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# Correlation Study of Sfrp2 And Β-Catenin With Colorectal Carcinogenesis

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***Abstract***
***Aim:*** *To explore the correlation of sFRP2 and β-catenin with colorectal carcinogenesis.*
***Methods:*** *Immunohistochemical staining was used to detect the expression of sFRP2 and β-catenin in 54 cases of colorectal cancer and 32 cases of normal colorectal mucosa, to detect the expression level and positive rate of sFRP2 and β-catenin. The expression of sFRP2 gene in colorectal cancer cell line HCT116 was up-regulated by plasmid transfection, it was verified by Western blot.Then we conduct CCK-8 method, wound-healing assay and Transwell assay.*
***Results:*** *The results of immunohistochemistry showed that the positive rate of sFRP2 expression in normal colorectal mucosa is higher than colorectal cancer group(P<0.05); However, The rate of β-catenin membrane expression deficiency and ectopic expression in colorectal cancer group is higher than normal colorectal mucosa(P<0.05). This study also found that the expression of sFRP2,and abnormal expression of β-catenin were significantly correlated with tumor stage and tissue differentiation(P<0.05), however, it was not associated with sex, age, tumor location, tumor size and metastasis(P>0.05).The expression of sFRP2 was negatively correlated with β-catenin membrane expression deficiency (r=-0.298, P=0.029) and ectopic expression(r=-0.308，P=0.023); There was a positive correlation between the β-catenin membrane expression deficiency and the ectopic expression (r=0.402, P=0.003). After plasmid transfection, the results are as follows: 1.the expression of sFRP2 significantly increased; 2.the proliferation rate of sFRP2 transfection group was significantly slower compared with the control group and the empty plasmid group; 3.sFRP2 significantly inhibited the migration ability of cells; 4.the control group through the membrane (195.39±8.68), sFRP2 transfection group (75.69±7.19), sFRP2 transfection group of cell membrane permeability were significantly less than the control group(P=0.00).*
***Conclusion:*** *The interaction between sFRP2 and Wnt/β-catenin pathway plays an important role in the progression of colorectal cancer. Up regulation of sFRP2 significantly inhibited the proliferation, migration and invasion of colorectal cancer cell line HCT116.*
***Keywords:*** *Colorectal cancer; sFRP2; β-catenin; HCT116*

## I. Introduction

In recent years, the incidence of colorectal cancer has become higher, and the incidence of it is in third place in the world, with a second fatality rate[1]. Secreted Frizzled-related proteins 2(sFRP2) is a type of sFRP family, belonging to secretory glycoproteins, which is similar to Frizzled protein, and has cysteine rich domain(CRD), which can be combined with Wnt protein competitively. Wnts is a secreted and lipid modified glycoprotein that plays a key role in embryonic development and promoting tissue homeostasis in adults. They regulate A wide range of cellular behaviors was regulated, including differentiation, proliferation, migration, survival, and self-renewal of stem cells[2-4].Abnormal Wnt signaling is associated with many diseases, especially cancer[5]. Reduction or disappearance of sFRP2 expression results in less competitive binding to Wnt protein, activation of the Wnt/ β-catenin pathway and abnormal expression of β-catenin, contributing to the development and progression of CRC(colorectal cancer)[6,7]. This study is mainly divided into two aspects: first, by immunohistochemical methods, to assess the positive rate of sFRP2 and its relationship with clinical pathology, the relationship β-catenin membrane expression deficiency and the ectopic expression with clinical pathology, and to investigate the correlation between them. Secondly, the expression of sFRP2 protein in colorectal cancer cell line HCT116 was transfected by liposome transfection, and the proliferation, migration and invasion experiments were carried out to the detect the changes before and after transfection.

