

# Role of FBXL20 in human colorectal adenocarcinoma

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**Abstract.** In the present study, in order to determine whether the *FBXL20* gene plays a role in the carcinogenesis of human colorectal adenocarcinoma, the mRNA expression levels of *FBXL20* were examined in 30 pairs of human colorectal adenocarcinoma tissues and corresponding adjacent normal colorectal tissues by quantitative real-time PCR (qRT-PCR). The pGPU/GFP/Neo-FBXL20 siRNA expression vector was transfected into the human colon carcinoma cell lines, SW480 and SW620. The mRNA levels of  $\beta$ -catenin, *c-Myc*, *cyclin D1*, *p53*, *PP2A*, *SET* and *E-cadherin* were determined by qRT-PCR, whereas the protein levels of  $\beta$ -catenin, *c-Myc*, caspase-3 and SET were examined by western blot analysis. MTT assay and flow cytometry were employed to assess cell proliferation, apoptosis and the cell cycle. As shown by qRT-PCR, *FBXL20* mRNA expression was upregulated in 76.7% of the tumor samples. Cell proliferation was inhibited by 33.3% in the SW480<sup>-FBXL20</sup> cells and by 22.7% in the SW620<sup>-FBXL20</sup> cells, compared to the corresponding control cells. Furthermore, the percentages of apoptotic cells were 21.3 and 17.1% in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells, whereas the percentages of G1 phase cells in the two cell lines were 90.13 and 78.13%, respectively. In addition, the mRNA expression of *SET* and *E-cadherin* was upregulated in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells, whereas that of  $\beta$ -catenin, *c-Myc*, *cyclin D1*, *p53* and *PP2A* was downregulated. Consistently, the protein expression of  $\beta$ -catenin and *c-Myc* was downregulated, whereas that of SET and caspase-3 was upregulated. Based on these results, we can conclude that FBXL20 plays an essential role in the carcinogenesis of human colorectal adenocarcinoma. Moreover, our data suggest that FBXL20 promotes carcinogenesis through the regulation of the Wnt signaling pathway and caspase activation.

## Introduction

Colorectal adenocarcinoma is one of the most common malignancies, of which the prevalence has been increasing recently. With the second highest incidence among all cancers, it is also the second most common cause of cancer-related mortality worldwide (1,2). The carcinogenesis of colorectal adenocarcinoma is a multi-step process that involves a number of genomic alterations. Understanding the molecular mechanism of this process may aid in cancer prevention, early diagnosis and effective treatment.

Using a subtractive cDNA library and cDNA microarray analysis, a total of 86 expressed sequence tags were previously identified from human colorectal adenocarcinoma tissues (3). In the present study, we mainly focused on the *FBXL20* gene (GenBank accession no., NM032875.2), which was identified using the BLASTn program of NCBI from one differentially expressed sequence tag (GenBank accession no., ES274070). This gene contains 10,381 base pairs, with a 1308-bp open reading frame. It is located on the human chromosome 17q21.2, predicted to encode a 436-amino acid protein containing an F-box motif, which is the key feature of F-box proteins (FBPs) (4,5). FBPs are defined by the presence of a 40-amino acid domain that can interact with other proteins. It was originally identified in cyclin F, therefore named F-box. Studies on different species have shown that FBPs play a crucial role in the ubiquitin-mediated degradation of cellular regulatory proteins (6). However, the function of FBXL20 remains unknown.

In order to determine whether FBXL20 is involved in the carcinogenesis of colorectal adenocarcinoma, the mRNA expression of *FBXL20* in human colorectal adenocarcinoma and normal colorectal tissues was determined by real-time PCR (RT-PCR). Two new cell lines, SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup>, in which the *FBXL20* gene was permanently silenced, were generated to investigate the role of FBXL20 in this pathogenic process.

## Materials and methods

**Collection of tissue samples.** This study was approved by the Medical Research Ethics Committee of Sichuan University, Chengdu, China. Written informed consent was obtained from the patients. Colorectal adenocarcinoma tissues and adjacent normal colorectal tissues were obtained from 30 patients at the West China Hospital of Sichuan University. Normal

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colorectal tissues were collected from the area at least 5 cm away from the edge of the tumor tissues. Pathological examination further confirmed the absence of cancer cells in the collected normal tissues, which were snap-frozen and stored in liquid nitrogen.

**Quantitative RT-PCR (qRT-PCR).** According to the manufacturer's protocol, total RNA from tumor and matched normal tissues was isolated using the TRIzol RNA isolation reagent (Invitrogen Life Technologies, USA). Total RNA was reverse-transcribed using the M-MuLV Reverse Transcriptase kit (Fermentas, Burlington, Canada). RT-PCR was performed using SYBR Premix ExTaq (Takara, Otsu, Japan), according to the manufacturer's instructions and related international standards (7). *GAPDH* was used as the endogenous control. PCR primers specific for *FBXL20* and *GAPDH* are shown in Table I.

**Cell culture.** The human colon adenocarcinoma cell lines, SW480 and SW620 (ATCC, USA), were cultured in DMEM (10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin) at 37°C with 5% CO<sub>2</sub>.

**Construction of the pGPU6/GFP/Neo-FBXL20 siRNA expression vector.** The synthesized 59-mer oligonucleotide containing the sequence specifically silencing the *FBXL20* gene was inserted into the pGPU6/GFP/Neo siRNA expression vector. The sense sequence of this oligonucleotide was as follows: 5'-CACCGCCAAATGCTTAGCCAATCTTTC AAGAGAAGATTGGCTAAGCATTTGGTTTTTTG-3'; and the antisense sequence of the oligonucleotide was as follows: 5'-GATCCAAAAAACCAAATGCTTAGCCAATCTTCTC TTGAAAGATTGGCTAAGCATTTGGC-3'. The two annealed complementary oligonucleotides were subsequently inserted into the *Bam*HI/*Bbs*I site of the pGPU6/GFP/Neo siRNA expression vector. The engineered pGPU6/GFP/Neo-FBXL20 siRNA expression vector was verified by restriction digestion and then sequenced by the Beijing Genomic Institute (Beijing, China). The pGPU6/GFP/Neo negative control siRNA vector was provided by GenePharma Company (Shanghai, China).

