Original Article

Anti-apoptosis Effect of Decoy Receptor 3 in Cholangiocarcinoma Cell Line TFK-1

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Abstract

Background: Decoy receptor 3 (DcR3) is a protein with anti-apoptotic effect that belongs to the tumor necrosis factor receptor superfamily. DcR3 is highly expressed in a variety of malignant tumors including cholangiocarcinoma and its expression was found to be related to the clinical stage, the invasion, and the metastasis of the tumor. This *in vitro* study aimed to investigate the effect of downregulated expression of DcR3 on cell viability, cell apoptosis, and cell cycle in cholangiocarcinoma cell line TFK-1.

Methods: Three different cell lines were cultured: human cholangiocarcinoma TFK-1, human biliary epithelial carcinoma HuCCT-1, and human cholangiocarcinoma RBE. The cholangiocarcinoma cell line with the highest expression of *DcR3* was selected for further investigation. The expression of *DcR3* was silenced/knocked down by transfection with DcR3-siRNA in the selected cell line. Various biological phenotype parameters such as cell viability, apoptosis, and cell cycle were observed.

Results: The mRNA and protein levels of DcR3 were measured in the three cell lines, and TFK-1 was selected. After the treatment with DcR3-siRNA for 48 h, *DcR3* mRNA and protein expression in the treatment group were 38.45% ($P < 0.01$) and 48.03% ($P < 0.05$) of that of the control, respectively. It was found that the cell viability decreased to 61.87% of the control group ($P < 0.01$) after the downregulation of DcR3 in cholangiocarcinoma cell line TFK-1 by transfection with DcR3-siRNA, while the percentage of apoptotic cells was 2.98 times as compared with the control group ($P < 0.05$). Compared with the control group the ratio of G_0/G_1 increased, and the ratio of G_2/M decreased in the treatment group. However, the differences were not statistically significant.

Conclusions: The effect of *DcR3* on the growth and apoptosis of cholangiocarcinoma has been demonstrated. DcR3 is not only a predictive marker for malignant tumor but it is also likely to be a potential target for cancer gene therapy. Further studies should focus on exploring the binding ligand of DcR3, the signaling pathway involved, and the molecular mechanism for the regulation of DcR3 expression in cholangiocarcinoma.

Key words: Apoptosis; Cell Cycle; Cholangiocarcinoma; Decoy Receptor 3; TFK-1

Introduction

Cholangiocarcinoma is one of the most common malignant tumors worldwide, which accounts for 2% of all known malignant tumors. It has the second highest incidence among malignant tumors with hepatobiliary origin, and the highest incidence was seen in Asia.[1-3] Decoy receptor 3 (DcR3) is a protein with anti-apoptotic effect that belongs to the tumor necrosis factor (TNF) receptor superfamily. *DcR3* is highly expressed in a variety of malignant tumors, including cholangiocarcinoma, and its expression was found to be related to the clinical stage, the invasion, and the metastasis of the tumor.[4] Studies have shown that DcR3 can competitively bind to the Fas ligand (FasL), TNF-like ligand 1A (TL1A), LIGHT, and other ligands, thus hindering the apoptosis induction of the corresponding ligand and demonstrating its anti-apoptotic effect.[5-8] Downregulation

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of *DcR3* expression in different tumor cells can induce apoptosis and decrease the invasion ability of the tumor cells, suggesting that DcR3 plays an important role in the process of tumor growth and metastasis.

In this study, the expression of *DcR3* was downregulated in the cholangiocarcinoma cell line TFK-1. The effects of DcR3 on cell viability, cell apoptosis, and cell cycle were observed.

Methods

Cell lines, reagents, and instruments

Cell lines used in the study included human cholangiocarcinoma TFK-1, human biliary epithelial carcinoma HuCCT-1, and human cholangiocarcinoma RBE. These three kinds of cells were donated by Academy of Military Medical Sciences, China.

