Int J Clin Exp Pathol 2016;9(11):11188-11196
www.ijcep.com /ISSN:1936-2625/IJCEP0031119

Original Article

Lentivirus-mediated RhoT1 down-expression inhibits pancreatic cancer cells proliferation, migration and invasion, and induces apoptosis

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Received April 24, 2016; Accepted September 8, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: The aim of this study was to construct a lentivirus vector targeting RhoT1 gene and to investigate the functional role of RhoT1 in pancreatic cancer cell line BXPC3. Firstly, we constructed a lentivirus plasmid of RNA interference targeting RhoT1. The expression of RhoT1 mRNA and protein was analyzed by qRT-PCR and Western blot. The CCK-8 cell proliferation assay, flow cytometry, wound healing assay and transwell assay were adopted to assess the effect of RhoT1 on cell proliferation, apoptosis and invasion ability. In vitro functional study indicated that down-expression of RhoT1 could significantly inhibit the proliferation, migration, and invasion of BXPC3 pancreatic carcinoma cells while promoting apoptosis (all $P<0.01$). In conclusion, Recombinant lentivirus vector expressing human RhoT1 gene was successfully constructed and pancreatic cancer cell line with stable shRNA expression targeting RhoT1 gene was established, which could provide a cell model for the further experiment, and our data indicated that RhoT1 shRNA played an important role in the inhibition of pancreatic cancer cells.

Keywords: Pancreatic cancer, RhoT1, RNA interference, lentiviral vector

Introduction

Pancreatic cancer ranks fourth in cancer-related causes of death [1]. It was estimated that 43140 Americans were diagnosed and 36800 patients died of pancreatic cancer in the United States in 2010 [2]. Despite extensive clinical and scientific efforts, the prognosis of this disease has not improved significantly over the last 20 years [2, 3]. The median survival time is 6 months after diagnosis without treatment [4]. Cancer cell invasion and metastasis are critical steps in cancer progression, and are the main causes of poor survival in patients with pancreatic cancer.

To date, the molecular mechanism of metastasis has not been well expounded. Cancer cell metastasis involves a series of changes in cell behavior, and leads to local tissue invasion, migration through extracellular matrix, entries into the vascular or lymphatic system. Studies have shown that the Rho family of GTPases regulates cell cytoskeleton organization, adhesion, migration and proliferation and thereby promotes the migration of tumor cells [5]. Rho GTPases contains more than twenty genes in humans, of which RhoA, RhoB, RhoC, Rac1 and Cdc42 are the best characterized members of Rho GTPases. Proteins of the Rho GTPase family can stimulate cell cycle progression and regulate gene transcription, suggesting that these Rho GTPases may have pro-oncogenic properties [6]. Furthermore, abnormal expressions of those proteins have been discovered in advanced malignant tumor and metastases with prognostic relevance in diverse cancers. However, the underlying molecular mechanism is not fully understood. The classical Rho GTPases have been reported to contribute to most steps of tumor cell invasion and metastasis by regulating the cycling between inactive GDP-bound and active GTP-bound forms, However, it should be stressed

that some Rho GTPase members have not been studied as intensively in human tumor or in mouse tumor models. Atypical members of the Rho GTPase family have additional sequence elements, which indicate that they can demonstrate other regulatory mechanisms. Among these members, we will focus our attention on RhoT1, which belongs to the mitochondrial Rho GTPase family. RhoT1 protein is classified as atypical GTPases first reported by Fransson in 2003 [7]. Previous studies have indicated that RhoT1 is required for controlling anterograde transport of mitochondria and the subcellular distribution. Recently, a study published in Cell Reports showed that mitochondrial phenotype associated with superoxide production which could promote the tumor cell migration and invasion. Therefore, mitochondrial superoxide scavenging has thus been proposed as promising target to prevent tumor progression and metastasis [8]. Previously, we used Agilent Gene Chip technology to detect gene expression differences between pancreatic cancer tissues and adjacent non-cancerous tissues, to screen the differentially expressed genes associated with pancreatic cancer. The results showed that there was a significant difference in the expression of RhoT1 between cancer and paracancerous tissues [9]. However, little is known about molecular mechanism of RhoT1 in cancer progression. We are interested in knowing whether the RhoT1 is similarly involved in the development of cancer.

