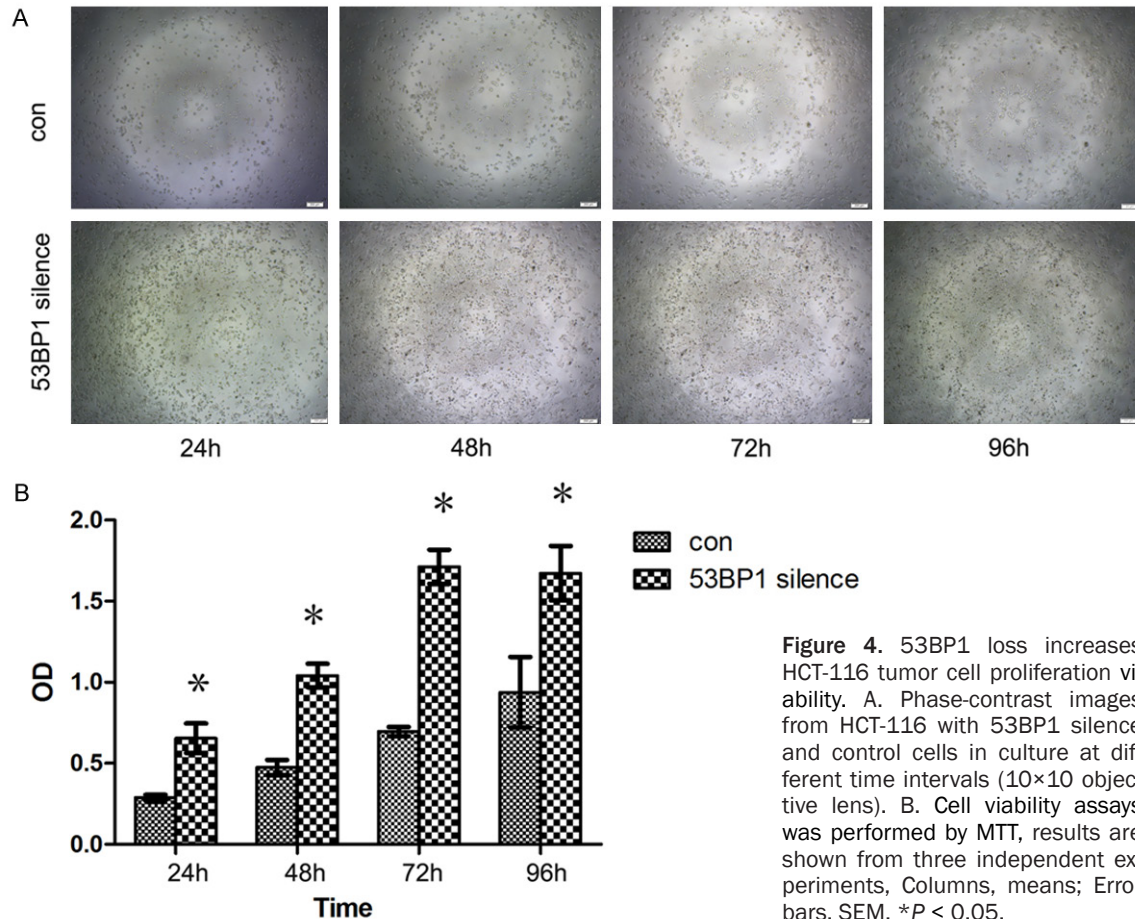


Figure 4. 53BP1 loss increases HCT-116 tumor cell proliferation viability. A. Phase-contrast images from HCT-116 with 53BP1 silence and control cells in culture at different time intervals (10×10 objective lens). B. Cell viability assays was performed by MTT, results are shown from three independent experiments, Columns, means; Error bars, SEM, * $P < 0.05$.

inner reason for some poor survival factors as tumor size, tumor location.

Silencing of 53BP1 by plasmids-mediated RNA interference in HCTH-116 cells

To evaluate the role of 53BP1 on the proliferation, apoptosis and cell cycle distribution in colorectal cancer cells, we established stable HCT-116 cell lines with shRNA-mediated knock-down of 53BP1. The transfection efficiency was confirmed by western blot and quantitative reverse-transcription PCR analysis (**Figure 3**). Our result showed the expression of 53BP1 protein in HCT-116 was downregulated after cell transfection.

