



Short Communication

The Inhibition of Cell Proliferation of Bile Duct Scar Fibroblasts in Miniature Pigs by 5-fluorouracil and its Mechanism

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ABSTRACT

The purpose of this study was to investigate the effect of 5-fluorouracil (5-FU) on the expression of TGF- β 1, cyclinD1, and CDK4 in fibroblasts of miniature pig bile duct scar model cultured *in vitro*. The bile duct scar fibroblasts were replicated by incision and anastomosis in miniature pigs and bile duct scar fibroblasts were obtained. After cell identification, the bile duct scar fibroblasts were given different concentrations of 5-FU (0, 0.001, 0.01, and 0.1 mmol/L). After 48 h of intervention, CCK-8 method was used to detect the cell proliferation level of each group; Western blot was used to detect the expression of TGF- β 1, cyclinD1, and CDK4 protein in each group of cells; and real-time polymerase chain reaction was used to detect the expression levels of TGF- β 1, cyclinD1, and CDK4 mRNA in each group of cells. The results showed that after 5-FU intervention, the proliferation of bile duct scar fibroblasts was inhibited, and the expressions of TGF- β 1, cyclinD1, CDK4 mRNA and protein in the cells were down-regulated ($P < 0.05$), showing concentration-dependence. In conclusion, 5-FU may have a role in inhibiting bile duct scars. One of the mechanisms may be related to its ability to inhibit the expression of TGF- β 1, cyclinD1, and CDK4 in bile duct scar fibroblasts.

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Authors' Contributions

SD conceived and designed the study, data collection and analysis and wrote the paper. YJ, JX and XZ were responsible for data collection and analysis. PH, JL and LZ wrote and edited the manuscript. JT reviewed and edited the manuscript.

Key words

5-fluorouracil (5-FU), miniature pig, bile duct scar, fibroblast, TGF- β 1, cyclinD1, CDK4.

Benign stenosis of the hilar bile duct mainly refers to the stenosis of the upper common hepatic duct and the left and right extrahepatic ducts (Maeda *et al.*, 2019). Benign bile duct stenosis means the bile duct fibrous tissue hyperplasia and scar contracture caused by biliary tract surgery, calculus, inflammation, and infection. The excessive proliferation of benign bile duct scar fibroblasts, which can lead to the excessive secretion of collagen, is a core link of benign bile duct scar stenosis (Geng *et al.*, 2005; Xu *et al.*, 2003).

Transforming growth factor- β 1 (TGF- β 1) is currently known as the closest and most representative growth factor to scar formation, and it is closely related to the occurrence and development of scars, and also affects scar contracture (Fang *et al.*, 2019; Wang *et al.*, 2020). TGF- β 1 is a multifunctional cytokine with an autocrine mechanism and the ability to regulate cell proliferation, differentiation, and expression of the intercellular matrix (Chi *et al.*, 2018; Ji *et al.*, 2019). The continuous expression of TGF- β 1 can

lead to the subsequent secretion and overexpression of TGF- β 1 through the inflammatory mediators produced during the healing process of bile duct injury and the autocrine mechanisms, thus stimulating the production and excessive deposition of extracellular matrix (ECM). ECM stimulates fibroblasts to secrete more TGF- β 1, forming a positive feedback effect, and eventually resulting in hyperplasia of local tissue scars and the thickening of the bile duct wall fibrosis, scar contracture and stenosis.

One study has found that the transcriptional levels of cyclinD1 and CDK4 in hypertrophic scar fibroblasts are elevated (Gong *et al.*, 2016). It is speculated that the abnormal expression of cyclinD1 and CDK4 is closely related to the occurrence of pathological scars.

Drug injection for the treatment of pathological scars is more and more accepted due to its simple operation, low cost and positive effects. Fluorouracil (5-FU) and other drugs are commonly used in clinical practice. 5-FU is a uracil metabolite. It is first converted into fluorouracil deoxynucleoside and then into uracil deoxynucleotide by enzymatic reaction in the body (Barsotti and Ipata, 2002). One study has shown that 5-FU has a certain effect in treating pathological scars (Hietanen *et al.*,

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2019). However, the mechanism with which 5-FU treats pathological scars is unclear.

The purpose of this study was to investigate the effect of 5-FU on the expression of TGF- β 1, cyclinD1, and CDK4 in fibroblasts of a miniature pig bile duct scar model cultured *in vitro* in order to reveal the therapeutic mechanism of 5-FU.