Therefore, we have carried out this study to construct the recombinant lentiviral vector containing human RhoT1 gene and to examine its expression in 293T ceils, which can offer experimental data for illuminating molecular mechanisms of pancreatic tumorigenesis, tumor development, invasion and metastasis, and identification of candidate molecular marker for prognostic prediction and potential target of gene therapy.

Materials and methods

Cell culture

PANC-1, SW1990, CAPANC-1, BXPC3 pancreatic carcinoma cell lines were obtained from the Chinese Academy of Sciences in Shanghai. Monolayer cultures of cells were cultured in ATCC-formulated RPMI-1640 base medium containing 10% fetal bovine serum. The cells were maintained at 37°C and in a 5% CO_2 humidified atmosphere.

Materials

DH5a competent cell was obtained from Transgene Biotek Ltd (Bollaram, India), Plasmid DNA Extract kit was obtained from Omega Bio-Tek Inc (Norcross, GA, USA), Liposome transfection reagent kit was obtained from Invitrogen Corporation (Carlsbad, CA, USA), Lentivirus was purchased from Shanghai Genechem Inc. (Shanghai, China). PLKO.1-EGFP (lentivirus core plasmid), VSV-G and pol/gag were obtained from Addgen Company (Cambridge, MA, USA), 293T cell was obtained from ATCC (American type culture collection, Manassas, VA, USA). LipofectAMINE 2000 was from Invitrogen (Carlsbad, CA, USA).

Lentivirus vector construction for RNAi

The lentivirus vector system is composed of the vectors PLKO.1-EGFP, VSV-G and pol/gag. The plasmid PLKO.1-EGFP stably expressed siRNA and a marker (DS5000). The vectors VSV-G and pol/gag contain virus package imperative elements. The siRNA sequences targeting the human RhoT1 (Gene ID, 303351) transcript were designed using the software developed by Ambion Inc. (Foster, CA, USA). Recombinant lentiviruses were produced by transfecting 293T cells with the lentiviral expression plasmid CN362. 293T cells (66105) were cultured in a 10-cm petri dishes with polybrene (1 mg/ml) (GIBCO, USA). Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) when the cell density reached a confluency of 90%. 293T cells were maintained at 37°C in a humidified incubator of 5% CO_2. Infectious lentiviruses were harvested at 48 hours post-transfection and then concentrated. The infectious titer was determined by real-time quantitative PCR. The experiment is divided into three groups: siRNA group, blank plasmid group and negative control groups.

RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from patient specimens by the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Germany). Quantitative Real-time RT-PCR analysis was done as described. Primer sets used were as