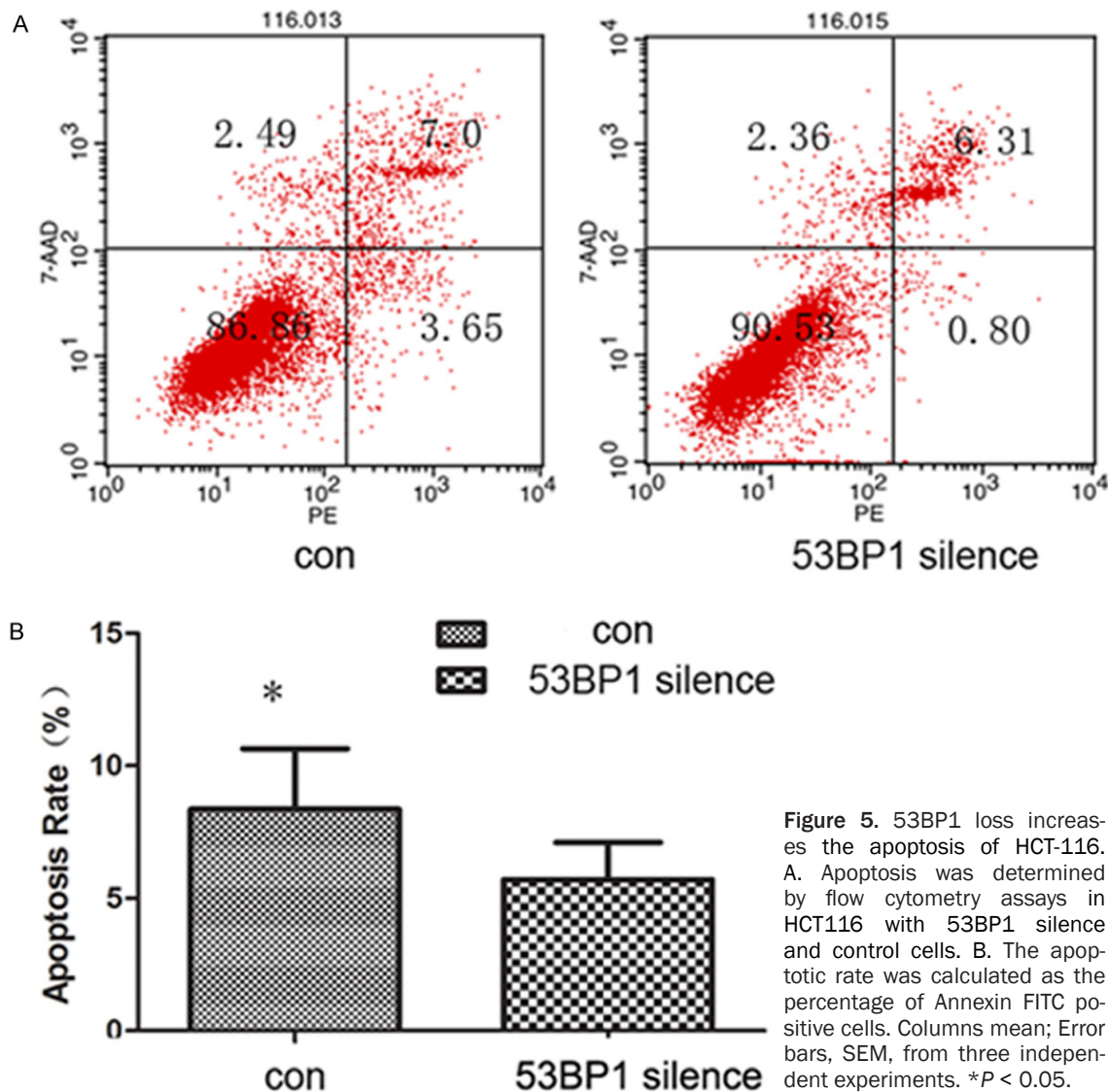
53BP1 silencing increases tumor cells proliferation and decreases apoptosis

The influence of 53BP1 silencing on cell viability was evaluated by the MTT assay. HCT-116 cell lines with 53BP1 silencing exhibited significantly increased growth rates compared with

that of control group at 24, 48, 72 and 96 hs ($P < 0.0001$) (**Figure 4A, 4B**). The influence of 53BP1 silencing on cell apoptosis was further detected via flow cytometry. The results were shown in **Figure 5A, 5B**. The apoptosis rates in 53BP1 knockdown cells and the control cells were $6.41\% \pm 1.41\%$ and $9.51\% \pm 2.29\%$ respectively ($P < 0.01$). Taken together, these findings suggest the 53BP1 silencing obviously promote cell proliferation and diminish tumor cell apoptosis.

