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Original Article

Biological effects of RNAi targeted inhibiting Tiam1 gene expression on cholangiocarcinoma cells

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Abstract: Objective: To investigate the characteristics of Tiam1 gene expression in human cholangiocarcinoma tissues and benign bile duct tissues, and to analyze the correlations between Tiam1 gene expression and the degree of tumor differentiation, invasive and metastatic abilities. To explore the effect of targeted inhibiting Tiam1 gene expression on proliferation and migration activity of human cholangiocarcinoma cells. Methods: Expression of Tiam1 in 83 cases of cholangiocarcinoma tissues and 25 cases of benign bile tissues was detected using immunohistochemistry. The clinical data of patients with cholangiocarcinoma were collected. The correlations between Tiam1 gene expression and the clinicopathologic features in patients with cholangiocarcinoma were analyzed. The human cholangiocarcinoma RBE cells were divided into 3 groups. Cells in experimental group and control group were respectively transfected with Tiam1 shRNA lentiviral vectors and negative shRNA lentiviral control vectors. Cells in blank group received no treatment. Real-time PCR endogenesis was used to verify Tiam1 gene expression. Cell cycle experiments and MTT assay were used to measure cell proliferation activity. Transwell test was used to detect cell migration activity. Results: The negative rate Tiam1 protein expression in cholangiocarcinoma tissues was significantly higher than that in benign bile tissues ($P<0.001$). Tiam1 protein expression in cholangiocarcinoma tissues had correlations with cholangiocarcinoma differentiation degree, TNM stage and lymph node metastasis ($P<0.05$), and had no significant correlations with gender, age and distant metastasis ($P>0.05$). Real-time PCR detection indicated that Tiam1 expression of experimental group was significantly lower than that in control group and blank group ($P<0.05$), demonstrating that Tiam1 shRNA was effective on Tiam1 gene silencing in RBE cells. Cell cycle experiment showed that the percentage of S phase in cell cycle in experimental group was lower than that in control group and blank group ($P<0.05$), demonstrating that after the down-regulation of Tiam1 gene expression, the speed of cell proliferation was inhibited. MTT assay results showed that the total growth speed in experimental group was significantly lower than that in control group and blank group ($P<0.05$), indicating that the proliferation activity of cholangiocarcinoma cells was inhibited after targeted inhibition of Tiam1 gene expression. Transwell detection results showed that the metastasis rate in experimental group was significantly lower than that in control group and blank group ($P<0.05$), demonstrating that targeted inhibition of Tiam1 gene expression could significantly inhibit migration ability of RBE cells. Conclusion: Tiam1 expression significantly increased in cholangiocarcinoma tissues, and increased along with the degree of malignancy of cholangiocarcinoma. Targeted silencing Tiam1 expression could inhibit proliferation and migration activity of cholangiocarcinoma cells.

Keywords: Cholangiocarcinoma, Tiam1, RNA, lentiviral vector

Introduction

Cholangiocarcinoma accounts for about 3% in all malignant tissues of digestive system [1]. In recent years, the incidence rate of cholangiocarcinoma shows increasing trend year by year along with the progress of diagnostic level worldwide. In USA, there are 5,000 new cases of cholangiocarcinoma each year [2, 3]. In Europe, there are 10,000 new cases of cholangiocarcinoma each year [4]. In Asia, especially in China, the incidence rate of cholangiocarcinoma shows increasing trend year by year [5-7, 12]. The present induction range of cholangiocarcinoma includes malignant tumors originated from the intrahepatic and extrahepatic bile duct, with characteristics of high degree of malignancy, insidious onset, early subclinical

and very difficult to discover. So its surgical resection rate is low, with low postoperative survival rate [8-11]. There are data showing that even intraoperative rapid pathological slices have no cancer tissue residues in margin of bile duct, it is difficult to achieve radical resection. Simultaneously, in comprehensive treatment methods of cholangiocarcinoma, except surgical treatment, radiotherapy and chemotherapy are less effective, which are not routine treatment methods for cholangiocarcinoma. These above factors caused extremely bad curative effects of cholangiocarcinoma, with only 5% of 5-year survival rate [13]. Most patients die from infectious complications caused by hepatic function failure or biliary obstruction within one year. Therefore, the mechanism of genesis, development and metastasis of cholangiocarcinoma has become the one of the present research hotspots.

One of the important reasons why cholangiocarcinoma has high malignancy extent and poor prognosis is its high invasion and metastasis ability. The expansive growth of intrahepatic bile duct cell carcinoma and primary hepatic carcinoma is not the same, which is often manifested in the invasive growth along the bile duct and metastasis following the lymphatic system. Hilar cholangiocarcinoma develops into type IV due to rapid infiltration of tumor cells up along the bile duct, losing the opportunity of radical surgery. The characteristics of cholangiocarcinoma easy to invade and metastasize to nerve bundles, lymph nodes and peripheral vascular determine its high recurrence rate and poor efficacy. If a factor related to cholangiocarcinoma invasion and metastasis is found, a new method of delaying cholangiocarcinoma development or even cure cholangiocarcinoma might be offered.

T lymphoma invasion and metastasis inducing factor 1 (Tiam1) was isolated and determined from BWS147 high invasive mutant of murine T cell lymphoma using the principle of proviral insertion causing mutation by Habets et al. [14] in The Netherlands Cancer Institute in 1994. It was recognized as a proto-oncogene. The main structural functional domains of Tiam1 protein include carboxy-terminal DH (Dbl homology) and 2 pleckstrin Homology (PH) functional domains. Tiam1 protein playing a role in tumor malignant behavior is thought to have correlations with the above 2 structural functional domains at present. DH is a composition of 238 amino acid residues (1042-1280), which plays the role of guanine nucleotide exchange factors (GEF). It acts on Rho GTPase, catalyzing ornithine GDP-GTP conversion, regulating cytoskeletal reorganization and functioning in polarization of cell morphology, thus playing the role of regulating cell activity and metastasis [15].

Tiam1 gene has a close relation to the invasion and metastasis ability of tumors. Firstly it manifests in its regulation of the cytoskeleton. Tiam1 can promote cell integrin aggregation by activating rac, thus realizing regulating actin cytoskeleton, affecting the assembly and movement of cytoskeleton and inducing invasion and metastasis of tumor cells [16-18]. In addition, the effect of Tiam1 on tumor invasion and metastasis ability manifests in involving in cell membrane ruffling in PH functional domain. Cell membrane ruffling is an index of tumor cell activity and metastasis. Tiam1 induced membrane ruffling plays an important role in tumor metastasis [19-21]. Simultaneously, the invasion and metastasis ability of tumor cells has close correlations with its motility ability and adhesive capacity to extracellular matrix. Tiam1 involves in regulating the affinity of adhesion molecules by activating Rho, and involves in assembly of adhesion complexes. This will promote integrin-mediated interaction between atypical cells and between cells and matrix, and finally play the role of promoting tumor cell invasion and metastasis [22-26]. Tiam1 protein is the regulator of proliferation and apoptosis of a variety of tumor cells [27-29]. Overexpression of Tiam1 protein can play the role of promoting tumor cell proliferation and inhibiting tumor cell apoptosis by inhibiting pro-apoptotic effect or enhancing transcription and synthesis of anti-apoptotic proteins.