## II. Materials And Methods

### 2.1. Cells and tissues

Colorectal cancer cell line HCT116 is derived from the laboratory of Subei People's Hospital of Jiangsu province.Google biotechnology helps build the pcDNA3.1 plasmid that contains the full length of the sFRP2 protein was builded by Wuhan Servicebio Technology company. Colorectal tissue specimens were collected from Subei People's Hospital of Jiangsu province during 2015-2017 which were confirmed by surgery or endoscopic biopsy.32 cases of normal colorectal mucosa specimens (normal tissue of patients without colorectal cancer), 54 cases of colorectal cancer specimens.All patients had not received radiotherapy and chemotherapy, and clinical and pathological features as shown in table 2.

### 2.2. Main reagent

Rabbit anti human sFRP2 polyclonal antibody(Abcam);Rabbit anti human β-catenin antibody (Santa Cruz); SP Kit(KeyGEN BioTECH); Western Blot Kit(KeyGEN BioTECH); CCK-8 Kit(Dojindo); Trypsin(KeyGEN BioTECH); fetal bovine serum(Hangzhou Sijiqing); DMEM(Hyclone); LiPofectamine$^{TM}$3000(Invitrogen); Transwell chamber(Corning Corstart); Matrigel matrix(BD).

### 2.3. Immunohistochemistry(SP)

We determined the staining of sFRP2 and β-catenin according to the following criteria: (1) sFRP2: ①staining intensity: 0 points(without staining), 1 points (pale yellow), 2 (brownish yellow), 3 points(tan). ②According to the positive expression rate of cells: less than 25% was 0 points, 26% to 50% was 1 points, 51% to 75% was 2 points, greater than 76% was 3 points.The sum of both is greater than 3 points, which is positive, Otherwise it is negative.(2) β-catenin: Cells appear brown granules are positive expression.Cytoplasmic positive expression: the positive number of cytoplasm in the same kind of cell is greater than 10%, and the same is for the nucleus. Both of them are called cytoplasm and nucleus positive expression, which is called ectopic expression. Membrane normal expression: the cell membrane dyeing degree is more than 70%, less than or equal to 70% is membrane expression deficiency; membrane expression deficiency and the ectopic expression are collectively called abnormal expression[8].

### 2.4. The methods of cell culture and transfection

Culture conditions: DMEM(10% fetal bovine serum) and incubator(37℃ and 5%CO2). PcDNA3.1 plasmid was transfected with LiPofectamine$^{TM}$3000 in the logarithmic phase of cell growth. The experiment was divided into 3 groups: control group(no transfection), empty plasmid group(blank plasmid was transfected) and sFRP2 transfection group. The transfection process was carried out according to the transfection reagent instruction.

### 2.5. Western blot analyses

Total lysates of treated cells were prepared using RIPA buffer containing1× Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. Total proteins (50μg) from each lysate were separated by SDS/PAGE and transferred onto PVDF membranes, and then probed with the indicated antibodies using standard protocols.

### 2.6. CCK-8

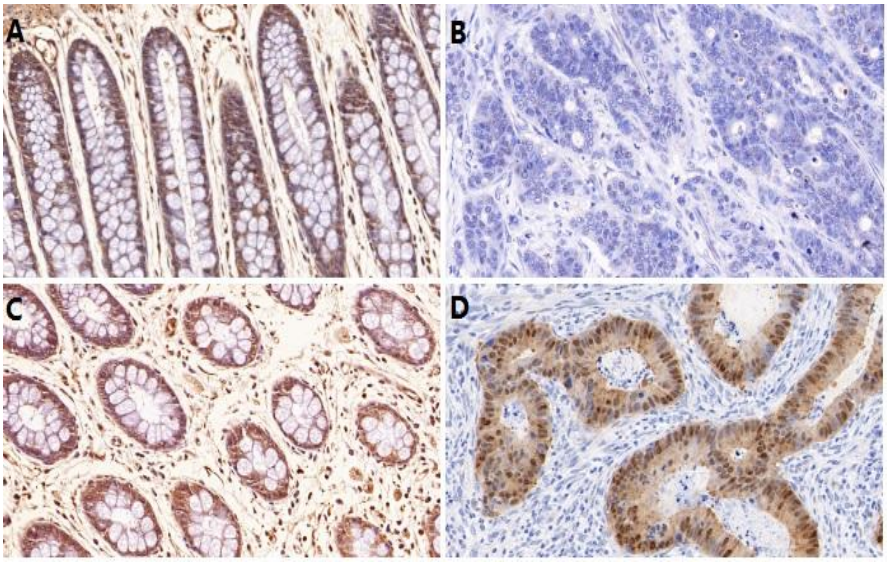
1× 10^4 cells were inoculated on the 96-well microplates and counted according to the CCK-8 kit product manual. In short, 10 L CCK-8 solutions were added to each pore and incubated for 1 hours, then absorbance was measured at 450 nm, and the experiment was repeated three times.