53BP1 loss induces the accumulation of S phase

The influence of 53BP1 silencing on the HCT116 cell cycle was detected via flow cytometry. As shown in **Figure 6A, 6B**, cells with 53BP1 silencing showed obviously increase of accumulation of cells in the S phase compared with that control group ($26.87\% \pm 12.91\%$ versus $11.92\% \pm 4.88\%$, $P < 0.05$). Meanwhile, the accumulation of cells in the G0/G1 phase was also decreased in the 53 GP1 knockdown



group compared with control group, although there was no difference between the two.

53BP1 loss decreases up and down-stream proteins expression

53BP1 protein consists of two Tudor structural domains and a C-end BRCT domain. DNA double-strand breaks can induce the strong phosphorylation of 53BP1, and in turn phosphorylate histone H2AX (gamma-H2AX, γ -H2AX) and other proteins to join in the damage repair by Tudor domain. Meanwhile, 53BP1 can also regulate the cell cycle G1/S, S and G2/M checkpoints and phosphorylate P53 to induce apoptosis by activating cell cycle checkpoint kinase 2 (checkpoint kinase-2, CHK2) through the C-terminal end [6]. To explore the mechanism

of 53BP1 loss in inducing tumor cell proliferation, we detected related protein expression in up and down-stream by western blot analysis, and found the expressions of γ -H2AX, CHK2 and P53 in 53BP1 loss HCT-116 cells were obviously decreased compared with control cells. The results were shown in **Figure 7A, 7B**.

Discussion

53BP1 was first thought as an activator of tumor-suppressor gene p53 to induce apoptosis [19], and it has recently been found that 53BP1 also played a critical role in maintaining cell genomic stability and preventing tumor development through participating in the DNA damage repair [14, 20]. As early as 2005, Ward et al. found that 53BP1^{-/-} mice exhibited nearly