Materials and methods

Four healthy male experimental miniature pigs of clean grade were selected and randomized into a normal control group and a bile duct scar model group, with 2 pigs in each group. The pigs were put in fast for 12 h before the operation. They were intravenously anesthetized with 2.5% sodium pentobarbital solution (45 mg/kg body weight). A midline incision was made in the upper abdomen, the skin and subcutaneous tissue were cut, and the common bile duct was exposed. In the bile duct scar model group, the common bile duct with a length of about 1 cm was freed near the duodenum. It was cut transversely for about 1/3 of its circumference, and was sutured with Dexon thread. The abdomen was sutured layer by layer. In the normal control, the abdomen was sutured after opening, without opening the common bile duct. Both groups were fed as routines after 12-h fasting. The bile duct tissues of the bile duct scar model group and the normal control group were taken one week after the operation, washed with phosphate buffer solution (PBS) three times, and cut into 2 mm³ tissue pieces, transferred to the culture flask and placed in a 5% carbon dioxide incubator at 37°C. After 4-h incubation, the cells adhered well, and DMEM medium (containing 20% inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate) was added to subculture the adherent cells. Fibroblasts were isolated and purified using differential adherence, and passaged when the cells grew to 90% confluence. Vimentin cells were immunofluorescence-positive, and cytokeratin cells were immunofluorescence-negative. The third-generation fibroblasts that fit the characteristics of fibroblasts were used for this experiment.

The third-generation fibroblasts of the normal bile duct from the normal control miniature pigs were selected as Group A. The third-generation fibroblasts of miniature pig bile duct scar in the bile duct model group were selected as Group B (0 mmol/L 5-FU), Group C (0.001 mmol/L 5-FU), Group D (0.01 mmol/L 5-FU) and Group E (0.1 mmol/L 5-FU).

CCK-8 method to detect cell proliferation. Keloid fibroblasts (cell density 2×10^4 /well) in the logarithmic growth phase of Groups A, B, C, D and E were inoculated into 96-well culture plates (100 μ L/well). After incubating for 24 h in a 5% carbon dioxide incubator at 37°C, the

5-FU with the corresponding concentration was added to incubate for 48 h according to the different groups (the same amount of sterile physiological saline was added to Group A), and the CCK-8 reagent solution was added to incubate at 10 μ L/well for 2.5 h. The culturing was then terminated. It was shaken up. The optical density (OD) value of each well was measured at 450 nm wavelength on an automatic quantitative microplate reader. The background deduction strategy was used to eliminate errors. Each experiment was repeated three times, and the average OD value was calculated for comparison. A higher OD value indicated stronger proliferative activity of the cell.

Expression levels of TGF- β 1, cyclinD1, and CDK4 protein were detected by Western blot method. The cells in the logarithmic growth phase of each group were used for the experiment, and the total RNA of each group of cells was extracted. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. After blocking overnight with 5% skim milk TBST solution, the membranes were washed, and the corresponding primary antibodies TGF- β 1, cyclinD1, and CDK4 (1:500) were added, and the secondary antibodies Rabbit Anti-Mouse IgG secondary antibody (HRP) (1:5000) were added. They were developed and analyzed on a chemiluminescence imager after color development by ECL, with β -actin as an internal reference. The experiment was repeated three times to ensure the accuracy of the experimental results.

Semi-quantitative RT-PCR was used to analyze the expression of TGF- β 1, cyclinD1, and CDK4 mRNA in each group of cells. The amplification conditions were predenaturation at 94°C for 5 min, denaturation at 94°C for 30 s, extension at 72°C for 1 min, and cycling 30 times, and finally extension at 72°C for 8 min to terminate the reaction. The forward primer of TGF- β 1 is 5'-GCCCTGG-ACCAACTATTGC-3', and the reverse primer is 5'-GCACTTGCAGGAGCGCA-3'. The forward primer of cyclinD1 is 5'-GAGACCATCCCCCTGACGGC-3', and the reverse primer is 5'-TCTTCCTCCTCGGC-GGC-3'. The forward primer of CDK4 is 5'-TGATGCGCCAGTTTCTAAGAGG-3', and the reverse primer is 5'-GGTCGGCTTCAGAGTTTCCACA-3'. The forward primer of β -actin is 5'-GACAACGGCTCC-GGCATGTGCA-3', and the reverse primer is 5'-TGAG-GATGCCTCTCTTGCTCTG-3'. The ABI Stepone software v 2.3 qRTPCR system was used to collect data and analyze the results. The melting curve was a single peak type, indicating good primer specificity. With β -actin as an internal reference, the relative expression of the target gene was calculated using the $2^{-\Delta\Delta C_t}$ method. The experiment was repeated three times and the average was taken.

Data analysis was performed using SPSS 25.0 statistical software (SPSS Inc., Chicago, IL, USA). The

measurement data were expressed as mean \pm standard deviation. Comparisons among multiple groups were analyzed by one-way variance with LSD-t test. The $P < 0.05$ indicated that the difference was statistically significant.