### 2.7. Wound-healing assay

When HCT116 cells were inoculated in 6 holes, fused to 80%-90%, they were scratched lightly along the straight line at the center axis of the pore plate, and then removed by PBS. Serum-free medium was used for culture, observing and taking pictures in 0,48 hours. The experiment was repeated three times.

### 2.8. Transwell assay

The bottom membrane of Transwell chamber is covered by Matrigel matrix (filter pore size is 8μm).The upper chamber: serum-free DMEM (containing cells), lower chamber: DMEM(10% fetal bovine serum), incubated for 24 hours. After the cells in upper chamber was removed, fixed in paraformaldehyde for 15 min, stained for 15 min. 5 eyes (100 times) were randomly selected for cell count, and their mean values were obtained.




**2.9. Statistical analyses**

Apply SPSS21.0 statistics software. The *t* test was applied to measurement data, the $X^2$ test was applied to enumeration data, and Spearman analysis was used to examine the correlation. Independent sample *t* test or single factor analysis of variance were used for comparison between groups. If the *P* value is less than 0.05, the difference is statistically significant.

## III. Results

***3.1. Immunohistochemistry(SP)***

***3.1.1 Expression of sFRP2 and β-catenin in colorectal tissues***

Immunohistochemical staining was performed on normal and cancerous tissues.In normal colorectal mucosa, sFRP2 and β-catenin were expressed on the cell membrane (Fig 1-A and 1-C). Low expression or no expression of sFRP2 in colorectal carcinoma (Fig 1-B), however, the abnormal expression of β-catenin increased (Fig1-D). The positive rate of sFRP2 in 32 normal tissues was 100% , 25.93% in 54 cancer tissues, and the difference betweeen them was statistically significant (*P*=0.00). The rate of β-catenin membrane expression deficiency was 0% in 32 normal tissues,53.70% in cancer tissues relatively, and the difference betweeen them was statistically significant (*P*=0.00). There was significant difference about β-catenin ectopic expression between two groups(*P*=0.00), normal tissues(0%) VS cancer tissues(61.11%%). The results are shown in table 1.

**Figure1** Expression of sFRP2 and β-catenin in colorectal tissues(SP×400).
A. Normal tissues(sFRP2); B. Colorectal cancer tissue(sFRP2); C.Normal tissues(β-catenin); D.Colorectal cancer tissue(β-catenin).

**Chart 1** Expression of sFRP2 and β-catenin in colorectal tissues

| Histologic type | sFRP2(+) | Abnormal expression of β-catenin | |
|---|---|---|---|
| | | Membrane expression deficiency(+) | Ectopic expression (+) |
| Normal tissues n=36 | 32（100%） | 0（0%） | 0（0%） |
| Colorectal cancer tissue n=54 | 14（25.93%） | 29（53.70%） | 33（61.11%） |
| *P-value* | *P=0.00* | *P=0.00* | *P=0.00* |

***3.1.2 The relationship between sFRP2, β-catenin and clinical features of colorectal cancer***

As shown in Table 2， their expression was significantly correlated with tumor stage (*P*=0.02, *P*=0.01, *P*=0.02) and tissue differentiation (P=0.02, P=0.02, P=0.01) (*P*<0.05), they were irrespective related to sex (*P*=0.76, *P*=1.00, *P*=0.58), age (*P*=0.76, *P*=0.59, *P*=0.58), tumor site (*P*=0.53, *P*=0.58, *P*=0.39), tumor size (*P*=1.00, *P*=0.58, *P*=0.39), and whether there was metastasis (*P*=0.53, *P*=0.10, *P*=0.15).