**Establishing the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines.** According to the Lipofectamine 2000 (Invitrogen Life Technologies) manufacturer's instructions, the SW480 and SW620 cells were transfected with 1 µg of the pGPU6/GFP/Neo-FBXL20 siRNA expression vector. After 48 h, the transfected SW480 and SW620 cells were treated with 800 µg/ml G418 (Sigma, St. Louis, MO, USA). After 14 days, monoclonal cells were cultured in the presence of 400 µg/ml G418. The new cell lines generated from the corresponding monoclonal cells were named SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup>. The SW480<sup>-negative control</sup> (SW480<sup>-NC</sup>) and SW620<sup>-negative control</sup> (SW620<sup>-NC</sup>) cell lines were generated by the transfection of the pGPU6/GFP/Neo negative control siRNA vector into the SW480 and SW620 cell lines. SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells were regarded as the experimental group, SW480<sup>-NC</sup> and SW620<sup>-NC</sup> cells were regarded as the negative group, and SW480 and SW620 cells were regarded as the blank group in the following experiments.

**MTT assay.** MTT assay was performed to examine cell proliferation. The experiment was repeated three times. Cells in the logarithmic phase were cultured in 96-well culture plates. Three groups were set up as follows: the blank, the negative and the experimental group. Each group contained six replicates. The MTT reagent (20 µl) (Sigma) was added at four time-points (one, two, three and four days). After the cells were cultured for an additional 4 h, the medium was removed, and 150 µl of DMSO were added into each well. The plate was read at the absorbance wavelength of 490 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

**Flow cytometry.** Cell apoptosis was measured by flow cytometry (FACS Aria II cell sorter; BD Biosciences, San Jose, CA, USA). The experiment was repeated three times. Cells in the logarithmic phase were collected and cultured in six-well culture plates. Three groups (each in triplicate) were set up as follows: the blank, the negative and the experimental group. Briefly, cells were collected 48 h post-plating, after being washed twice with PBS. The cells were suspended in 400 µl of binding buffer (BestBio, Shanghai, China), after which 5 µl of Annexin V-FITC were added followed by gentle agitation. The mixture was incubated for 10 min at room temperature. After incubation, 10 µl of PI were added into the mixture, followed by 10 min of incubation on ice. Cell apoptosis was then measured by flow cytometry. For the cell cycle assay, the cells were collected 60 h post-plating and washed twice using ice-cold PBS, after which the cells were fixed in 75% ice-cold ethanol at -20°C for 48 h. Finally, the cell cycle assay was performed using the Cell Cycle Detection kit (BestBio).

**RT-PCR for the detection of the mRNA expression of  $\beta$ -catenin, SET, E-cadherin, c-Myc, cyclin D1, p53 and PP2A.** Real-time PCR was performed as stated. Quantitative real-time PCR primers of these genes are shown in Table I.

**Western blot analysis for the detection of the protein expression of  $\beta$ -catenin, SET, c-Myc and caspase-3.** The cells were lysed in ice-cold RIPA buffer (Beyotime, China). The total protein concentration was determined using an Enhanced BCA Protein assay kit (Beyotime). The equivalent amount of protein was then separated by 12% SDS-PAGE and was transferred onto PVDF membranes (Bio-Rad). After blocking in Tris-buffered saline (TBS) containing 5% non-fat milk, the membranes were incubated with primary antibodies (at 4°C for 12 h, followed by incubation with horseradish peroxidase (HRP) conjugated anti-rabbit antibody (all from Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) at room temperature for 1 h. Signals were detected on a gel imaging system using the ECL western blotting substrate (Thermo Scientific, USA). The expression of  $\beta$ -actin was used as the loading control.

**Statistical analysis.** The results presented in the current study are the means  $\pm$  standard error of the mean (SEM). Statistical analysis was performed using the Student's t-test, the Fisher's exact probability test and ANOVA with SPSS17.0 software.  $P < 0.05$  was considered to indicate a statistically significant difference.

Table I. Quantitative real-time PCR.

Name	Sequences	Product location	Product length (bp)
<i>FBXL20</i>	Forward: 5'-gtcttcgtggatgtcttgag-3' Reverse: 3'-gtgcctgagttggaacagaac-5'	544-564 695-674	152
<i>GAPDH</i>	Forward: 5'- ggaaggtgaaggtcggagt-3' Reverse: 3'-ctggggaagtaactggagt-5'	107-125 223-205	117
<i>β-catenin</i>	Forward: 5'-gctggtgacagggaagacatc-3' Reverse: 3'-ggtagtccatagtgaggcgaac-5'	1628-1648 1743-1721	116
<i>E-cadherin</i>	Forward: 5'- ttgctactggaacaggacact-3 Reverse: 3'-ggagatgtattggagggaaggtc-5'	1845-1866 1998-1976	154
<i>c-Myc</i>	Forward: 5'-tcaagaggcgaacacacac-3' Reverse: 3'- ggccttttcattgtttcca-5'	1631-1550 1740-1721	110
<i>cyclin D1</i>	Forward: 5'-gtggcctctaagatgaaggaga-3' Reverse: 3'-ggaagtgtcaatgaaatcgtg-5'	534-555 702-681	169
<i>PP2A</i>	Forward: 5' -agttggccaaatgtgtctcc -3' Reverse: 3'-gagttgcggtacaaggaagg-5'	1129-1148 1273-1254	145
<i>SET</i>	Forward: 5'-gctcaactccaaccacgac-3' Reverse: 3'- attactgttcggtcactcct-5'	389-407 508-488	120
<i>p53</i>	Forward: 5'-tagtgtggtggtgcctatgag-3' Reverser: 3'-agtgtgatgatggtgaggatgg-5'	524-545 652-631	129

Table II. FBXL20 expression and patient characteristics.