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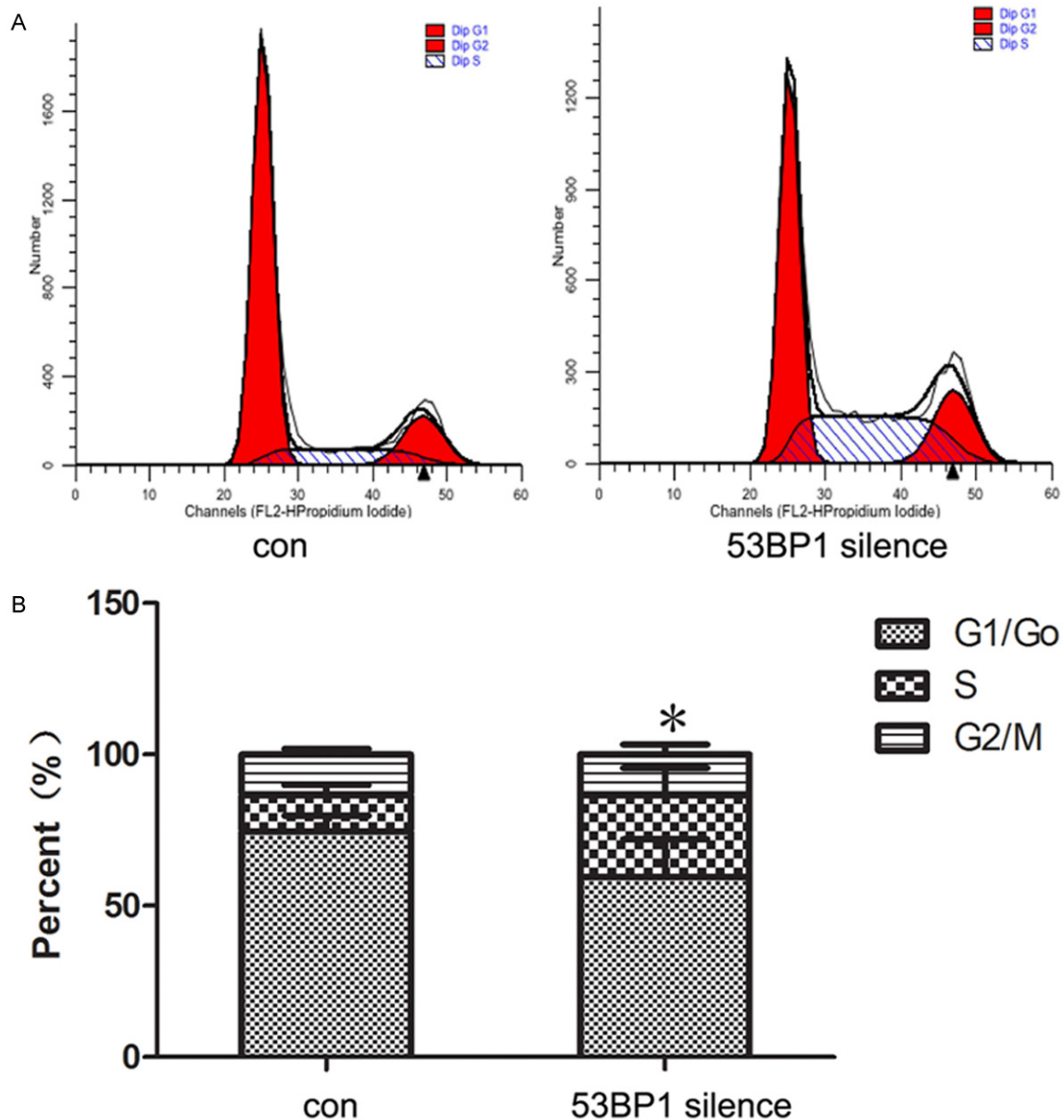


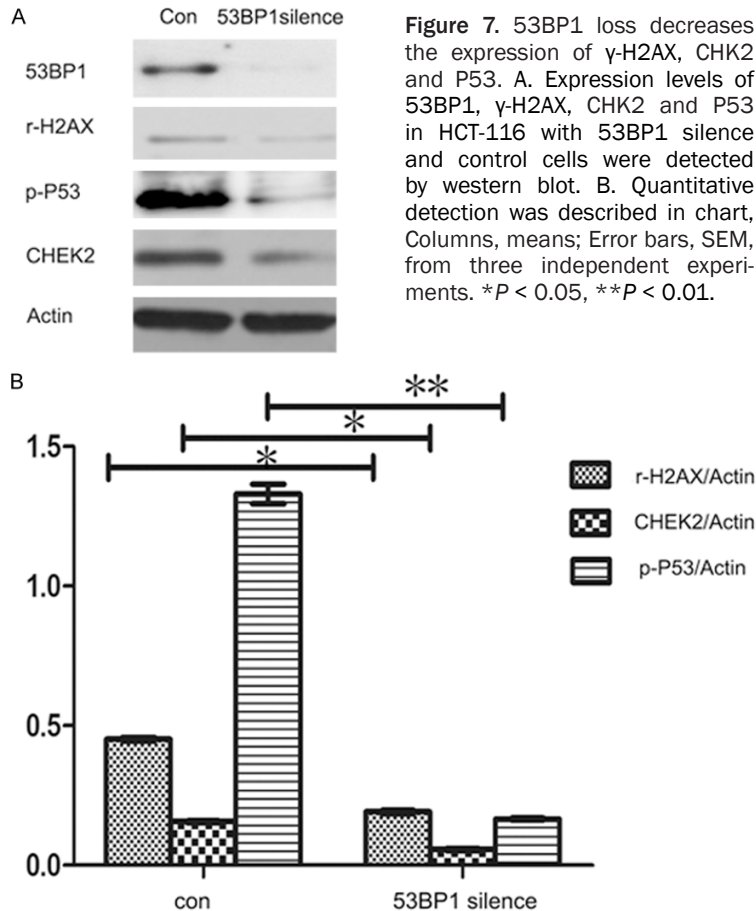
Figure 6. 53BP1 loss induces the accumulation of S phase. Cell cycle distribution was determined by flow cytometry assays in HCT116 cells before and after 53BP1 silence. A. Cells arrested in S phase. B. Histogram plots of cell cycle distribution, Columns, mean; Error bars, SEM, from three independent experiments. * $P < 0.05$.