The following reagents were used in this study: 1640 medium and fetal bovine serum (Gibco, California, USA); RNA extraction kit, RNA reverse transcription kit, and SYBR® Green polymerase chain reaction (PCR) Real-Master Mix (Tiangen, Beijing, China); negative siRNA and DcR3-SiRNA (RIBOBIO, Guangzhou, China); Lipo2000 (Invitrogen, California, USA); CCK-8 kit (DOJINDO, Kyushu, Japan); Annexin V-FITC cell apoptosis detection kit and cell cycle detection kit (Beyotime, Shanghai, China); rabbit anti-DcR3 polyclonal antibody (CST, Boston, USA); mouse anti-β-actin monoclonal antibody, horseradish peroxidase (HRP)-labeled goat anti-mouse, and goat anti-rabbit IgG antibodies (ZSGB-BIO, Beijing, China); and bicinchoninic acid (BCA) Protein Quantification Kit and enhanced chemiluminescence (ECL) Luminescence Kit (Thermo, Waltham, USA).

Instruments used in the study were flow cytometry (Guava® easyCyte, Merck, Germany), real-time PCR (CFX96, BioRad, USA), and chemiluminescence gel imaging system (FluorChem FC3, Protein Simple, USA).

Cell culture and transfection

The culture medium used for the 3 cholangiocarcinoma cell lines was 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were cultured in an incubator with 5% CO_2 at 37°C.

TFK-1 cells were inoculated in 6-well plates one day before the transfection, and the inoculum was 7.5×10^4/ml. The amount of negative siRNA and DcR3-siRNA was 50 nmol/L; transfection was carried out following the manufacturer's instructions for Lipo2000 reagent. Cells were harvested after an incubation time of 48 h.

Real-time quantitative polymerase chain reaction

RNA was extracted from the cells using the total RNA extraction kit, and 1 μg of RNA was used to synthesize cDNAs using the reverse transcription kit; the cDNAs were stored at −20°C. The reaction system of quantitative PCR (qPCR) was 20 μl, including cDNA 1 μl, upstream and downstream primers 1 μl each, SYBR® Green PCR Real-Master Mix 10 μl, and RNase-free H_2O to make the total volume to 20 μl. The following conditions were used for the qPCR reaction: 95° predenaturation 15 min, 95° denaturation 10 s, 55° annealing 20 s, 72° extension 30 s, and a total of 40 cycles. Melting curve (i.e., the dissociation curve) was used to check the amplification purity of the target gene. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the internal reference. The $2^{-\Delta\Delta CT}$ method was used for data processing.

For *GAPDH*, the forward primer was 5'-ACCACAGTCC ATGCCATCAC-3', the reverse primer was 5'-TCCACCAC CCTGTTGCTGTA-3'. For *DcR3*, the forward primer was 5'-GGTACCAGGAGCTGAGGAGTGT-3', the reverse primer was 5'-CCTTGGTGTCGGACCCCA-3'.

Western blot analysis

Cells were collected, and the whole cell lysis buffer was added. The supernatant was collected after centrifugation at 14,000 ×*g* for 10 min, and the protein concentration was determined by the BCA assay. The electrophoretic protein samples were prepared and separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (5% gel for sample concentration and 10% gel for protein separation). Protein samples were routinely electrophoresed and transferred to a polyvinylidene fluoride (PVDF) membrane.

The PVDF membrane was blocked with 5% skimmed milk at room temperature for 1 h and then incubated with the primary antibody at an appropriate concentration (DcR3 1:5000) at 4°C overnight. The second antibody, HRP-labeled goat anti-mouse, and goat anti-rabbit IgG antibodies (both 1:5000), was added and incubated for 1 h at room temperature. The membrane was then washed with tris-buffered saline/0.1% Tween-20 for 10 min for three times.

After adding the ECL luminescent reagent for 30 s, the signal was detected using a chemiluminescent gel imaging system (Protein Simple, USA). The semi-quantitative gray scale analysis was performed using Image J software (NIH, Bethesda, USA) to calculate the ratio of the total absorbance of each protein band to the corresponding total absorbance value of the internal reference.

Statistical analysis

All experimental data were expressed as mean ± standard error (SE). Data with a normal distribution were analyzed using SPSS19.0 statistical software (SPSS Inc., Chicago, IL, USA), and the one-way analysis of variance (ANOVA) was used for intergroup comparison. A $P < 0.05$ was considered as statistically significant.