Tiam1 is found to express highly in a variety of tumor tissues and tumor cells such as lymphoma, pancreatic cancer, breast cancer, bladder cancer, lung cancer *etc*. The concentration of Tiam1 protein is also found to have close correlations with tumor proliferation and migration activity [30, 31]. After Tiam1 gene was introduced into the human breast cancer cell line in cell experiments by some researchers, the invasion and migration ability of objective cancer cells was found to be increased [32]. Some other scholars observed proliferation and metastasis ability after employing Tiam1 anti-

sense oligonucleotide to inhibit Tiam1 gene in human lung cancer cells and obtained the results of significant reduction [33]. Tiam1-cDNA was introduced into NIH3T3 cell, large amount of membrane ruffling was found in the treated tumor cells. Animal experiments were implemented using these treated tumor cells, the tumor was successfully inoculated and the presence of metastasis was observed [34].

The ability of Tiam1 regulating tumor invasion and metastasis ability is the result of involving the interaction of multiple genes and multiple paths, which may be related to cell signal transduction strength, signal environment, cell source, type and degree of differentiation. The expression of Tiam1 gene in cholangiocarcinoma, the correlations between Tiam1 expression and cholangiocarcinoma invasion and metastasis, as well as the probable mechanism are not reported at present. To explore the correlations between cholangiocarcinoma and Tiam1 gene might bring new hope to the patients with cholangiocarcinoma treated by various therapies.

We found that in early experiments, Tiam1 expression in cholangiocarcinoma tissues was significantly higher than that in para-carcinoma tissues and benign bile duct tissues, moreover, Tiam1 expression was related to cholangiocarcinoma differentiation degree, lymph node metastasis and TNM stage. We also discovered that Tiam1 expressed highly in RBE of cholangiocarcinoma cells. Based on the above investigations, we envisage that: since the invasion and metastasis of cholangiocarcinoma had close correlations with Tiam1 gene, the invasion and metastasis ability of cholangiocarcinoma could be reduced by inhibiting Tiam1 gene expression. This might induce apoptosis of cholangiocarcinoma. The probable effective paths of Tiam1 were explored by silencing Tiam1 gene expression in cholangiocarcinoma cells and observing the changes of relevant genes expression to seek a new target for clinical diagnosis and treatment of cholangiocarcinoma.

Materials and methods

Tissue source of patients with cholangiocarcinoma

Sample collection: 83 cases of cholangiocarcinoma samples were paraffin-embedded specimens of bile duct cancer after radical resection offered by Hunan Provincial People's Hospital. 25 cases of benign bile duct epithelium samples were paraffin-embedded specimens of bile duct tissues with hepatolithiasis treated by hepatectomy, without significant manifestation of cholangitis (non-emergency surgery, without preoperative fever or significant abdominal pain, blood leukocytes and neutrophils ratio in the normal range). There were 40 cases of male (48.2%) and 43 cases of female (51.8%) in the 83 cases of patients. The ages were between 27 and 79, with the median age of 55. All the patients were not treated with radiotherapy or chemotherapy. There were 32 cases with lymph node metastasis and 15 cases with distant metastasis, including other organ metastasis of liver, pancreas, omentum, *etc*. According to histopathological grading of Bloom-Richardson classification standard: there were 38 cases of poor differentiation (45.8%), 32 cases of moderate differentiation (38.6%) and 13 cases of high differentiation (15.7%). According to the tumor location, they were divided into 21 cases in hepatic portal (23.2%), 9 cases in middle or lower section of common bile duct (10.6%) and 53 cases in intrahepatic bile duct (66.2%). According to 2011 NCCN Hepatobiliary TNM stage, there were 12 cases of stage I (14.5%), 16 cases of stage II (19.3%), 35 cases of stage III (42.2%) and 20 cases of stage IV (24.1%). There were 11 cases of male (44%) and 14 cases of female (56%) in control group. The ages were between 29 and 78, with median age of 54. All the pathological examination of the resection specimens indicated benign bile duct tissues with inflammatory cell infiltration. All of the slices were strictly and definitively diagnosed and reviewed by two pathologists of high qualification. All the samples were fixed using 10% formalin solution, embedded in paraffin, and sliced into 4 μm serial sections.

Cholangiocarcinoma cell lines

RBE human cholangiocarcinoma cell line was purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cell culture conditions were: 5% CO_2, 37°C, RPMI-1640 medium containing 10% fetal bovine serum in conventional culture, saturated humidity.

RBE human cholangiocarcinoma cell line was divided into experimental group, negative con-

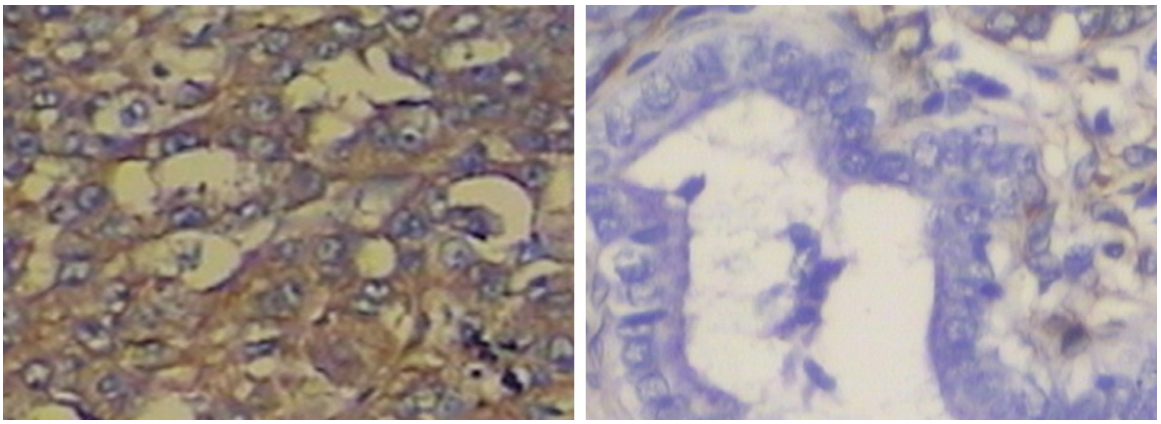

Figure 1. Expression of Tiam1 protein in cholangiocarcinoma tissues (×400) (Left: cholangiocarcinoma, right: benign bile duct tissues).

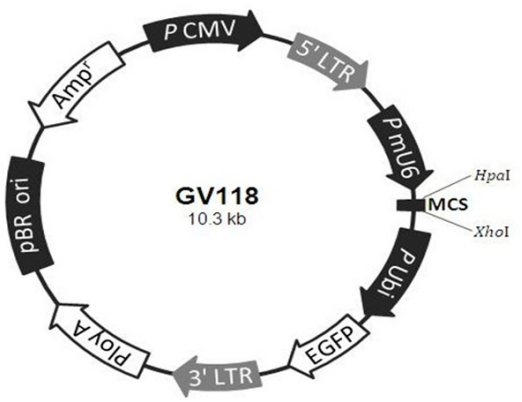

Figure 2. Lentiviral vector system GV118.

trol group and blank control group. According to multiplicity of infection (MOI), cell line in experimental group was divided into high MOI group and low MOI group.