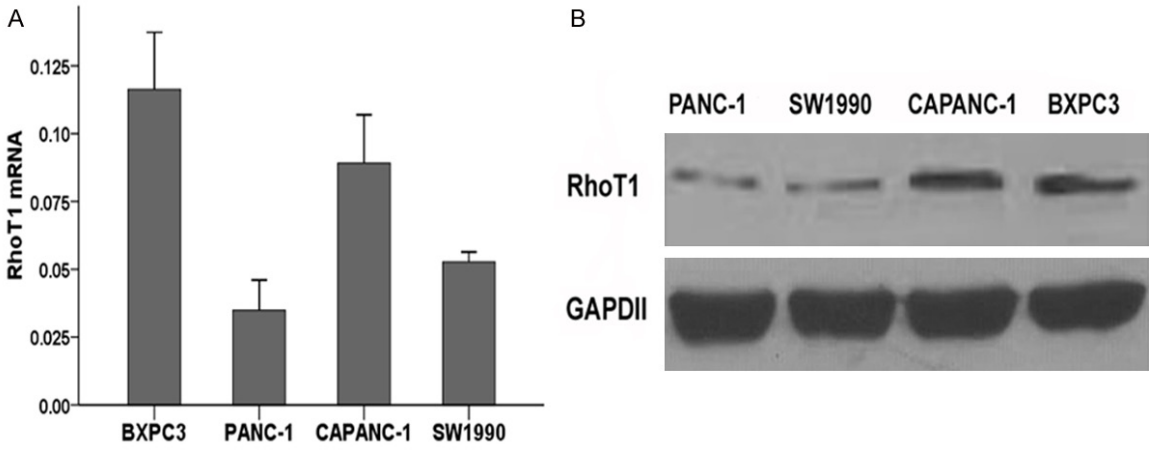

Figure 1. Representative Real-time quantitative PCR (A) and Western blot analysis (B) of RhoT1 expression in pancreatic cancer cell PANC-1, SW1990, CAPANC-1 and BXPC3.

follows: Primer (+): 5'-CAAGGTCGGGCAGGAAGAG-3'; Primer (-): 5'-TAGAAGGCACAGTCGAGG-3'.

Western blot analysis

Western blot analysis was performed as we previously described [10]. Briefly, cells were scraped in lysis solution containing protease and phosphatase inhibitors. Total cellular proteins (10-15 μg) were resolved on 10% SDS--polyacrylamide gel electrophoresis and transferred onto nitrocellulose filter membrane (Millipore Corporation, Billerica, MA, USA). After blocking the membrane was probed by immunoblot using antibodies for RhoT1 (1:1000, Abcam company, Cambridge, MA, USA) and GAPDH (1:1500, Cell Signaling Technology, Inc. Danvers, MA, USA) at 4°C overnight. Immunodetection was performed with the ECL Western Blotting KIT (Millipore Corporation, Billerica, MA, USA). The band images were detected with an enhanced chemiluminescence system (YHMED, shantou, guangdong, China).

CCK-8 cell proliferation test

Cell proliferation speed was analyzed by CCK-8 (Dojindo, Gaithersburg, MD) according to the manufacturer's protocol. Briefly, cells were seeded at a approximately density of 1×10^4 in 96-well plates. CCK-8 reagent was added at 0, 12, 24, 48 and 72 h. The plates were incubated for 1 h at 37°C in 5% CO_2. The absorbance values at 450 nm were measured with a microplate reader (Labsystems, Helsinki, Finland).

Cell apoptosis detection

Cell apoptosis detection was detected with Annexin V-FITC/propidium iodide (PI) according to the manufacturer's protocol. Briefly, Cells were trypsinized and counted, seeded at 1×10^5 cells/well. Harvest the cells after the incubation period and wash in cold phosphate-buffered saline (PBS). The cells were re-suspended in binding buffer and incubated for 15 min at room temperature in the dark after Annexin V/FITC solution and propidium iodide solution were added. Cells were subsequently analyzed by a flow cytometer (Becton Dickinson, USA).

Migration and invasion assay

For cell migration and invasion assays, the transwell chamber (8 μm pore size polycarbonate membrane; Corning, USA) was placed into a 24-well plate and used to analyze the cell motility. The upper surface of the membrane was pre-coated with matrigel. The cells in each group were detached by trypsinization, re-suspended in serum-free medium, and then added to the upper chamber at 1×10^5 cells/well. Subsequently, 10% FBS was added to the lower chamber as a chemoattractant. After 24 hours of incubation, the non-motile cells on the upper chamber were scraped away with cotton swabs, and invading cells that migrated to the lower chamber were fixed with paraformaldehyde and stained with crystal violet hydrate solution (JRDUN Biotechnology Co., Ltd., Shanghai, China). The number of cells was photographed and counted under an inverted microscope.