Results

High-level mRNA and protein expression of decoy receptor 3 detected in the TFK-1 cell line

The expression of *DcR3* was detected by qPCR (for mRNA) and Western blot (for protein) in three cell lines: HuCCT-1, TFK-1, and RBE. A higher level of *DcR3* mRNA [Figure 1a]

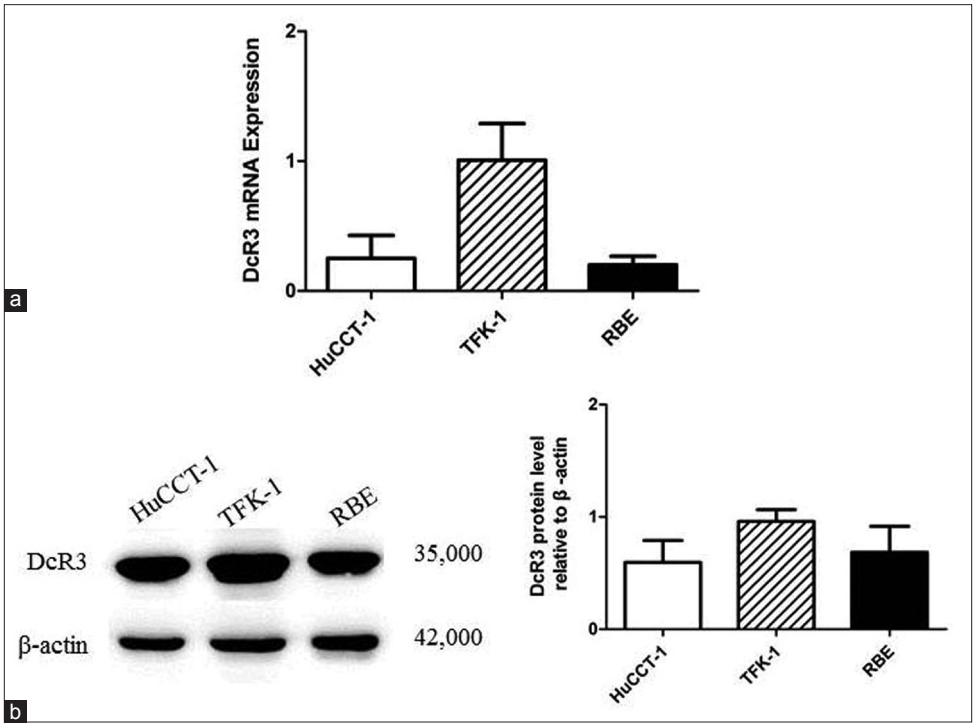

Figure 1: Expression of *DcR3* mRNA and protein in HucCCT1, TFK-1, and RBE cell lines. (a) Relative expression levels of *DcR3* mRNA detected by real-time quantitative polymerase chain reaction. (b) DcR3 protein assessed by Western blot analysis. The data were normalized to β-actin and expressed as mean ± standard error (n = 3). DcR3: Decoy receptor 3.

and protein [Figure 1b] expression was found in the TFK-1 cells than the other 2 cell lines (the HuCCT-1 and the RBE cell lines). Therefore, TFK-1 was selected for further investigation.

The mRNA and protein expression of decoy receptor 3 decreased after decoy receptor 3 siRNA treatment

Since TFK-1 had the highest expression of *DcR3* at both the mRNA and protein levels among the 3 cholangiocarcinoma cell lines tested [Figure 1], it was selected for cell transfection during the follow-up experiments.

TFK-1 cells cultured *in vitro* were used for transfection with negative siRNA and DcR3-siRNA. After TFK-1 cells were treated with negative siRNA and DcR3-siRNA for 48 h, the expression of *DcR3* mRNA and protein was detected by qPCR and Western blot in the control group, the negative group, and the treatment (DcR3-siRNA) group. Results showed that after the treatment with DcR3-siRNA for 48 h, *DcR3* mRNA and protein expression in the treatment group were 38.45% (F=51.356, $P < 0.01$; Figure 2a) and 48.03% (F=16.848, $P < 0.05$; Figure 2b) of that of the control, respectively.

Downregulation of decoy receptor 3 expression reduced cell viability

TFK-1 cells were treated with negative siRNA and DcR3-siRNA for 48 h; cell viability was detected using the CCK-8 kit. It was found that the cell viability of the treatment group was 61.87% of that of the control group, which was statistically significantly lower (F=5.994, $P < 0.01$; Figure 3). These results showed that when the expression of *DcR3* was knocked down in TFK-1 cells, cell viability decreased.