Experimental group 1, namely low MOI group (knock down 1, KD1 group) was transfected by lentivirus GV118-Tiam1 shRNA expression vectors (MOI = 20).

Experimental group 2, namely low MOI group (knock down 2, KD2 group) was transfected by lentivirus GV118-Tiam1 shRNA expression vectors (MOI = 40).

Negative control group (NC group) was transfected with GV118-Tiam1 shRNA negative control vectors.

Blank control group (CON group) was not treated.

Immunohistochemistry

Tiam1 protein expression in all the cholangiocarcinoma tissue samples and benign bile duct tissue samples was detected using immunohistochemical method. The steps were listed as follows. The paraffin sections with thickness of 4 μm per each were heated at 65°C for 3 hours. Xylene was used to dewax for 3 times, each time for 8 min. Graded alcohol (100%-90%-80%-70%) was used to remove xylene and hydrate, each stage for 2 min. Then the slices were washed using PBS 3 times with each time of 3 min. The resultant samples were put into 3% H_2O_2 at room temperature (RT) for 30 min. The serum was removed to add Tiam1 antibody dropwise (1:200 dilution) on the slice at 4°C in wet box overnight. Then the slices were washed by PBS 5 min for 3 times. The secondary antibody working solution was added at 37°C for incubation for 30 min, then PBS was used for washing 5 min for 3 times. Streptavidin was added at 37°C for incubation for 10 min, then PBS was used for washing 5 min for 3 times. DAB substrate working solution was added dropwise onto the slices. The slices were observed under microscopy and tap water was sued for controlling color reaction. The slices were counterstained using hematoxylin and dehydrated by alcohol. Xylene was used for transparent. Neutral gum was used for sealing. PBS was used for negative control.

Result determination: Tiam1 protein positive products mainly were localized in the cytoplasm, showing brown-yellow granules in the cytoplasm. The double-blind method was used

Table 1. Expression of Tiam1 protein in various tissue

Tissue type	N	Positive (%)	Negative	*P*
Cholangiocarcinoma tissue	83	66 (9.5)	17	<0.001
Benign bile duct tissue	25	8 (32)	17	

for Tiam1 protein result determination, and the scores were based on the proportion of positive reaction cells was and the strength of reaction. The scores were divided into 2 parts: one was the strength of staining. Colorless was counted as 0, faint yellow as 1, claybank as 2 and tan as 3. The other was the number of positive cells under the same objective lens vision. 1-10% was counted as 1, 11%-50% as 2, 51%-75% as 3 and >75% as 4. The 2 kinds of scores were multiplied, 3 was counted as "+", 4 as "++", and "+++" for over 5. "+~+++" represented positive expressions.

Human Tiam1 gene specific RNA interfering lentivirus vectors and cell lines transfection

The lentivirus vector system of the study was GV118 (**Figure 2**), purchased from Shanghai Genechem Company. This lentivirus vector system is constituted by three plasmids including pGC-LV vector, pHelper 1.0 vector and pHelper 2.0 vector, which can targeted silence Tiam1 expression in RBE cells of cholangiocarcinoma cell line. The effect of Tiam1 gene on RBE cells proliferation, invasion and metastasis function was observed *in vitro*.

10 Tiam1 target sequences were synthesized in accordance with the principle of RNA interference sequences design, according to Tiam1 sequence (GeneBank, NM_003253) and Invitrogen website (http://rnaidesigner.invitrogen.com/rnaiexpress/design.do). After the assessment of Shanghai Genechem Company, one of the Tiam1 siRNA target sequences were selected (**Table 3**). siRNA sequence was confirmed not to have other homologous sequences after BLAST search in Genebank database. Oligonucleotide double strands (ds-oligo) were synthesized by Shanghai Genechem Company (**Table 4**).

Double digestion linearized vector pGC-LV and DNA fragment was reacted in appropriate buffer using T4 DNA ligase at 16°C overnight. The connected product pGC-LV-Tiam1 shRNA was used for transformation experiments. PCR positive bacteria liquid was used for sequencing analysis. The correct transformant was used for experiments.

293T cells at good state were selected as lentiviral packaging cell lines. 293T cells were spread 24 h before experiment. The cell density was controlled at about 80% during transfection. When the cells were spread, the culture dish could be gently shaken to let the cells evenly distributed in the dish. The extracted high-quality plasmid (including lentiviral packaging pHelper 1.0 vector, pHelper 2.0 plasmid and lentivirus vector plasmid pGC-LV) was taken for transfection. After transfection, it was incubated at 37°C, 5% CO_2 for 48 h.

Fluorescence method was used to detect GV118 lentivirus titer. The steps were as follows. The day before the assay, the needed cells were plated in 96-well plate, each well added 4×10^4 cells, with a volume of 100 μL. According to the expected titer of the virus, 7~10 sterile Ep tube were prepared. 90 μL of serum-free medium was added to each tube. 10 μL virus stock to be determined was added to the first tube, after mixing, 10 μL resultant liquid was added to the second tube. The same operation was continued till the last tube. The needed well was selected to abandon 90 μL medium. Then 90 μL diluted virus solution was added. Then it was put into incubator. After 24 h, 100 μL complete medium was added. Be careful not to blow the cells. After 4 days, florescence expression condition was observed. The number of florescent cells decreased along with the increase of dilution.

Detection of Tiam1 mRNA expression in RBE Real-time using PCR assay in each group

4 days after infection, Tiam1 mRNA expression in each group was detected. The effect of target silencing Tiam1 gene was evaluated. Software Beacon Designer 2 was used for the design of Tiam1 primer and GAPDH primer. The information of the primer that was synthesized by Shanghai Genechem Company was as follows (**Table 5**):

The extraction of total RNA was in accordance with the Trizol instruction of Invitrogen Company). RNA reverse transcription was implemented to acquire cDNA. Real-time PCR detection of the program is set to two-step method: pre-denaturation at 95°C for 15 s, then dena-

Table 2. The relationship between the Expression of Tiam1 protein in cholangiocarcinoma and its clinical and pathological parameters

	Groups	*n*	Tiam1 protein (%)	*P*
Gender	Male	40	33 (82.5)	0.516
	Female	43	33 (76.7)	
Age	>55 years	31	25 (80.6)	0.844
	≤55 years	52	41 (78.8)	
Differentiation degree	High/moderately differentiated	45	31 (68.9)	0.009
	Medium-low/low differentiation	38	35 (91.1)	
TNM stage	Stage I, II	28	17 (60.7)	0.002
	Stage III, IV	55	49 (89.1)	
Lymph node metastasis	No	51	37 (72.5)	0.047
	Yes	32	29 (90.6)	
Distant metastasis	No	68	52 (76.5)	0.266
	Yes	15	14 (93.3)	

Table 3. Targeted sequences of TiamI gene for RNAi

Species	Target Seq	GC%
Human	GGAGATGAGATTCTTGAGA	42.11%

turation at 95°C for 5 s, annealing extension at 60°C for 30 s, with a total of 45 circulations. Absorbance value in each extension phase was recorded. After the end of PCR, changes in fluorescence of melting curves can be plotted for each sample in the process of variability. Relative expression (RQ) of target gene in the samples was calculated using ΔΔCT method: $RQ = 2^{-\Delta\Delta CT}$ (CT showed that real-time fluorescence intensity of the reaction was significantly larger than the number of the background value cycles.) ΔCt = Tiam1 Ct value-GAPDH Ct value. $-\Delta\Delta Ct$ = mean of $\Delta Ct_{(\text{for each group of QBC939})}$-$\Delta Ct_{(\text{for each sample})}$. $2^{-\Delta\Delta Ct}$ reflected relative expression levels of Tiam1 sample of QBC group relative to each sample.