**Chart 2** The relationship between sFRP2, β-catenin and clinical features of colorectal cancer

| Parameters | n | sFRP2(+) | Abnormal expression of β-catenin | |
|---|---|---|---|---|
| | | | Membrane expression deficiency(+) | Ectopic expression (+) |
| Gender | | | | |
| Male | 25 | 6 | 13 | 14 |
| Female | 29 | 8 | 16 | 19 |
| *P* value | | 0.76 | 1.00 | 0.58 |
| Age (years) | | | | |
| <50 | 24 | 7 | 14 | 16 |
| ≥50 | 30 | 7 | 15 | 17 |
| *P* value | | 0.76 | 0.59 | 0.58 |
| Tumor site | | | | |
| colon | 33 | 10 | 19 | 22 |
| rectum | 21 | 4 | 10 | 11 |
| *P* value | | 0.53 | 0.58 | 0.39 |
| Tumor size | | | | |
| <5cm | 23 | 5 | 11 | 13 |
| ≥5cm | 31 | 9 | 18 | 20 |
| *P* value | | 1.00 | 0.58 | 0.58 |
| TNM stage | | | | |
| I/II | 20 | 9 | 6 | 8 |
| III/IV | 34 | 5 | 23 | 25 |
| *P* value | | 0.02 | 0.01 | 0.02 |
| Histologic type | | | | |
| poorly differentiated | 11 | 6 | 2 | 3 |
| Well and moderately differentiated | 43 | 8 | 27 | 30 |
| *P* value | | 0.02 | 0.02 | 0.01 |
| Metastasis | | | | |
| - | 33 | 10 | 21 | 23 |
| + | 21 | 4 | 8 | 10 |
| *P* value | | 0.53 | 0.10 | 0.15 |

### *3.1.3 The correlation between them*

Spearman analysis was used to test the correlation. The correlation between the expression of sFRP2 and the membrane expression deficiency of β-catenin was statistically significant (P=0.029), negatively correlated (r=-0.298). The correlation between the expression of sFRP2 and the ectopic expression of β-catenin was statistically significant (P=0.023), negatively correlated (r=-0.308). It was also found that the correlation between ectopic expression of β-catenin and the the membrane expression deficiency of β-catenin was statistically significant (P=0.003), positively correlated (r=-0.402). As shown in Table 3.

**Chart 3** Correlation between expression of sFRP2 and beta -catenin in colorectal cancer

| | Membrane expression deficiency of β-catenin | | Ectopic expression of β-catenin | |
|---|---|---|---|---|
| | + | - | + | - |
| sFRP2 | | | | |
| + | 4 | 10 | 5 | 9 |
| - | 25 | 15 | 28 | 12 |
| | *r*=-0.298 | *P*=0.029 | *r*=-0.308 | *P*=0.023 |
| Ectopic expression of β-catenin | | | | |
| + | 23 | 10 | | |
| - | 6 | 15 | | |
| | *r*=0.402 | *P*=0.003 | | |

***3.2. Western blot analyses***

The results of Western blot showed that the expression level of sFRP2 increased after transfection. β-catenin used as a reference. As shown in figure 2.