Downregulation of decoy receptor 3 expression increased apoptosis

Same as the experiments described above, TFK-1 cells cultured *in vitro* were transfected with negative siRNA and DcR3-siRNA. After 48 h of treatment, Annexin V-FITC/PI kit was used to detect apoptosis. TFK-1 cells were treated with negative siRNA and DcR3-siRNA for 48 h. Apoptosis was detected using the Annexin V-FITC/PI kit and results showed that the percentage of apoptotic cells in the treatment group was 2.98 times of that in the control group (F=27.957, $P < 0.05$; Figure 4); the difference was statistically significant. Based on the findings of this experiment, it has been demonstrated that apoptosis increased when knocking down the expression of *DcR3* in TFK-1 cells.

Downregulation of decoy receptor 3 expression affected the cell cycle

TFK-1 cells were treated with negative siRNA and DcR3-siRNA for 48 h. The cell cycle was examined by PI staining. Results showed that compared with the control group, the ratio of G_0/G_1 increased and the ratio of G_2/M decreased in the treatment group. However, the differences were not statistically significant [Figure 5].

Discussion

Cholangiocarcinoma is originated from the epithelium of the bile duct with a high degree of malignancy. In recent years, the incidence of cholangiocarcinoma has been

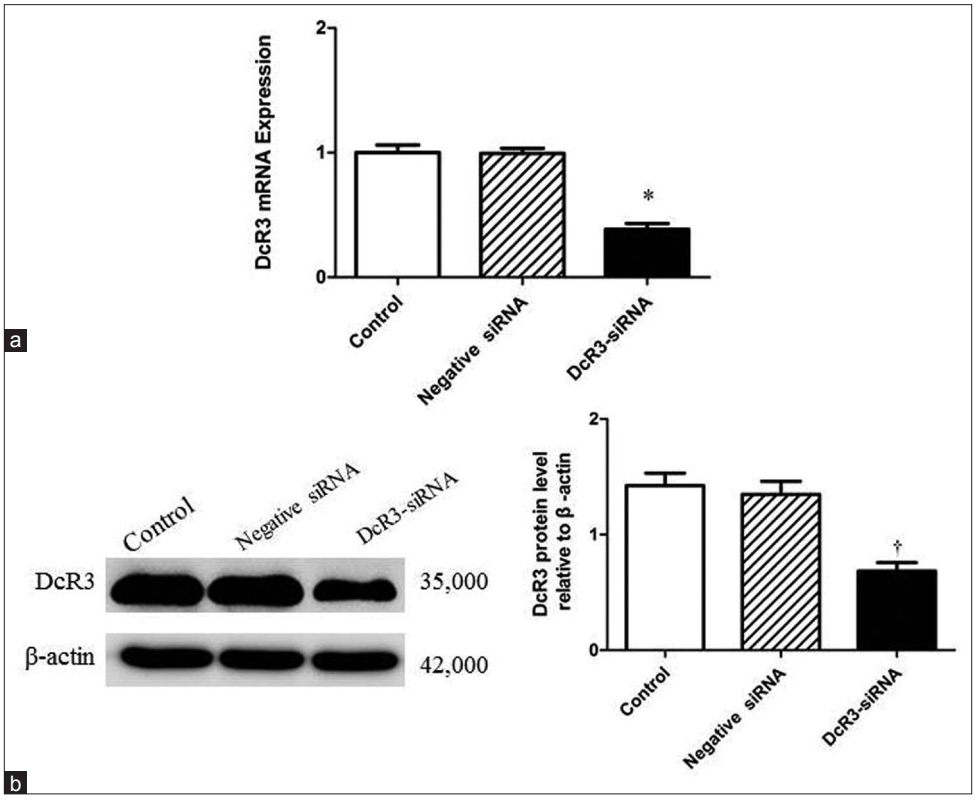

Figure 2: Expression of *DcR3* mRNAs and protein in TFK-1 cells 48 h after DcR3- siRNA treatment. (a) Relative expression levels of *DcR3* mRNA detected by real-time quantitative polymerase chain reaction. (b) DcR3 protein assessed by Western blot. The data were normalized to β-actin and expressed as mean ± standard error ($n = 3$). *$P < 0.01$; †$P < 0.05$ versus control group. DcR3: Decoy receptor 3.