Cell cycle experiment

Intracellular DNA content was detected using flow cytometry PI staining. It could divide of each phase of the cell cycle into G1/G0 phase, S phase and G2/M phase. The percentage of each phase could be calculated by observing the results. In this experiment, 5 days after human cholangiocarcinoma cell line was infected by lentivirus, the cell confluence reached 80%. The samples were extracted for cell cycle experiment. The steps were as follows: When the 6 cm dish cell growth coverage reached 80%, simultaneously the cell did not enter entered the growth plateau to be confirmed, the supernatant was abandoned. D-hanks liquid was used to wash the cells then trypsin was used for digestion. Finally complete medium was used to stop digestion reaction. The cells were collected into 5 mL centrifuge tube, each group with 3 wells (In order to ensure a recombinant cell number on the machine, the cell number ≥1000000/ processing was required). Then it was centrifuged at 1200 rpm for 5 min. The supernatant was abandoned. PBS pre-cooled at 4°C (pH = 7.2~7.4) was used for washing the cells. Then the cells were precipitated and centrifuged at 1500 rpm for 5 min. Then the cells were collected and fixed in 70% alcohol at 4°C for more than 1 h. The resultant cells were centrifuged at 1500 rpm for 5 min. The fixation liquid was abandoned. The cells were washed by PBS, and then centrifuged. The preparation of cell staining solution was 40×PI mother liquor (2 mg/mL): 100×RNase mother liquid (10 mg/mL): 1×PBS = 25:10:1000. Cell staining: according to the number of cells, appropriate cell staining solution was added for re-suspending to ensure that the cells through rate reached 200~350 cells/s when on machine.

Detection of cell proliferation capacity using methyl thiazolyl tetrazolium (MTT) assay

Microplate reader was used to observe absorbance at 490 nm which indirectly reflected the proliferation of cells. In this experiment, 5 days after lentivirus was used to infect RBE human cholangiocarcinoma cell line, samples were extracted for MTT assay. The steps were as follows: cells in logarithmic phase were digested by trypsin and resuspended using complete medium to prepare into cell suspension. The number of cells was counted. The growth speed of cells was observed to decide the cell density spreading on the plate according to the growth speed (mostly 2000 cells/well). The liquid was

Table 4. The information of ds-oligo synthesis

NO.	5'	STEM	Loop	STEM	3'
TIAM1/GV118-RNAi#1-a	T	caGGAGATGAGATTCTTGAGA	CTCGAG	TCTCAAGAATCTCATCTCCTG	TTTTTTC
TIAM1/GV118-RNAi#1-b	TCGAGAAAAAA	caGGAGATGAGATTCTTGAGA	CTCGAG	TCTCAAGAATCTCATCTCCTG	A

Table 5. primers sequence table of Real-time PCR

No.	Primer	Name of primer	Sequence information	Amplified bands
1	Up-stream	*GAPDH*-F	TGACTTCAACAGCGACACCCA	121 bp
2	Down-stream	*GAPDH*-R	CACCCTGTTGCTGTAGCCAAA	
3	Up-stream	*TIAM1*-F	CTGTATTATGCTGACCGCTTC	248 bp
4	Down-stream	*TIAM1*-R	TAGTGCTCCTCGCTCTCC	

added into the wells, each well for 100 μL, each group for 3-5 wells, with a total of 5 96-well plates. The cells were observed continuously for 5 days. The key during the process was to ensure the cells added in the wells were consistent. Then the cells were cultivated in incubator at 37°C, 5% CO_2. 10 μL 5 mg/mL MTT was added into each well from the 2nd day of planking to 4 h before the end, without changing medium. After 4 h, the culture solution was abandoned, 100 μL DMSO was added into each well to end reaction. After 5-10 min of shaking with shaker, microplate reader was used to detect OD value at 490 nm. The data were plotted.

Transwell cell migration assay

Cell migration is an important index of tumor metastasis. By detecting cell migration capacity, tumor metastasis ability. Transwell chamber in corning transfer experiments kit is composed of 24-well tissue culture plates and 12-well cell culture inserts. The intercalant contains a polycarbonate membrane with 8 μm pore size. Transfer cells migrate and adhere to the bottom of the polycarbonate membrane. In this experiment, after lentivirus infected RBE human cholangiocarcinoma cell line, the samples were extracted to implement Transwell cell migration assay. The steps: were as follows. The needed chamber number was confirmed, then the chambers were placed into a blank 24-well plate. 100 μL serum-free medium was added into the chamber and placed in the incubator for 1~2 h. Cell suspension preparation: Number of cells were counted. Then the medium in the chambers was abandoned. 600 μL medium containing 30% FBS was added into the lower chamber of the chamber. Serum-free medium was diluted according to a certain ratio, then 100 μL resultant cell suspension (containing 100,000 to 200,000 cells) was added into each chamber. The chambers were transferred to the lower chamber containing 30% FBS medium. Then 4-24 chambers were incubated in incubator. The chamber was flipped onto the absorbent paper. After the clean medium was abandoned, the non-metastatic cells were removed using cotton swab. 400 μL staining solution was added into 24-well plate. The chamber was soaked in staining solution for 20 min. The cells were stained and transferred under lower surface of the membrane. Then the chamber was soaked in a large cup, and then washed and aired. Microscopy was used to photograph. The resultant cells were then dissolved by 10% acetic acid and detected using OD570.

Statistical analysis

SPSS 17.0 was used for statistical analysis. Completely randomized design diversity means were compared using ANOVA analysis. $P<0.05$ was considered statistically significant.

Results

Tiam1 protein expression in cholangiocarcinoma tissue

The positive rate of Tiam1 protein expression in cholangiocarcinoma tissue was significantly higher than that in benign bile duct tissue ($P<0.001$). The protein product was mainly distributed in cytoplasm. Positive expression was brown yellow (**Table 1**; **Figure 1**).