Expression	Gender		Age		Dukes' Stage				Differentiation degree		
	Male	Female	≥50	<50	A	B	C	D	High	Middle	Low
Upregulation	13	10	13	10	2	8	10	3	3	19	1
Downregulation	5	2	4	3	0	6	1	0	0	6	1

## Results

**mRNA expression level of FBXL20 in human colorectal adenocarcinoma tissues.** The mRNA expression level of *FBXL20* was upregulated in 76.7% of the tumor samples (23 out of 30 samples), which was statistically significant ( $P=0.000$ , by the one-sample t-test). Compared to the adjacent normal colorectal tissues, 14 samples exhibited a fold-change of  $>2$ , ranging from 2.445 to 77.35 (Fig. 1). Of note, the *FBXL20* expression in older patients ( $>50$  years old) was significantly higher than that in younger patients ( $<50$  years old) ( $P=0.047$ ). No significant correlation was found between *FBXL20* mRNA expression and gender ( $P=0.815$ ), Dukes' stage ( $P=0.284$ ) or differentiation degree ( $P=0.643$ ) by the ANOVA test (Table II).

**mRNA expression levels of FBXL20 in SW480-<sup>FBXL20</sup> and SW620-<sup>FBXL20</sup> cell lines.** The mRNA expression level of *FBXL20* in the SW480-<sup>FBXL20</sup> cell line was significantly lower than that in the SW480 cell line. The reduction rate was 77.19%, which was statistically significant as shown by the ANOVA test ( $P=0.002$ ). The *FBXL20* expression level in the SW620-<sup>FBXL20</sup>

cell line was significantly lower than that in the SW620 cell line. The reduction rate was 68.12%, which was statistically significant as shown by the ANOVA test ( $P=0.004$ ) (Fig. 2).

**Cell proliferation.** The cell proliferation of the SW480-<sup>FBXL20</sup> and SW620-<sup>FBXL20</sup> cells was significantly inhibited, compared to that of the blank and the negative group, measured by MTT assay. The inhibition rate was 33.3% in the SW480-<sup>FBXL20</sup> cell line and 22.7% in the SW620-<sup>FBXL20</sup> cell line (Fig. 3). The inhibition was statistically significant as shown by the ANOVA test.

**Cell apoptosis and cell cycle.** After the cells were plated for 48 h, the apoptotic rates of the SW480-<sup>FBXL20</sup>, SW480-<sup>NC</sup> and SW480 cells were 21.3, 13.3 and 12.7%, respectively, measured by flow cytometry. The apoptotic rate of the SW480-<sup>FBXL20</sup> cells was significantly higher than that of the SW480 cells ( $P=0.003$ ) (Fig. 4A). The apoptotic rates of the SW620-<sup>FBXL20</sup>, SW620-<sup>NC</sup> and SW620 cells were 17.1, 10.32 and 9.9%, respectively. The difference between the SW620-<sup>FBXL20</sup> and SW620 cells was statistically significant ( $P=0.036$ ) (Fig. 4B).

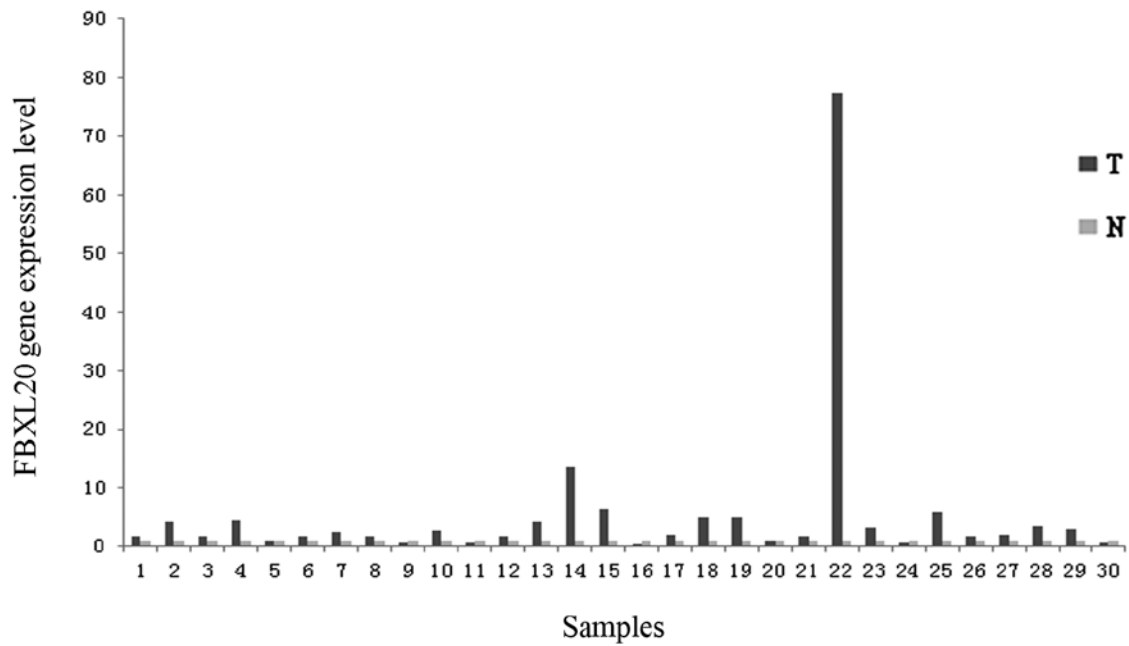


Figure 1. *FBXL20* is overexpressed in human colorectal cancer. qRT-PCR analysis of *FBXL20* expression in human colorectal adenocarcinoma samples (T) and matched normal samples (N). *GAPDH* was employed as the loading control.