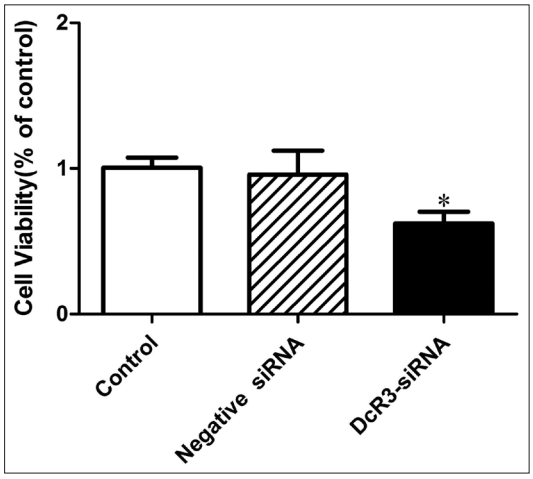

Figure 3: The change of cell viability after DcR3-siRNA transfection in TFK-1 cells. Cellular viability detected by CCK-8 in TFK-1 cells 48 h after DcR3-siRNA treatment. The data were expressed as mean ± standard error ($n = 7$). *$P < 0.01$ versus control group. DcR3: Decoy receptor 3.

steadily increasing, while there is no effective treatment. Searching for new treatments through studying the pathogenesis of cholangiocarcinoma is one of the applicable approaches to improving the prognosis of patients with cholangiocarcinoma. Studies have shown that the *DcR3* gene is overexpressed in a variety of malignant tumors, and its expression is closely related to the clinical stage, the volume of the tumor, the degree of tumor differentiation, and lymph node metastasis.[9,10]

The high expression of *DcR3* also plays an important role in the carcinogenesis and progression of cholangiocarcinoma. In one immunohistochemical study including 45 cases of cholangiocarcinoma and 15 cases of normal bile duct tissues adjacent to the cancerous tissue, the authors found that using the streptavidin–peroxidase (SP) method 29 (64.4%) of the 45 cholangiocarcinoma cases had a positive DcR3 expression, but all the normal tissue samples were negative.[11]

All these evidences indicated that DcR3 can be used as a marker for early diagnosis of tumors. In this study, we screened three cell cholangiocarcinoma lines, HuCCT-1, TFK-1, and RBE. Since TFK-1 had the highest levels of mRNA and protein expression of *DcR3*, it was selected for further investigation. In subsequent experiments, we found that after interference with DcR3-siRNA, the mRNA and protein expression of *DcR3* were both decreased to 38.45% and 48.03%, respectively, and the differences were statistically significant compared with the control group.

DcR3 is an anti-apoptotic protein that competitively binds to FasL, TL1A, LIGHT, and other ligands that induce apoptosis.[5-7] It is well known that the growth of tumor is caused by imbalanced cell proliferation and apoptosis. The key step of tumor-killing by the immune system is to induce the binding of apoptotic ligand and its receptor that leads to tumor cell apoptosis. By blocking the binding between these ligands and the corresponding receptors, such as Fas, DR3, and LTβR, DcR3 hampers