*Correlation between Tiam1 protein expression and clinical pathological features in cholangiocarcinoma tissue (**Table 2**)*

Tiam1 protein expression had correlations with degree of differentiation, TNM stage and lymph node metastasis of cholangiocarcinoma ($P<0.05$), and had no significant correlations

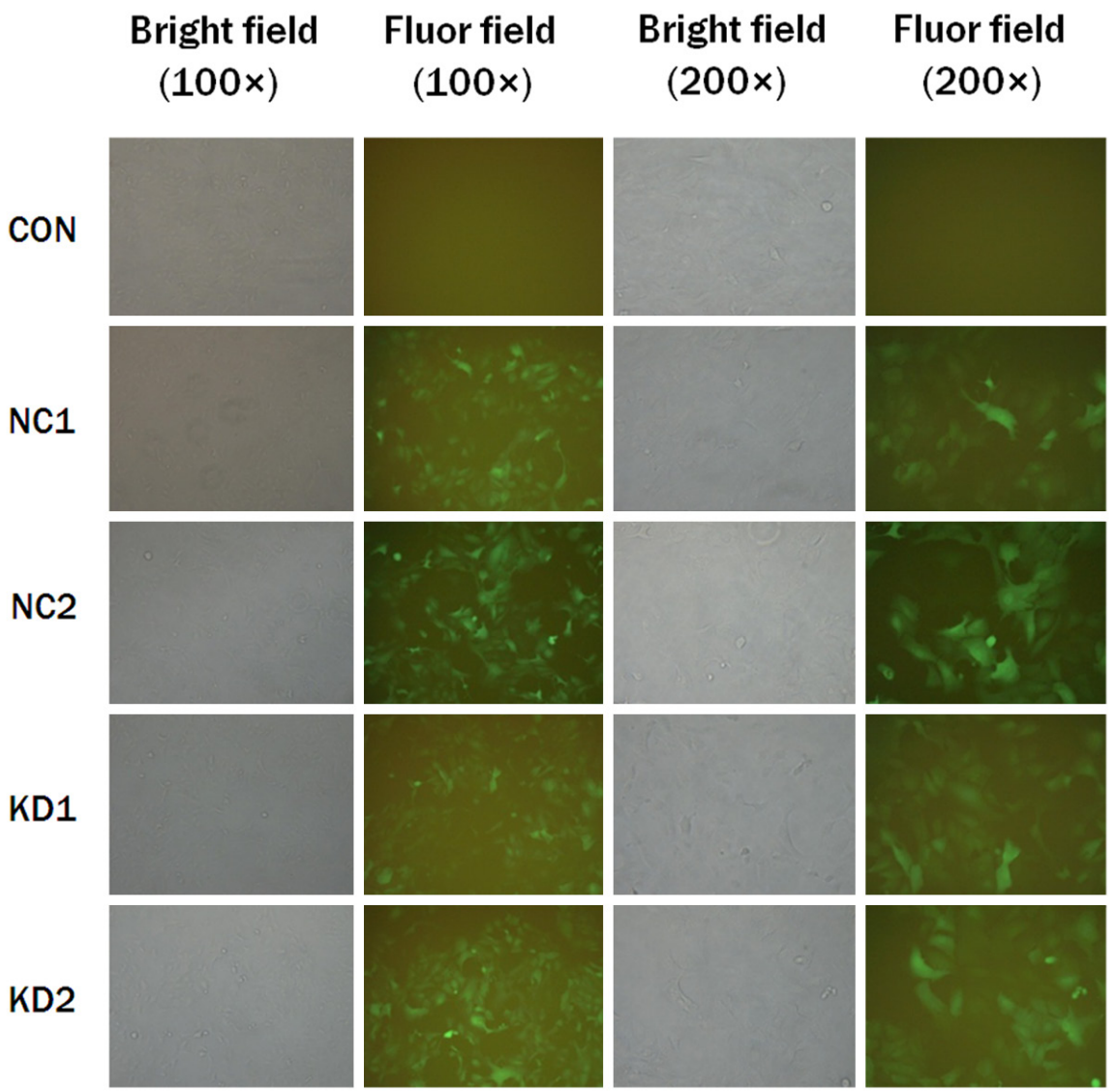

Figure 3. RBE cells displaying green fluorescence after infection of lentivirus (CON: uninfected lentivirus RBE cells; NC: infected Negative control lentivirus RBE cells; KD: infected interfering sequence RBE cells).

with gender, age, and distant metastasis ($P > 0.05$).

Efficiency assessment of lentiviral vector transfecting RBE cells

72 h after RBE human cholangiocarcinoma cells were transfected by lentiviral particles respectively at MOI = 20 and 40, gene expression was observed using fluorescent microscope. Enhanced green fluorescent particles could be observed (**Figure 3**). The transfection efficiency was above 80%. The growth condition of RBE cells was good, indicating that the transfection condition was set stably. The cells were successfully transected and could be used in the follow-up experiments.

Tiam1 RNA expression in RBE cells in each group

Fluorescence quantitative Real-time PCR was used to detect Tiam1 RNA expression in RBE cells in each group. Quantitative PCR amplification curves (**Figure 4**) displayed each of the samples had been entered into the amplification plateau, indicating that the experimental parameters and conditions set accurately.

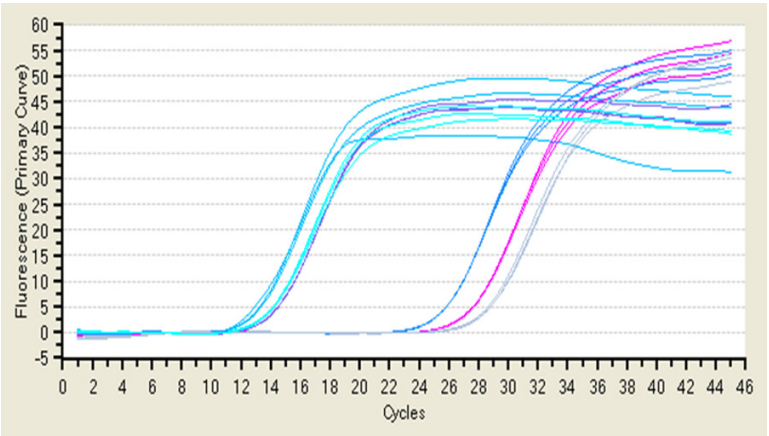

Figure 4. Amplification curve of real-time PCR.

Tiam1 and amplification product dissolving curve (**Figure 5**) of standardized internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed no impure peaks, indicating a single amplification product.

There was significant difference of Tiam1 mRNA expression among the 4 groups (P<0.05) (**Table 6**). In the GV118-Tiam1 shRNA lentiviral particles transfected cells of experimental group (KD1, KD2), Tiam1 mRNA expression was significantly lower than that in negative control (NC) group and blank control (CON) group (P<0.05) (**Figure 6**). This indicated that GV118-Tiam1 shRNA lentiviral vector was effective on silencing Tiam1 gene in RBE cells. There was no significant difference of Tiam1 mRNA expression between NC group and CON group (P = 0.218). In experimental groups, Tiam1 mRNA expression in low MOI (KD1) group was significantly higher than that in high MOI (KD2) group (P<0.05).

Cell cycle distribution detected by flow cytometry

Flow cytometry was used to analyze the RBE cell cycle distribution in each group. The results showed that ratios of cells in stage S in the 3 group were respectively 35.69%, 36.92% and 23.22% (**Table 7**). Statistical analysis showed that in the RBE cells in interference group of targeted silenced Tiam1, the ratio of stage S in cell cycle was significantly lower than that in NC and CON group, with significant difference (P<0.05). This indicated that after the down-regulation of Tiam1 gene, the speed of cell proliferation was inhibited. Simultaneously, in the experimental results, the presence of apoptotic peak was not observed, indicating that Tiam1 gene did not lead to the occurrence of apoptosis after disruption (**Figure 7**).