2-fold tumor incidence and decreased survival time compared with 53BP1^{+/+} mice [6]. Morales et al further showed that knockdown of 53BP1 could induce chromosomal deficiency as clonal deletion, swap and polyploidy formation [21]. These results suggest the loss of 53BP1 results in genomic instability/DNA damage, and involved in tumor development. Subsequently, a number of clinical studies have found that there exists different levels of 53BP1 loss in tumor tissue, and it has been correlated with

tumor progression, poor response and prognosis in many different cancers [15, 17, 22, 23].

However, until now it is uncertain whether the loss of 53BP1 contributes to the disease development and poor prognosis in colorectal cancer. Thus, in present study, 53BP1 expression was evaluated in tumor tissues from 95 colorectal cancer specimens, and clinical pathological and survival information were analyzed and compared. Our study revealed there was

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no revelation between 53BP1 expression and patients' age, gender, tumor grade, tumor size and stage. Interestingly, it was exhibited that low expression of 53BP1 was closely associated with tumor location (right side). Further in survival analysis we observed the low expression of 53BP1 revealed decreased survival time compared with high expression in subgroups as male, tumor size > 5 cm, tumor located at right side, T stage as T3-T4, N0, clinical stage as early stage I-II. However, if the tumor located at left side, tumor size was < 5 cm, clinical stage was advanced stage as III-IV; there seemed no influence of loss of 53BP1 on survival. This result was consistent with Bouwman's study in breast cancer, which showed that 53BP1 loss exhibited worse survival in early stage triple-negative breast cancer without metastasis [22]. Consequently, it is indicated that 53BP1 loss might be one of earlier adverse events associated with tumor progression [3].

Moreover, we investigated the potential influence of 53BP1 on tumor cell proliferation and

apoptosis through in vitro study. After silencing 53BP1 expression in HCT 116 cancer cell lines, we found 53BP1 loss could significantly promote tumor cell proliferation and inhibit tumor apoptosis. These results are consistent with the previous observation that downregulation of 53BP1 can obviously reduce the doubling time of breast cancer cell and enhance breast cancer cell proliferation [17, 24].

However, the potential mechanism that 53BP1 loss induces tumor proliferation is unclear until now. According to the characteristic of 53BP1 protein, it consists of two Tudor structural domains and a C-end BRCT domain and participates in DSB repair through the former domain. DNA double-strand breaks can induce the strong phosphorylation of 53BP1, and in turn phosphorylate histone H2AX and other proteins to

join in the damage repair [25, 26]. Through the latter C-terminal end, 53BP1 can regulate the cell cycle G1/S, S and G2/M checkpoints and phosphorylate P53 to induce apoptosis by activating CHK2 [6]. Thus, We investigated cell cycle distribution by flow cytometry and detected the related protein expressions in up and down-stream by western blot analysis, and found 53BP1 loss obviously increased the accumulation of S phase in cell cycle, reduced the expressions of γ -H2AX that represents the extend of cell damage, CHK2 and P53. Thus, our findings suggest 53BP1 can influence the tumor proliferation, apoptosis and cell cycle distribution through affecting the expressions of up and down-stream protein as γ -H2AX, CHK2 and P53.

In conclusion, our results showed that 53BP1 loss was associated with poor survival in colorectal cancer through clinical data analysis. Furthermore, we found 53BP1 loss could inhibit colorectal cancer cell apoptosis and

induce proliferation. The underlying mechanism could be aberrant expression of 53BP1 interferes related protein expressions, such as γ -H2AX, CHK2 and P53, to change cell cycle distribution and induce tumor cell into proliferation phase. This maybe the reason 53BP1 loss leads to poor survival in colorectal cancer. Although its molecular mechanism remains to be further clarified, our findings suggests 53BP1 represents a candidate biomarker for predicting prognosis and disease development in colorectal cancer.

Disclosure of conflict of interest

None.

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