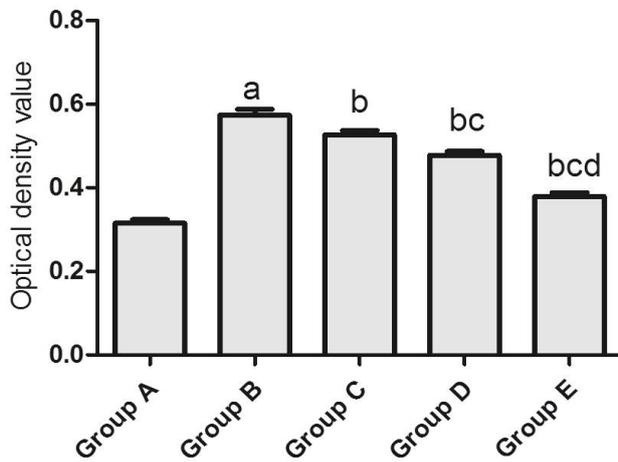


Fig. 1. The effect of 5-FU on the OD value of bile duct scar fibroblasts in miniature pigs ($n=3$). Compared with group A, ^a $P < 0.05$; compared with group B, ^b $P < 0.05$; compared with group C, ^c $P < 0.05$; and compared with group D, ^d $P < 0.05$.

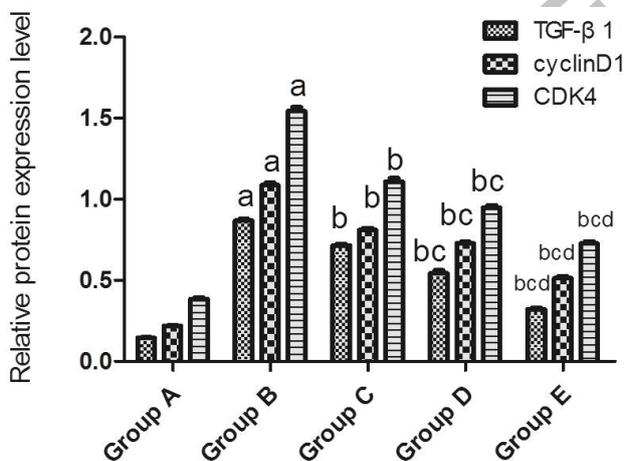


Fig. 2. The effect of 5-FU on the expression levels of TGF-β1, cyclinD1, and CDK4 proteins in miniature pig bile duct scar fibroblasts ($n=3$). Compared with group A, ^a $P < 0.05$; compared with group B, ^b $P < 0.05$; compared with group C, ^c $P < 0.05$; and compared with group D, ^d $P < 0.05$.

Results and discussion

After 5-FU concentration gradient acted on bile duct scar fibroblasts, the OD value decreased significantly as the 5-FU concentration increased, indicating a significant increase in inhibition, and the difference was statistically

significant ($P < 0.05$; Fig. 1). It is suggested that 5-FU can inhibit the proliferation of bile duct scar fibroblasts in miniature pigs. This conclusion is basically consistent with the results reported in previous study (Ophir, 1991).

Compared with Group A, the expression levels of TGF-β1, cyclinD1, CDK4 protein and mRNA in Groups B, C, D and E were increased ($P < 0.05$; Figs. 2, 3). It is suggested that the production of TGF-β1, cyclinD1 and CDK4 may play an important role in the process of bile duct scar formation in miniature pigs.

With the increase of 5-FU concentration, the expression levels of TGF-β1, cyclinD1, CDK4 protein and mRNA in cells decreased significantly ($P < 0.05$; Figs. 2, 3), showing concentration-dependence. It is speculated that the inhibition of the proliferation of bile duct scar fibroblasts in miniature pigs by 5-FU is related to the regulation of TGF-β1, cyclinD1 and CDK4.

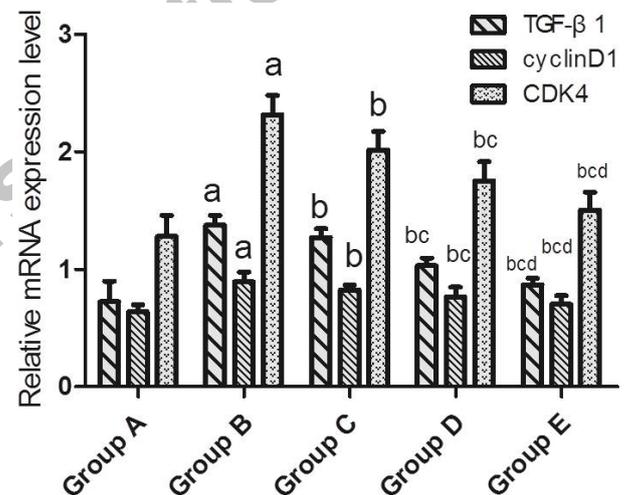


Fig. 3. The effect of 5-FU on the expression levels of TGF-β1, cyclinD1, and CDK4 mRNA in miniature pig bile duct scar fibroblasts ($n=3$). Compared with group A, ^a $P < 0.05$; compared with group B, ^b $P < 0.05$; compared with group C, ^c $P < 0.05$; and compared with group D, ^d $P < 0.05$.

Conclusion

The results of this study indicate that 5-FU can inhibit the proliferation of bile duct scar fibroblasts in miniature pigs, and the mechanism may be related to the down-regulation of TGF-β1, cyclinD1, and CDK4. However, whether 5-FU directly results in changes in TGF-β1, cyclinD1, and CDK4, or down-regulates the expression of TGF-β1 and then induces changes in cyclinD1 and CDK4 through TGF-β1 requires further study in future.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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