Detection of cell proliferative capacity using MTT assay

OD value in extracted samples was detected by MTT assay 1-5 days after RBE cells in each group were treated. Cell growth curve in each group was plotted according to detection result (**Figure 8**). In the growth curves, the curve in experimental group was placid, the total cell growth speed was significantly lower than that in CON and NC group (P<0.05). (In day 4 and day 5, the OD value in experimental group was significantly lower than that in CON and NC group (P<0.05), while there was no significant difference between CON group and NC group (P>0.05)). This indicated that after Tiam1 gene in RBE cells was target inhibited, the cell proliferation capacity was inhibited.

Detection of cell migration ability using Transwell

24 h after inoculation, metastatic cells in base of polycarbonate film was stained for observation. The results illustrated that metastatic cells could be observed in base of polycarbonate film of each group (**Figure 9**). The metastasis rate in experimental group was significantly lower than that in CON and NC group (P<0.05). There was no significant difference of metastasis rate between CON group and NC group (**Figure 10**). The results indicated that targeted inhibition of Tiam1 gene expression could significantly inhibit migration ability of RBE cells.

Discussion

Cholangiocarcinoma is not common in gastrointestinal tumors, but there is a growing trend of its incidence rate in recent decades. The etiological agent of cholangiocarcinoma is still not clear enough. In China, the possible causes include hepatitis virus infections, parasitic infections such as clonorchiasis infection, repeated biliary inflammation such as bile duct stones complicating cholangitis and reflux cholangitis, the influence of various carcinogens. Although

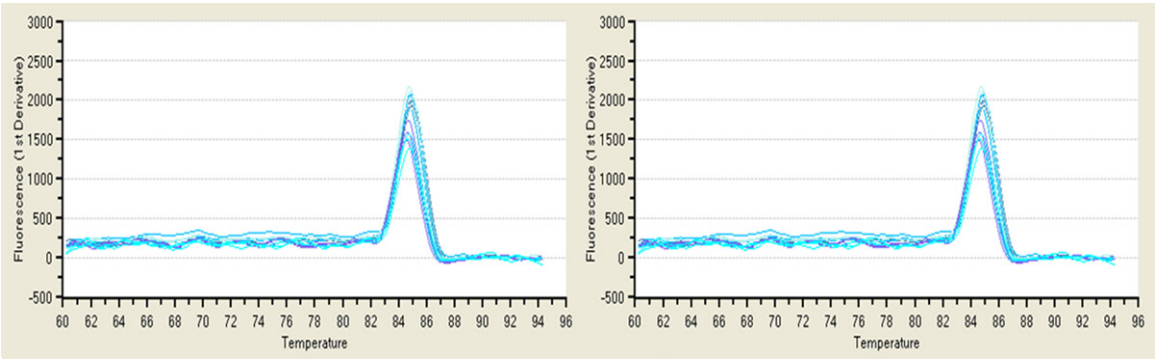

Figure 5. Amplification product dissolution curve (left: GAPDH, right: Tiam1).

Table 6. Relative expression of Tiam1 RNA of RBE cell in each group

Group	*n*	$2^{-\Delta\Delta Ct}$ ($\bar{X}\pm S$)	*F*	*P*
CON	3	0.918±0.045	84.222	0.000
NC	3	1.008±0.153		
KD1	3	0.073±0.013		
KD2	3	0.461±0.398		

the therapeutic effect of cholangiocarcinoma enhanced, the five year survival rate is still not satisfactory. Patients often die from biliary tract infections and hepatic failure. Due to the special anatomical structure of cholangiocarcinoma genesis, the attack of cholangiocarcinoma often reached terminal stage. So the chance of radical surgery is lost. The only therapy of radical cure at present is radical surgery based on early diagnosis. Therefore, early diagnosis of cholangiocarcinoma is especially important. For the diagnosis of cholangiocarcinoma, tumor marker of cholangiocarcinoma is helpful clinically. Tumor markers applied much include CEA, CA125, CA50 and CA199. But these markers may increase in other tumors or inflammations. For instance, CEA and CA125 are not specific tumor marker of cholangiocarcinoma. These 2 indexes might increase in the presence of other gastrointestinal cancers, gynecological cancer and bile duct lesions such as cholangitis and bile duct calculus. So the present applied tumor markers for the early diagnosis of cholangiocarcinoma are not ideal.

Since Tiam1 was first isolated and determined from high invasive mutant of murine T cell lymphoma by The Netherlands Cancer Institute in 1994, it has once become a hot spot in research field of cancer. Many researchers observed the role of Tiam1 gene in a variety of tumor development and progression. In some literatures, Tiam1 expressed high in lung cancer, liver cancer, nasopharyngeal cancer, gastric cancer, esophageal cancer, colorectal cancer and other malignant tumors. And Tiam1 gene was observed to have correlations with invasion and metastasis characteristics of various malignant tumors. But how Tiam1 gene expresses in cholangiocarcinoma and whether it has correlations with invasion and metastasis has not been reported. In this study, immunohistochemistry was used to detect the expression of Tiam1 in cholangiocarcinoma and benign bile duct tissue. The expression intensity of Tiam1 in cholangiocarcinoma relative to benign bile duct tissue was observed. The correlation between expression intensity of Tiam1 protein in cholangiocarcinoma and clinical pathological characteristics was also investigated in order to find the correlations between Tiam1 gene and the occurrence and development of cholangiocarcinoma, thus providing foundation and clues for treating cholangiocarcinoma. Tiam1 transmits pathways by Tiam1-Rac signal, and regulates life activities such as cell cycle progression, cytoskeletal reorganization cell migration and adhesion, playing an important role in the process of tumor growth, invasion and metastasis. Several studies have confirmed that Tiam1 was associated with proliferation, invasion and metastasis of malignant tumors including breast cancer, colorectal cancer, stomach cancer, skin cancer etc. [35-39].

RNA interference affects the function of the target gene by silencing of the target gene expression [40-42]. It is the preferred strategy of gene and protein function study in fundamental

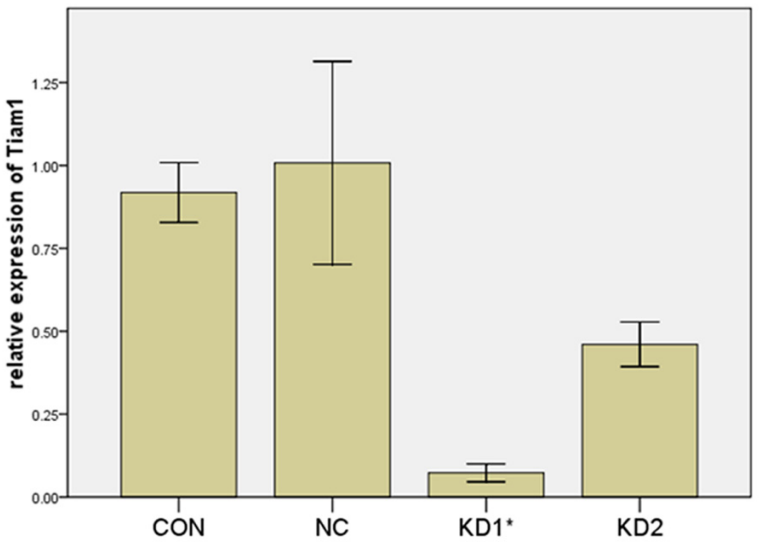

Figure 6. Relative Tiam1 mRNA level of each group cells determined by Real-time PCR. *P<0.05 vs. negative control group (NC), blank control group (CON) and high MOI group (KD2).