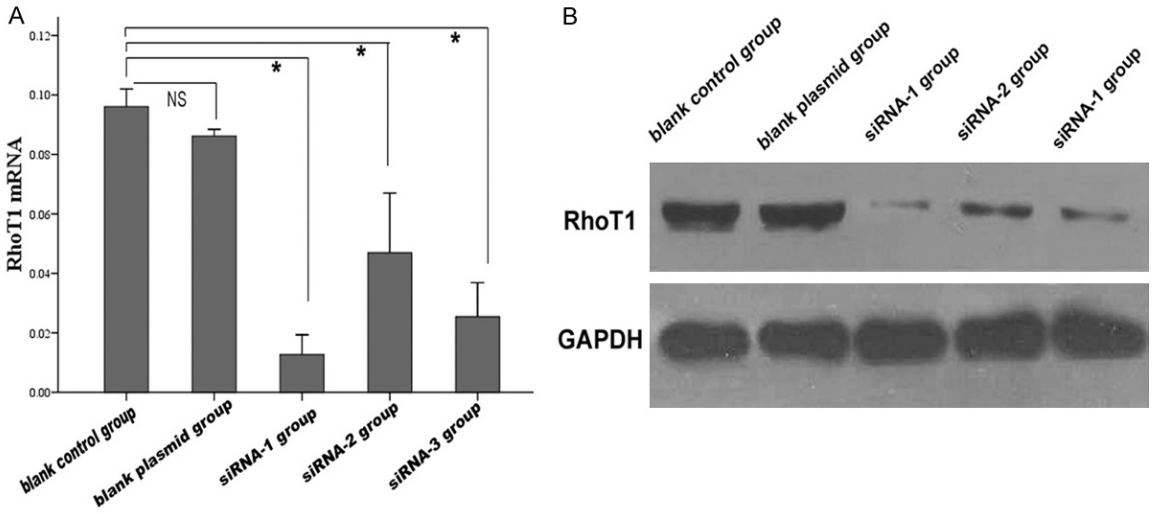

Figure 2. A. The mRNA expression level of RhoT1 by lentiviruses transfection was detected by qRT-PCR. The RhoT1 mRNA expression levels in siRNA groups were obviously decreased (siRNA-1, siRNA-2, siRNA-3 were 0.1327±0.03022, 0.49±0.09184, 0.2654±0.05327 respectively). *P<0.001, compared with blank control group and blank plasmid group. NS, non-significant. B. The protein expression level of RhoT1 by lentiviruses transfection was detected western blot. The RhoT1 protein expression levels in siRNA groups were obviously decreased (siRNA-1, siRNA-2, siRNA-3 were 0.0807±0.0227, 0.2101±0.05918 and 0.1170±0.03295 respectively), significantly decreased as compared with that of the blank control group and blank plasmid group (P<0.05).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Differences between groups were examined by one-way ANOVA with LSD post hoc test. Statistical analysis was conducted using the SPSS software package (SPSS Inc, Chicago, IL, USA, version 17.0). P< 0.05 was considered significant.

Results

RhoT1 gene expression of four strains of pancreatic cancer cell lines

Real-time quantitative PCR and Western blot were used to detect the expression of mRNA and protein of RhoT1 in pancreatic cancer cell PANC-1, SW1990, CAPANC-1 and BXPC3. The result showed that RhoT1 expression in BXPC3 cell was the highest (**Figure 1**), which would be subsequently chosen for the further experiment study.

Construction and identification of lentivirus vector interfering with RhoT1 gene

The gene fragments were obtained during double enzyme digestion of AgeII and EcoI. positive clone stripe size of 335 bp, negative clone stripe size of 322 bp. Results in line with expectations, it was verified that the target gene was correctly inserted into PLKO.1-EGFP lentivirus vector by endonuclease cutting, PCR and gene sequence analysis. DNA sequencing data showed that RhoT1 interference expression vector was constructed successfully. The recombinant lentivirus vectors were packaged and amplified in 293T cells to gain RhoT1 down-expression recombinant vectors. The recombinant was transferred into 293T cells and RhoT1 protein was identified. The expression of green fluorescence protein in infected 293T cell line was observed under a fluorescent microscope.