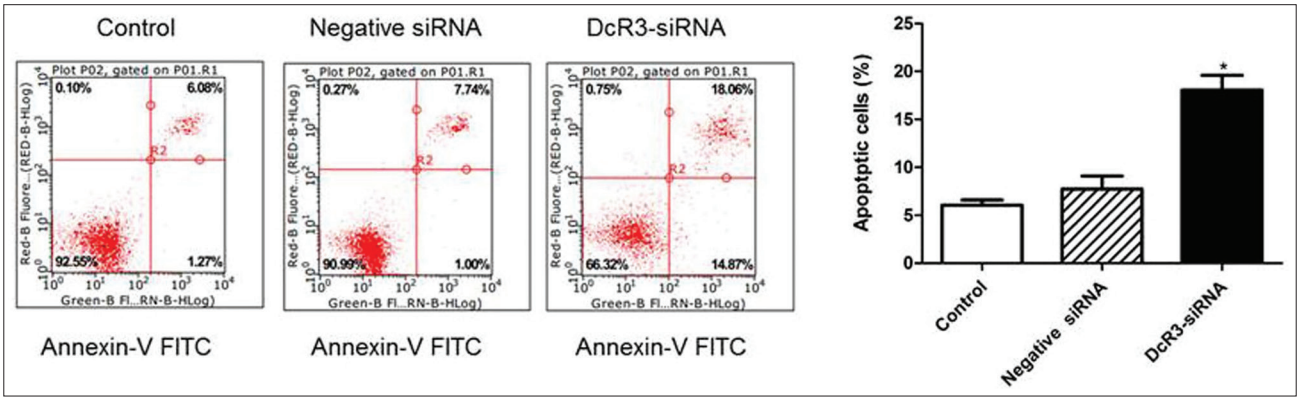

Figure 4: The apoptosis rate of TFK-1 cells after DcR3-siRNA transfection. Flow cytometric analysis of apoptosis detected by Annexin V-FITC/PI in TFK-1 cells 48 h after DcR3-siRNA treatment. The data were expressed as mean ± standard error ($n = 3$). *$P < 0.05$ versus control group. DcR3: Decoy receptor 3.

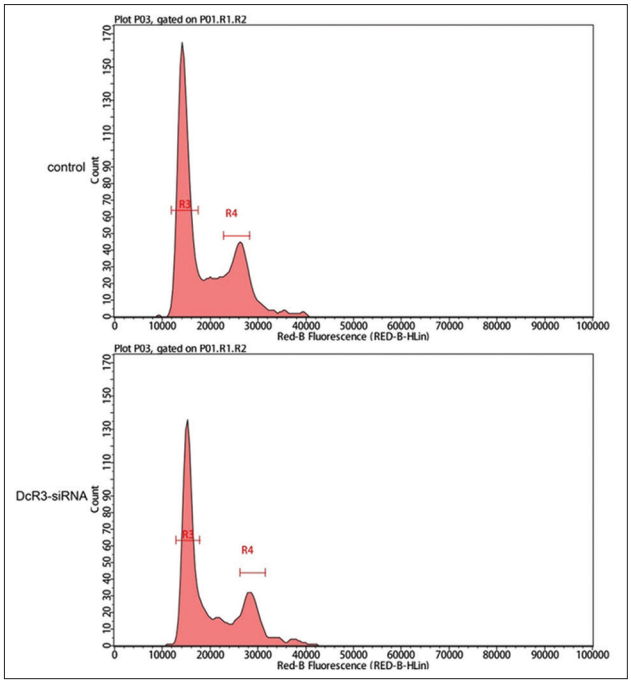

Figure 5: Changes of cell cycle after DcR3-siRNA transfection. Cell cycle was detected by cell cycle kit in TFK-1 cells 48 h after DcR3-siRNA treatment. R3: Ratio of G_0/G_1; R4: Ratio of G_2/M; $n = 3$. DcR3: Decoy receptor 3.

the initiation of the apoptosis pathway and achieves the effects of anti-apoptosis.

In this study, we found that after the interference of DcR3-siRNA, the proportion of apoptotic cells increased, and cell viability significantly decreased compared with the control. These results showed that downregulation of the expression of *DcR3* had an impact on the biological characteristics of cholangiocarcinoma cells, the excessive proliferation of the cholangiocarcinoma cells was inhibited and apoptosis of the cholangiocarcinoma cells increased. The effect of *DcR3* on the growth and apoptosis of cholangiocarcinoma has been demonstrated. DcR3 is not only a predictive marker for malignant tumor, but it is also likely to be a potential target for cancer gene therapy.

Until now, the molecular mechanism of the upregulation of *DcR3* expression has not been fully understood. Various experiments have shown that in different tumors and diseases, the anti-apoptotic effect of DcR3 is the result of the inhibited binding of FasL, TL1A, LIGHT, and other ligands to the corresponding receptors.[5,6] In cholangiocarcinoma, we have revealed the relationship between DcR3 and the growth and apoptosis of the tumor cells; the next steps will be to identify the binding ligand of DcR3 and to understand the molecular mechanism of the high expression of *DcR3*. Previous studies have confirmed that the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway and the mitogen-activated protein kinase (MAPK) pathway play important roles in the occurrence, development, and progression of malignant tumors,[12-16] but it has yet to be confirmed that the PI3K/Akt pathway and the MAPK pathway are involved in the regulation of *DcR3* expression in cholangiocarcinoma. Therefore, in our future studies, the signaling pathway and the molecular mechanism for the upregulation of *DcR3* expression in cholangiocarcinoma will be explored, which will provide the theoretical basis for further understanding of the relationship between DcR3 and cholangiocarcinoma.

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Conflicts of interest

There are no conflicts of interest.

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