Table 7. Distribution of cell cycle in each group cells detected by flow cytometry

Group	*n*	S ($\bar{x}$±s) (%)	F	*P*
CON	3	35.69±5.42	5.780	0.040
NC	3	36.92±7.55		
KD	3	23.22±1.74*		

*Compared with NC and CON group, P<0.05.

research field at present, and is now extending to the field of gene therapy widely [43-47]. In numerous transfer medium of RNA interference experiments, lentiviral vector plays as role because of the metaphase and non-metaphase. It is the ideal choice of RNA interference experiment due to its long-term expression *in vivo* and high security. In this study, lentiviral vector system containing HIV basic elements and other auxiliary components was selected to target silence Tiam1 gene expression in RBE cholangiocarcinoma cell line. The effect of Tiam1 gene on RBE cell proliferation, invasion and metastasis function.

In this study, we found that positive expression rate of Tiam1 was 79.5% in 83 cases of cholangiocarcinoma tissues, which was significantly higher than that in benign bile duct tissue. Moreover, Tiam1 protein expression had no correlations with gender, age, and distant metastasis, but had correlations with tumor differentiation, TNM stage and lymph node metastasis in patients with cholangiocarcinoma. Positive expression rate of Tiam1 in moderately-poorly/poorly differentiated cholangiocarcinoma tissues was significantly higher than that in high/moderately differentiated cholangiocarcinoma. Positive expression rate of Tiam1 in cholangiocarcinoma tissues of stage III and IV was significantly higher than that in cholangiocarcinoma tissues of stage I and II. Positive expression rate of Tiam1 in cholangiocarcinoma tissues with lymph node metastasis was significantly higher than that in tissues without lymph node metastasis. These results indicated that Tiam1 was closely associated with differentiation extent and invasion and metastasis capacity. Though Tiam1 expression in tumor tissues with distant metastasis was higher than that in tissues without distant metastasis, there was no significant difference. This might be related to too small sample size.

Malignant tumor proliferates unlimitedly and abnormally due to the deregulated cell division. Its cell cycle distribution is significantly different from normal cells. By observing cycle distribution of tumor cells, the proliferation capacity of tumor can be determined. Due to the different DNA content in different time phase, additionally DNA content in different time phase can be detected by flow cytometry, the length of cell cycle could be detected using flow cytometry. In this study, the ratio of RBE cells in stage S after Tiam1 gene was silenced by RNA was significantly lower than that in NC group and CON group in which Tiam1 gene was not affected. The result showed that Tiam1 gene involved in the process of promoting RBE cholangiocarcinoma cells proliferation *in vitro.* Moreover, Tiam1 gene changed the cell cycle distribution of cholangiocarcinoma cells. By inhibiting Tiam1 gene, cholangiocarcinoma cell proliferation could be inhibited.

In this study, MTT assay was implemented for 5 days after RBE cholangiocarcinoma cells in

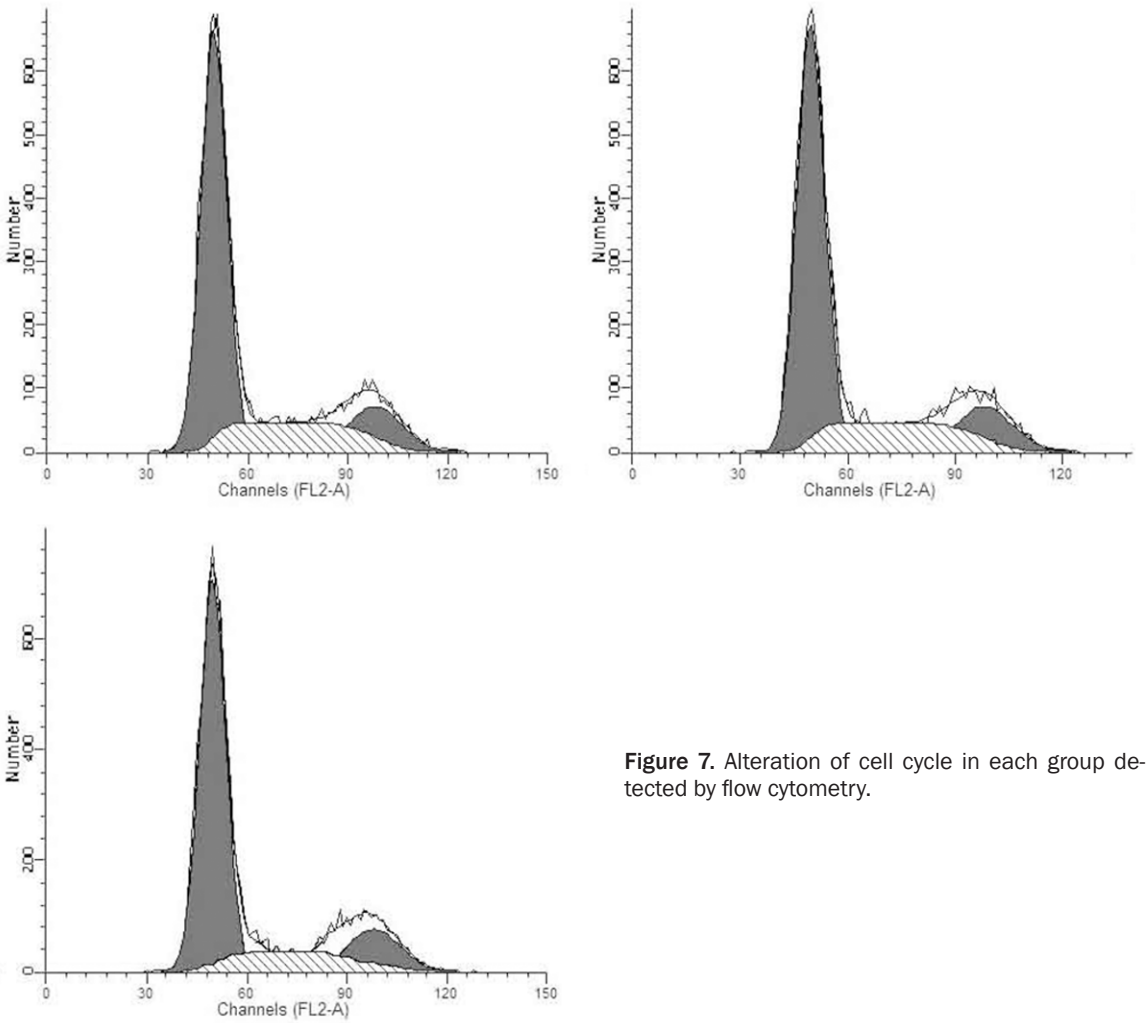

Figure 7. Alteration of cell cycle in each group detected by flow cytometry.