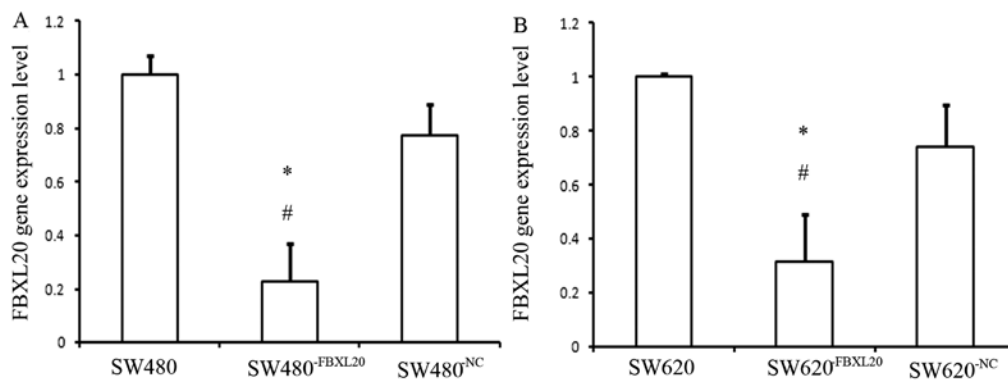


Figure 2. *FBXL20* is downregulated in SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells. (A) The mRNA expression of *FBXL20* was detected by qRT-PCR in the SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup> and SW480 cells. (B) The mRNA expression of *FBXL20* was detected by qRT-PCR in the SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620 cells. Statistical differences compared to the blank group are indicated as \**P*<0.05. Those compared to the negative group are indicated as #*P*<0.05. *GAPDH* was employed as the loading control.

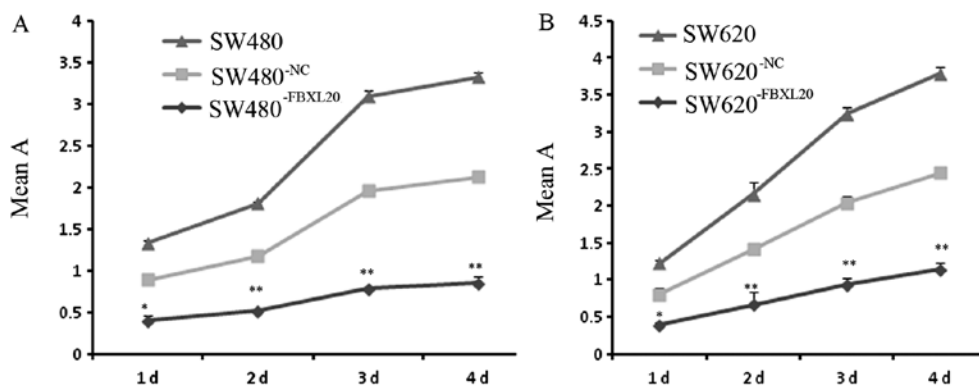


Figure 3. Growth inhibition of human colorectal cell lines by silencing *FBXL20* mRNA expression. (A) Cell proliferation curves of the human colorectal cells, SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup> and SW480. (B) Cell proliferation curves of the human colorectal cells, SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620. Human colorectal cell lines were incubated for the indicated time-periods (one, two, three and four days). As a measurement of cell viability, the relative absorbances (means  $\pm$  SD, *n*=6), obtained from MTT assay, are shown. Statistical differences, compared to the blank group, are indicated as \**P*<0.05 and \*\**P*<0.01.