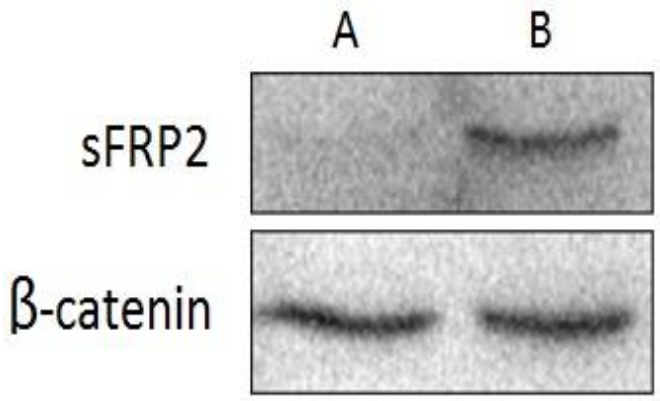



**Figure 2** Expression of sFRP2 in HCT116 cells before and after transfection. A.Before transfection. B.After transfection.

***3.3. CCK-8***

CCK-8 assay showed that the cell proliferation rate of sFRP2 transfection group was significantly slower than control group and empty plasmid group (Figure 3), indicating that sFRP2 inhibited the proliferation of cancer cells.

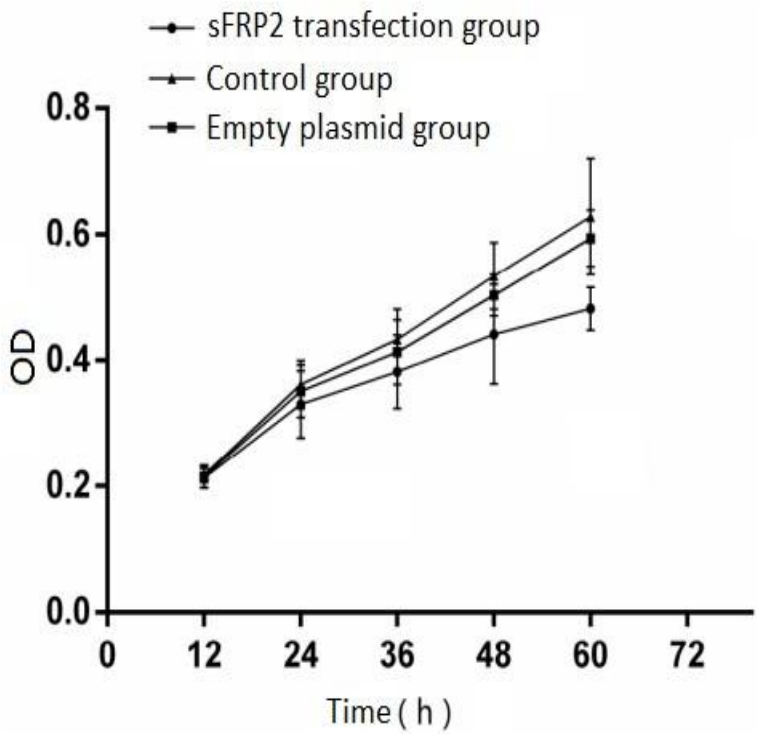



**Figure 3** Effect of sFRP2 on growth curve of colorectal cancer cell line HCT116

***3.4. Wound-healing assay***

To compare the effects of sFRP2 on the migration ability of HCT116 cells by comparing the distances of HCT116 cells scratched between the two straight lines in the sFRP2 transfection group, the empty plasmid group and the control group.The results showed that the distance between the two sides of the experimental group was far away from the other two groups, and the migration rate was slower, indicating that sFRP2 inhibited the migration of cancer cells. As shown in figure 4.