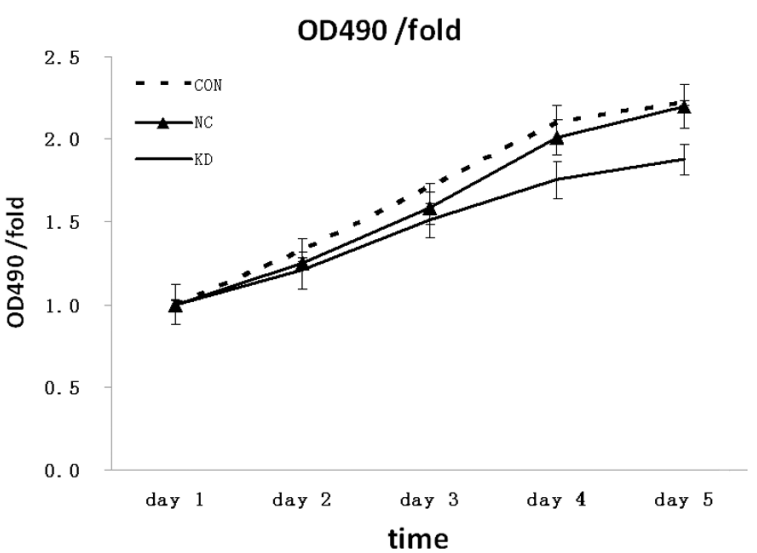

Figure 8. Growth curves of RBE cells in each group in different interval. *P<0.05 vs. negative control group and blank control group.

each group were treated. We found that compared to NC and CON group in which Tiam1 gene expression was not affected, the total growth speed of RBE cells with Tiam1 silenced and interfered by RNA was significantly lower, indicating that Tiam1 gene involved in the process of promoting RBE cholangiocarcinoma cells proliferation *in vitro.* Inhibiting Tiam1 gene expression could decrease the speed of cholangiocarcinoma cells *in vitro.*

All he above results showed that silencing Tiam1 gene expression significantly inhib-

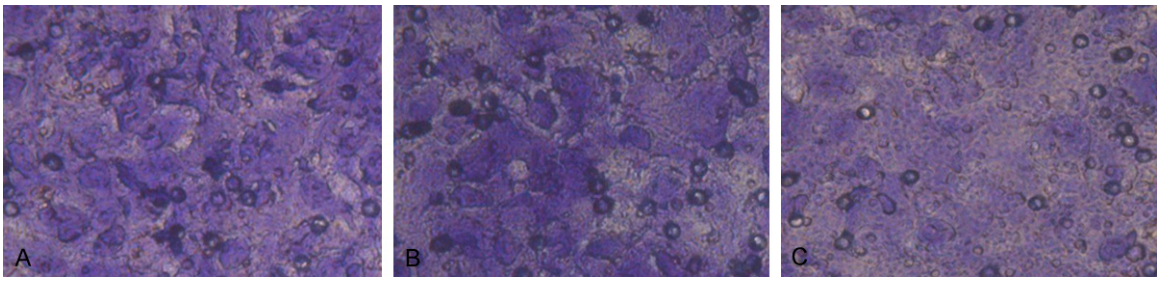

Figure 9. Influence of Tiam1 gene to migratory ability of RBE. A: Blank control group; B: Negative control group; C: Knock-down group.

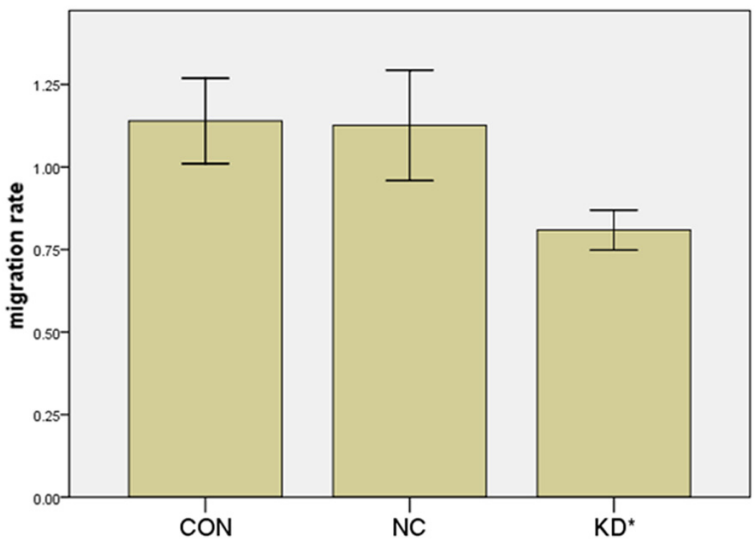

Figure 10. Comparison of migration rate of each group RBE cells. *P<0.05 vs. blank control group and negative control group.

ited the proliferation capacity and speed of RBE cholangiocarcinoma cells *in vitro*. This demonstrated that Tiam1 gene was the relevant gene of promoting cholangiocarcinoma cell proliferation. By inhibiting the expression of Taim1 gene, the cholangiocarcinoma cell proliferation could be inhibited.

Metastasis is one of the important manifestations of malignant tumor. The invasion and metastasis of malignant tumor is the result of the interaction among tumor cells, host cells and neighboring interstitial structures involving multiple mechanisms and steps. Tumor cell migration is an important indicator of tumor metastasis. Metastasis ability of tumor can be detected by cell migration.

When Tiam1 gene was first determined in 1994, it was taken seriously because in could enhance cell invasion capacity when transfected into T lymph cells [14]. Motility of tumor cells is closely related with the changes in the cytoskeleton. Tiam1 could promote cell integrin aggregation by activating rac, thus regulating specificity of actin cytoskeleton. And then it affects assembly and movement of cytoskeleton, inducing invasion and metastasis of tumor cells. Cell membrane fold is an important indicator of tumor cell motility and metastasis. The impact of Tiam1 on tumor invasion and metastasis ability manifests in that it can induce cell membrane fold with participation of PH functional area. The induced cell membrane fold plays an important role in tumor metastasis. Simultaneously, the invasion and metastasis capacity of tumor cells is closely related to its motility and adhesion ability to extracellular matrix. Tiam1 involves in regulating affinity of adhesion molecules and assembly of adhesion complexes by activating Rho, thus promoting integrin-mediated interaction between atypical cells and between cells and matrix, and finally playing its facilitating role in tumor cell invasion and metastasis. Transwell invasion assay detects migration ability of cells using the tendency of tumor cells to components such as some cytokines in serum. Transwell chamber is used to isolate the cell culture and the medium containing serum. The metastasis capacity of tumor is measured by observing metastatic cells migration and adhesion to the base of polycarbonate film. In this study, after 24 h of inoculation in Transwell,

tumor cell metastasis could be observed in each group. Compared to NC and CON group with non-interfered Tiam1, migration rate of RBE cell with silenced Tiam1 by RNA significantly decreased.

In summary, proliferation and metastasis of tumor cells is the most important cause of cancer hazard. In this study, Tiam1 gene in cholangiocarcinoma cells was targeted inhibited using RNA interference to observe its proliferation and metastasis capacity. The result showed that Tiam1 gene had promoting effect on the process of proliferation and metastasis in cholangiocarcinoma. Inhibiting Tima1 gene expression could slow down the process of proliferation and metastasis of tumor cells. Tiam1 protein expression might be a hint to the diagnosis of cholangiocarcinoma and be a target of gene therapy against cancer.

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Disclosure of conflict of interest

None.

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