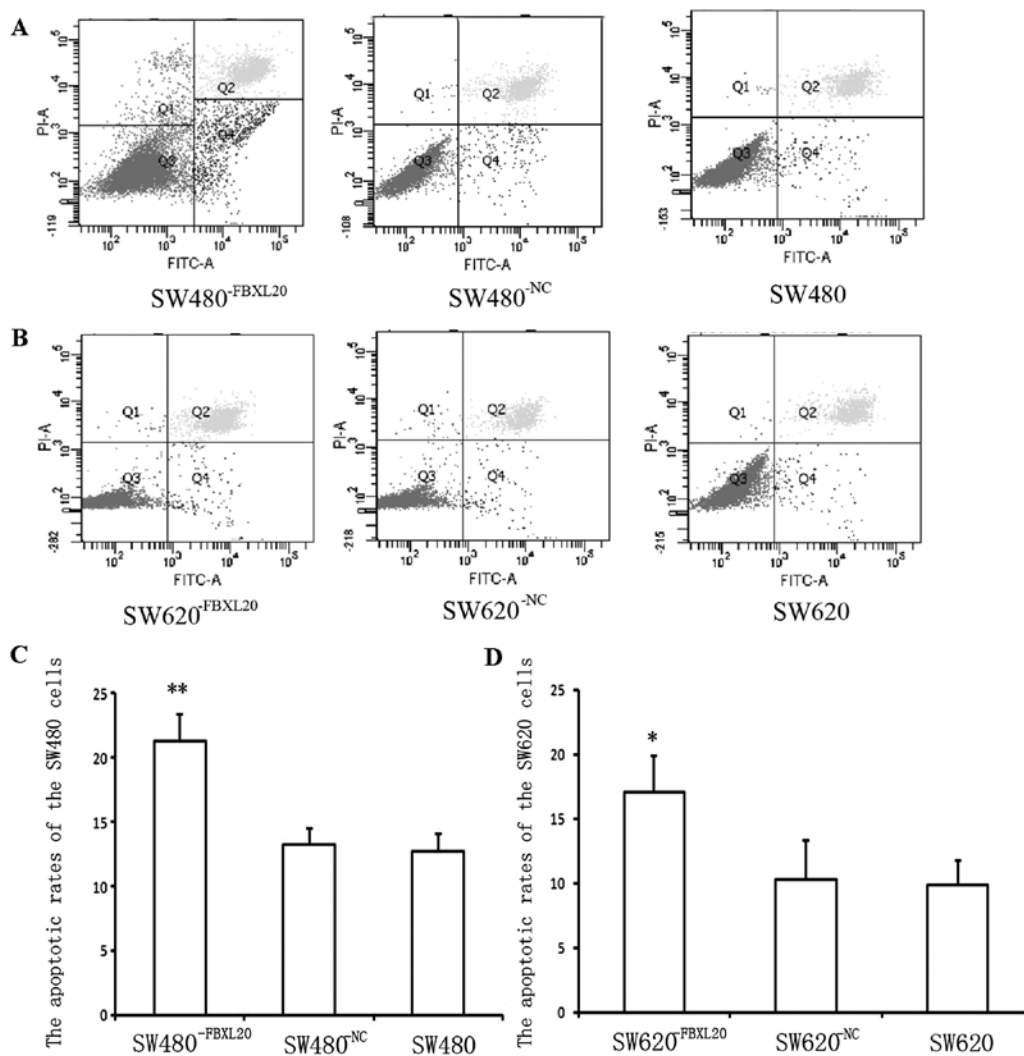


Figure 4. Cell apoptosis analysis of human colorectal cells by flow cytometry. Cells were harvested and stained with FITC and PI for cell apoptosis analysis. (A) The percentages of apoptotic SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup> and SW480 cells in one representative sample of each cell line. (B) The percentages of apoptotic SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620 cells in one representative sample of each cell line. (C) Average apoptotic rate of SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup> and SW480 cells. (D) Average apoptotic rate of SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620 cells. (C and D) Data are presented as the means  $\pm$  SD of triplicate cultures. Statistical differences compared to the blank group are indicated as \* $P < 0.05$  and \*\* $P < 0.01$ .

Cell cycle analysis showed that the percentages of the SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup> and SW480 cells in the G1/G0 phase were 90.13, 76.81 and 71.97%, respectively. The difference between the SW480<sup>-FBXL20</sup> and SW480 cells was statistically significant ( $P = 0.000$ ) (Fig. 5A). The percentages of the SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620 cells in the G1/G0 phase were 78.13, 60.22 and 58.33%, respectively. The difference between the SW620<sup>-FBXL20</sup> and SW620 cells was statistically significant ( $P = 0.001$ ) (Fig. 5B).

**mRNA expression levels of p53,  $\beta$ -catenin, PP2A, cyclin D1, c-Myc, E-cadherin and SET in SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines.** The mRNA expression levels of p53,  $\beta$ -catenin, PP2A, cyclin D1 and c-Myc were downregulated in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines, compared to those in the corresponding control cells. The respective reduction rates were 57.96, 40.13, 82.73, 84.25 and 84.54% for the SW480<sup>-FBXL20</sup> cells, which were all statistically significant as shown by the ANOVA test ( $P = 0.001$ ,  $P = 0.042$ ,  $P = 0.004$ ,  $P = 0.000$  and  $P = 0.000$ ). The respective reduction rates were

32.95, 25.06, 86.42, 60.95 and 74.57% for the SW620<sup>-FBXL20</sup> cells, which were all statistically significant as well, as shown by the ANOVA test ( $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$  and  $P = 0.000$ ). The expression of *E-cadherin* and *SET* was also upregulated in the engineered cell lines. The increase rates were 37.5 and 24.8%, respectively, for the SW480<sup>-FBXL20</sup> cells ( $P = 0.027$  and  $P = 0.009$ ), whereas for the SW620<sup>-FBXL20</sup> cells, the increase rates were 57.64 and 39.47%, respectively ( $P = 0.001$  and  $P = 0.000$ ) (Fig. 6).

**Protein expression levels of  $\beta$ -catenin, c-Myc, SET and caspase-3 in SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines.** The protein expression of  $\beta$ -catenin and c-Myc was downregulated in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines, compared to that in the SW480 and SW620 cell lines. The inhibition rates were 45.14 and 40.08% in the SW480<sup>-FBXL20</sup> cells ( $P = 0.029$  and  $P = 0.003$ ), whereas in the SW620<sup>-FBXL20</sup> cells, the numbers were 80.25 and 62.06 %, accordingly ( $P = 0.000$  and  $P = 0.000$ ).

The protein expression of caspase-3 and SET was upregulated in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines,