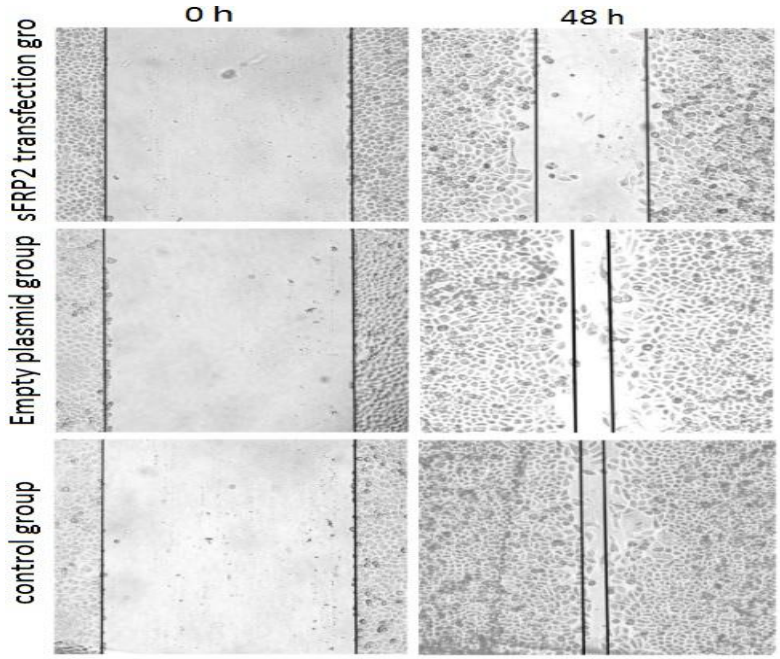



**Figure 4** Effect of sFRP2 on migration ability of colorectal cancer cell line HCT116

***3.5. Transwell assay***

Through the transwell assay, we found that the control group through the membrane (195.39±8.68), sFRP2 transfection group (75.69±7.19), sFRP2 transfection group of cell membrane permeability were significantly less than the control group($X^2$=25.46, $P$=0.00). As shown in figure 5.

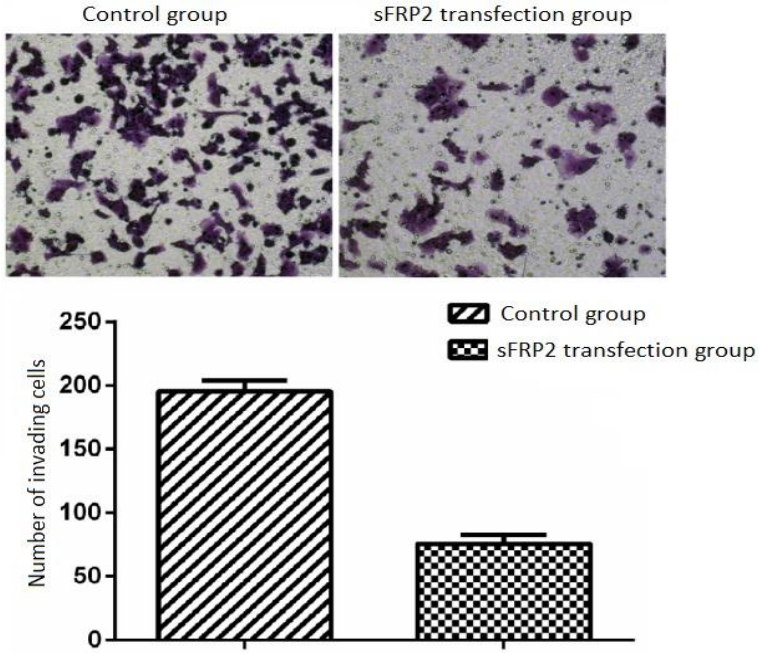



**Figure 5** Effect of sFRP2 on invasion ability of colorectal cancer cell line HCT116

## IV. Discussion

FRP2 is one of the members of the sFRP family. Because of its structural similarity to the Frizzed protein, contains CRD， on one hand, sFRP2 binds CRD to the Wnt protein by inhibiting the Wnt pathway by competing with Frizzed; On the other hand, sFRP2 and Frizzed combine to nonfunctional complexes, closed Wnt signaling pathway[9], inhibiting the occurrence of tumors. When the expression of sFRP2 is decreased, decreased binding to Wnt protein, and the Wnt pathway can not be blocked or closed, which leads to the activation of Wnt pathway and plays a role in promoting cancer[10,11].Studies have reported that sFRP2

methylation in colorectal cancer leads to decreased or silent expression of sFRP2[12-14]. Liu Ning et al. [15] found that the positive rates of sFRP2 were low to high: colorectal cancer (28.57%), colorectal adenoma (36.11%), non adenomatous polyps (95%), and normal colorectal mucosa (100%), which was basically consistent with our results by immunohistochemistry.We found that positive expression rate of sFRP2 in normal colorectal mucosa (100%) is more than colorectal cancer group (25.9%), with statistical significance (P<0.05).