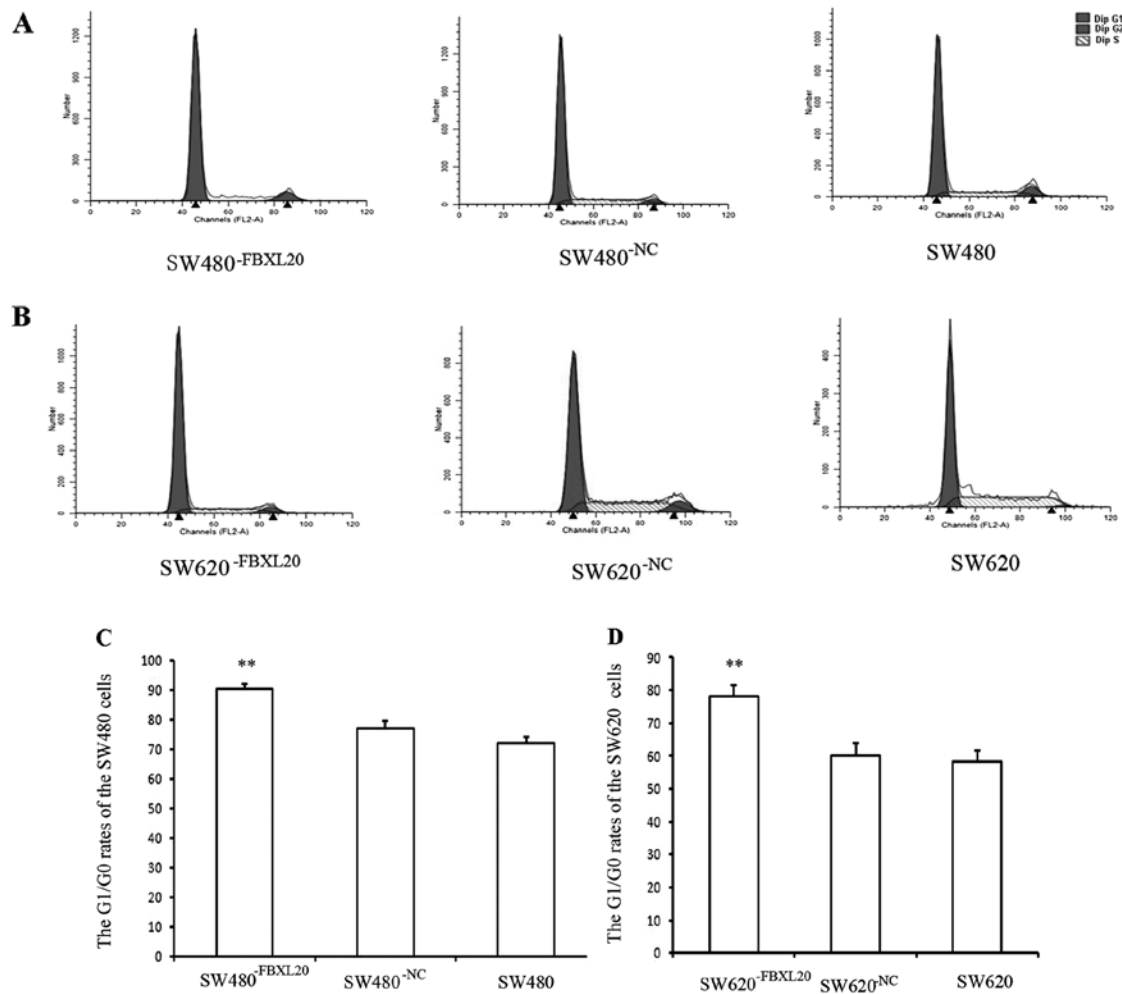


Figure 5. Cell cycle analysis of human colorectal cells by flow cytometry. (A) DNA frequency distribution of SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup> and SW480 cells in one representative sample of each cell line. (B) DNA frequency distribution of SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620 cells in one representative sample of each cell line. (C) Average percentages of the SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup> and SW480 cells in G1/G0 phase. (D) Average percentages of SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620 cells in G1/G0 phase. (C and D) Data are presented as the means  $\pm$  SD of triplicate cultures. Statistical differences compared to the blank group are indicated as \*P<0.05 and \*\*P<0.01.

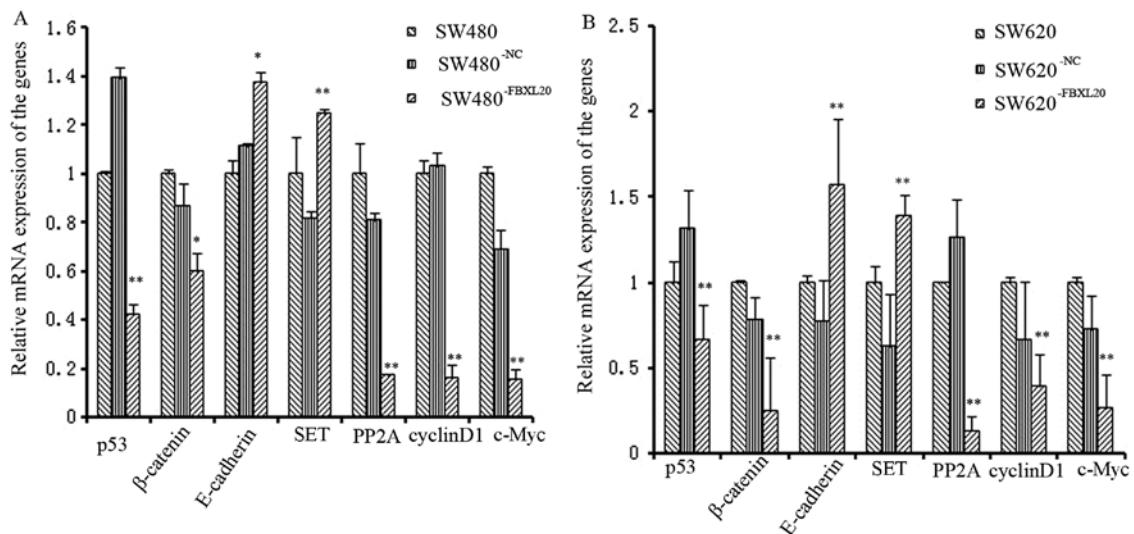


Figure 6. Downregulation of FBXL20 leads to the expression changes of the indicated genes in colorectal cells. (A) qRT-PCR analysis showed that the relative mRNA expression levels of  $\beta$ -catenin, c-Myc, cyclin D1, P53 and PP2A were downregulated, whereas those of E-cadherin and SET were upregulated in the SW480<sup>-FBXL20</sup> cells. (B) qRT-PCR analysis showed that the relative mRNA expression levels of  $\beta$ -catenin, c-Myc, cyclin D1, P53 and PP2A were downregulated, whereas those of E-cadherin and SET were upregulated in the SW620<sup>-FBXL20</sup> cells. Statistical differences compared to the blank group are indicated as \*P<0.05 and \*\*P<0.01. GAPDH was employed as the loading control. Data are presented as the means  $\pm$  SD of triplicate cultures.



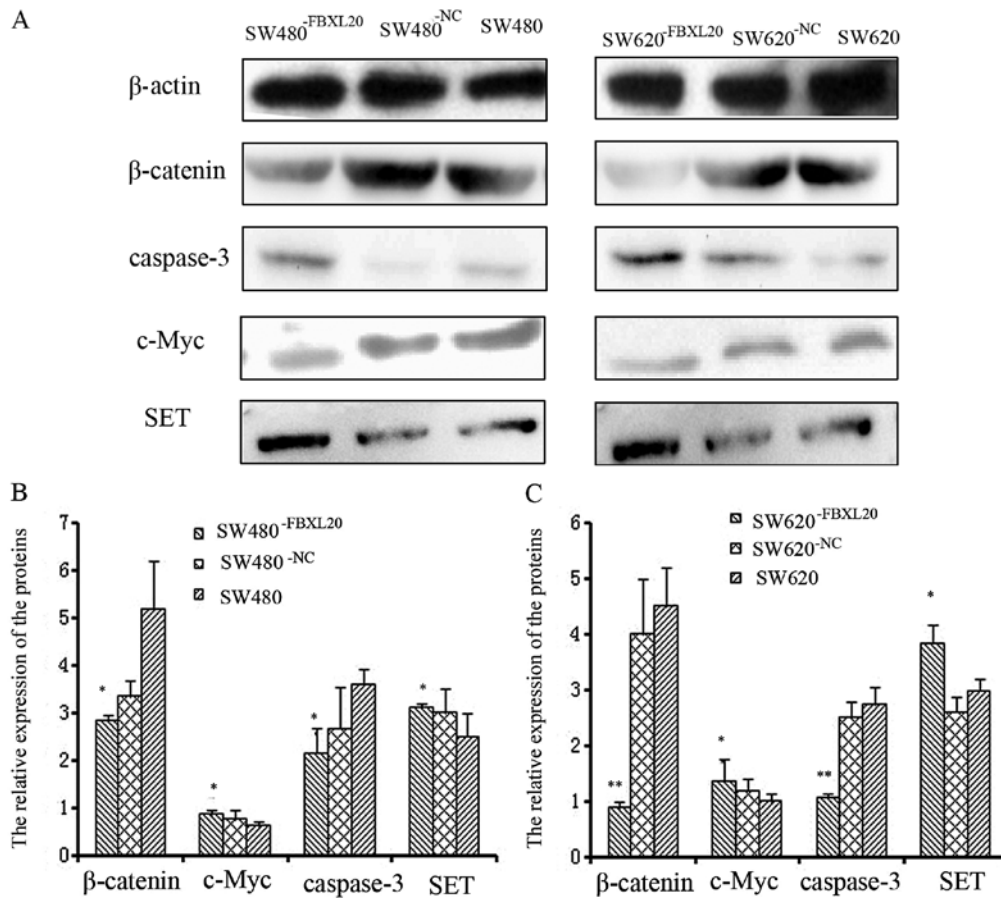


Figure 7. Downregulation of FBXL20 leads to expression changes in the indicated protein levels in colorectal cells. (A) Western blot analysis showing the protein expression levels of  $\beta$ -catenin, c-Myc, caspase-3 and SET in the SW480<sup>-FBXL20</sup>, SW480<sup>-NC</sup>, SW480, SW620<sup>-FBXL20</sup>, SW620<sup>-NC</sup> and SW620 cells. (B) Western blot quantification.  $\beta$ -catenin and c-Myc were downregulated, whereas caspase-3 and SET were upregulated in SW480<sup>-FBXL20</sup>. (C) Western blot quantification.  $\beta$ -catenin and c-Myc were downregulated, whereas caspase-3 and SET were upregulated in SW620<sup>-FBXL20</sup> cells. Statistical differences compared to the control group are indicated as \* $P < 0.05$  and \*\* $P < 0.01$ .  $\beta$ -actin was employed as the loading control. Data are presented as the means  $\pm$  SD of triplicate cultures.

compared to that in the SW480 and SW620 cell lines. The increase rates were 37.55 and 24.83% in the SW480<sup>-FBXL20</sup> cells ( $P = 0.019$  and  $P = 0.012$ ), whereas in the SW620<sup>-FBXL20</sup> cells, the numbers were 35.78 and 28.39%, accordingly ( $P = 0.025$  and  $P = 0.036$ ) (Fig. 7).

## Discussion

Among the 100 human FBPs identified so far,  $\beta$ -Trcp, Skp2 and Fbw7 are well characterized components with matched downstream substrates (8-10). A number of studies have reported that high levels of FBPs correlate with poor prognosis in several human malignancies, such as lung, renal, esophageal and colon cancer (11,12). Our results showed that the mRNA expression of *FBXL20* was significantly upregulated in human colorectal adenocarcinoma tissues, compared to that in the adjacent normal colorectal tissues, and its expression level closely correlated with the age of the patients. The upregulation rate in older patients ( $>50$  years old) was higher than that in younger ones ( $<50$  years old). It has been reported that sporadic colorectal carcinoma frequently appears in older individuals ( $>50$  years old), suggesting the possible role of *FBXL20* as a pathological factor in sporadic colorectal carcinoma. Of note, we did not observe any significant correlation between the *FBXL20* level and other patient characteristics,

such as gender, Dukes' stage and tumor differentiation; however, in one female patient, the *FBXL20* level in the tumor tissues was 77.35-fold higher than that in the adjacent normal colorectal tissues. Therefore, it is possible that *FBXL20* may be related to colorectal adenocarcinoma praecox. Therefore, we can conclude that *FBXL20* overexpression correlates with colorectal carcinoma progression, and that it may play a vital role in the pathogenesis of colorectal cancer.

In the present study, we showed that cell proliferation was significantly inhibited, whereas cell apoptosis was considerably increased in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells, compared to that in the corresponding control cells. In addition, a number of studies have reported that the migration and proliferation capacities of malignant cells were diminished when the FBP genes, such as RNF5, gp78 and Skp2, were silenced (13,14,15). Furthermore, von der Lehr *et al* (16) demonstrated that Skp2 activates the c-Myc gene by inducing the degradation of related proteins through the ubiquitin-proteasome pathway. It has also been reported that Skp2 can also induce p27 protein degradation through the ubiquitin-proteasome pathway (17). Based on our observation of inhibited proliferation and increased apoptosis in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells, it is likely that *FBXL20* has a similar function to other FBP members in regulating cellular activities.