Wnt/ β-catenin pathway is hot in the studies of the mechanism of colorectal cancer progression[16].When Wnt is activated, the β-catenin is not degraded and enters the nucleus and accumulates, and β-catenin appears to express membrane deletion and ectopic expression.It interacts with transcription factors, initiates transcription, regulates gene expression, and thus promotes the development and progression of tumors[17]. Liu Ning et al.[15] found that the rates of β-catenin membrane expression deficiency and the ectopic expression were low to high: normal colorectal mucosa (0%, 0%), non adenomatous polyps (0%, 0%), adenoma (11.11%, 30.56%), and colorectal cancer (52.38%, 64.29),.It was basically consistent with our results by immunohistochemistry, the rate of β-catenin membrane expression deficiency was low to high: colorectal cancer (53.7%), normal colorectal mucosa (0%),β-catenin ectopic expression:Colorectal cancer (61.1%) , normal colorectal mucosa (0%).There were statistically significant differences (P<0.05).

We found that the expression of sFRP2,and abnormal expression of β-catenin were significantly correlated with tumor stage and tissue differentiation($P$<0.05), however, it was not associated with sex, age, tumor location, tumor size and metastasis($P$>0.05), and this is consistent with the results of Liu Ning[15], Ma Siping[18] and Yu Haifeng[19].We also found that the expression of sFRP2 was negatively correlated with β-catenin membrane expression deficiency ($r$=-0.298, $P$=0.029) and ectopic expression(r=-0.308, $P$=0.023); There was a positive correlation between the β-catenin membrane expression deficiency and the ectopic expression ($r$=0.402, $P$=0.003).There were statistically significant ($P$<0.05), and consistent with related studies[15, 20].

We raised the expression of sFRP2 in HCT116 cells by plasmid transfection and verified the success of the transfection by Western blot analyses.The effect of sFRP2 on the proliferation of sFRP2 cells was detected by CCK-8, and the rate of cell proliferation was slowed down and the proliferation of HCT116 cells was inhibited when sFRP2 rised. Decreased expression of sFRP2 leads to activation of Wnt/ β-catenin pathway, and β-catenin go into the cytoplasm and nucleus ,and then accumulate. CyclinD1 promoter interactions in the LEF-1 locus, activating the transcription process, cyclin dependent kinase (CDKs) activated, induced phosphorylation, promoting gene transcription, inducing cell by G1 phase to S phase transition, and enter the proliferative phase[21,22].Therefore, the increase of sFRP2 expression, blocking the Wnt/β-catenin pathway, inhibits the proliferation of cells[23].Through Wound-healing assay, we found that sFRP2 inhibited the migration ability of cells.Through the transwell assay, we found that the number of sFRP2 transfected cells was significantly less than that of the control group, indicating that sFRP2 inhibited the invasion of HCT116 cells.But our experimental cells are single, and only one colorectal cancer cell line, so we still needs to test in other colorectal cancer cell lines.

Immunohistochemistry was first carried out in this study. Secondly, the plasmid transfer and cell function test were carried out.To investigate the effects of HCT116 on cell proliferation, migration and invasion, few studies have been reported.It is one of the focal points of clinical work that how to rapidly and accurately screen and diagnose colorectal cancer in the early stage. It is hoped that this study can provide a theoretical basis and direction for screening, diagnosis and treatment of colorectal cancer.

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