The FBP family members function through different signaling pathways. Shirane *et al* (10) found that  $\beta$ -TrCP can specifically degrade I $\kappa$ B, leading to the activation of the NF- $\kappa$ B signaling pathway. By contrast, a number of studies have reported that  $\beta$ -TrCP can specifically degrade  $\beta$ -catenin, therefore inhibiting the signal transduction of the Wnt pathway (18-20). In general, the function of FBPs has not been fully elaborated. The function of the *FBXL20* gene has not yet been elucidated. Numerous studies have confirmed that the development of colorectal cancer closely correlates with the activation of the Wnt signaling pathway (21). It has been demonstrated that the level of c-Myc, a downstream molecule in the Wnt signaling pathway, correlates with the proliferation capacity of neoplastic cells (22). In the present study, we also observed that c-Myc expression was downregulated in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines, compared to that in the control cells. Of note, the proliferation capacity of these cells were also reduced, as shown by MTT assay. These findings raised the possibility that the reduced proliferation capacity of SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells was caused by the downregulation of c-Myc expression. It is known that cyclin D1 is involved in cell cycle regulation, and it has been reported that the cyclin D1-CDK complex promotes cell cycle progression into the S phase (23). Our flow cytometry results showed that the percentages of the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells in the G1/G0 phase were higher than those of the control cells. Of note, cyclin D1 expression was downregulated in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells. Therefore, we conclude that the G1/G0 arrest in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines correlates with the downregulation of cyclin D1 expression.

$\beta$ -catenin is a key player in the signal transduction of the Wnt pathway. Together with the Tcf/lef transcription activator,  $\beta$ -catenin can activate the downstream targets, c-Myc and cyclin D1, in the nucleus (24). Our results showed that  $\beta$ -catenin expression was significantly decreased in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines, suggesting that the downregulation of c-Myc and cyclin D1 in these cells may be due to the decreased  $\beta$ -catenin expression.  $\beta$ -catenin also plays a role in E-cadherin-mediated cell adhesion in epithelial cells (25). Tian *et al* (26) reported that the aberrant expression of the E-cadherin/ $\beta$ -catenin complex is associated with a wide variety of human malignancies and fibrotic disorders. In our study, E-cadherin expression was significantly upregulated in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines, suggesting that the suppression of the Wnt signaling pathway in these cells may have resulted from E-cadherin upregulation, which then led to the downregulation of c-Myc and cyclin D1 expression through the inhibition of the nuclear translocation of  $\beta$ -catenin.

Our previous study showed that the *SET* gene is overexpressed in human colorectal adenocarcinoma, and that the inhibition of SET expression effectively suppresses cell proliferation and promotes cell apoptosis (27). SET can inhibit the tumor suppressor, PP2A (27,28). It has also been found that SET can increase the activity of the splitting enzyme in CYP17 and prompt the production of  $\gamma$ -interferon in natural killer cells by inhibiting PP2A expression. PP2A plays a crucial role in the cell cycle, DNA replication, signal transduction, cell differentiation and malignant transformation (28). It has been reported that  $\beta$ -catenin can be degraded by the Axin-APC-CK1-GSK3 $\beta$  complex. Moreover, Kim *et al* (25) found that

PP2A can dephosphorylate Axin, which in turn leads to the destabilization and degradation of Axin. In the present study, we observed that SET expression was upregulated, whereas PP2A expression was downregulated. Therefore, one possible explanation could be that the low level of  $\beta$ -catenin was caused by the accumulation of the Axin-APC-CK1-GSK3 $\beta$  complex, resulting from the decreased PP2A level.

FBXL20 expression was inhibited by 77.19% in the SW480<sup>-FBXL20</sup> and by 68.12% in the SW620<sup>-FBXL20</sup> cells, compared to that in the corresponding control cells. We hypothesized that the increased E-cadherin and SET expression may be caused by the silencing of the *FBXL20* gene, and that FBXL20 may play a role in the degradation of E-cadherin and SET. Low FBXL20 expression leads to the accumulation of E-cadherin, which in turn downregulates  $\beta$ -catenin. Low FBXL20 expression may also increase the expression of SET, which leads to the downregulation of PP2A. The downregulation of PP2A inhibits the degradation of Axin, leading to the downregulation of  $\beta$ -catenin and therefore a decreased  $\beta$ -catenin level in the nucleus. The decreased nuclear  $\beta$ -catenin level in cancer cells leads to the downregulation of c-Myc and cyclin D1, which reduces the proliferation capacity of the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells. Therefore, FBXL20 may be involved in multiple signaling pathways.

p53 is a tumor suppressor, of which many mutations have been found in more than 50% of malignancies (24). A number of studies have shown that p53 not only inhibits cell growth but also induces apoptosis. By contrast, we observed by flow cytometry that cell apoptosis was significantly increased, whereas the p53 expression level was significantly decreased in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines, indicating that this increased cell apoptosis was not induced by p53, directly. However, we did find that the protein level of activated caspase-3 was increased in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cell lines, suggesting that FBXL20 may activate caspase-3 directly or indirectly, which in turn induces apoptosis in the SW480<sup>-FBXL20</sup> and SW620<sup>-FBXL20</sup> cells. However, the underlying mechanism warrants further investigation.

In conclusion, our study shows that the *FBXL20* gene is overexpressed in human colorectal adenocarcinoma. Moreover, the inhibition of FBXL20 expression can effectively suppress cell proliferation and promote apoptosis in colorectal carcinoma cells, possibly by inducing the degradation of SET and E-cadherin through caspase activation. In conclusion, FBXL20 expression correlates with the pathogenesis of colorectal cancer. However, further studies are required to validate this correlation and to elucidate the underlying mechanism.

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