Lentivirus-mediated siRNA inhibited the expression of RhoT1 in Bxpc3 cells

The effect of siRNA on the mRNA and protein levels of RhoT1 was examined by real-time quantitative RT-PCR and western blot analysis by using GAPDH as internal controls, and then normalization processing was performed for data. The Bxpc3 cells transfected with the lentiviral vector-RhoT1-siRNA were taken as siRNA group, and those cells treated with the control virus and without any treatment

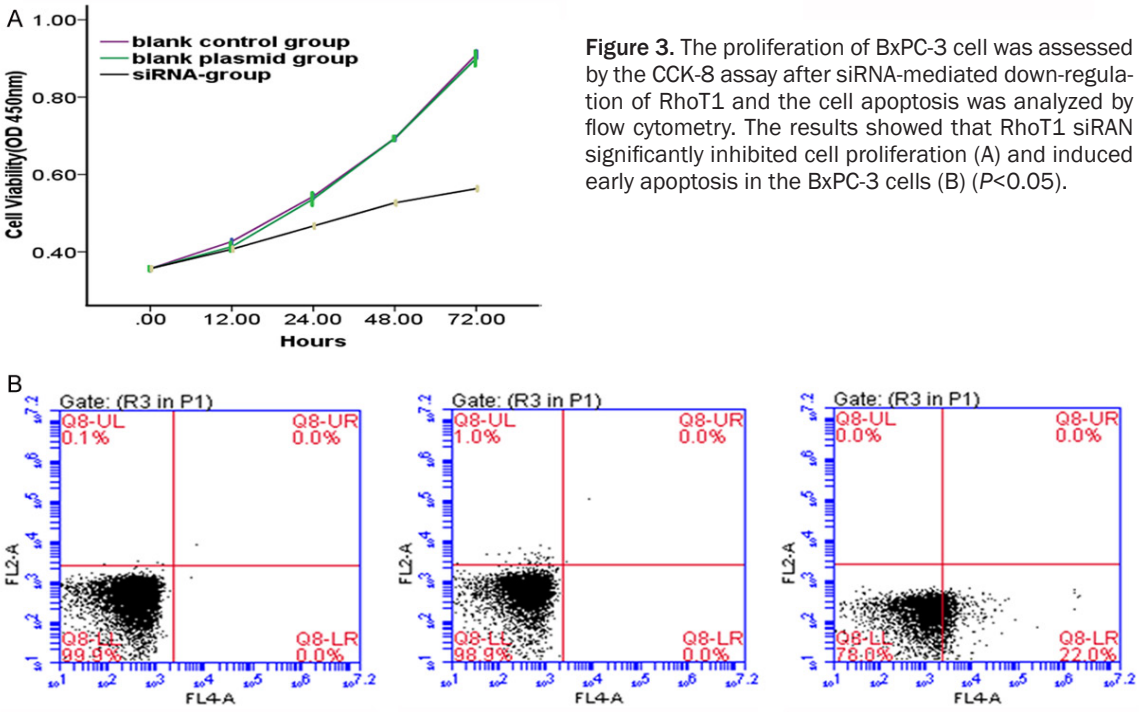

Figure 3. The proliferation of BxPC-3 cell was assessed by the CCK-8 assay after siRNA-mediated down-regulation of RhoT1 and the cell apoptosis was analyzed by flow cytometry. The results showed that RhoT1 siRAN significantly inhibited cell proliferation (A) and induced early apoptosis in the BxPC-3 cells (B) (P<0.05).

were taken as blank plasmid group and blank control group. Data was relative to the expression of GAPDH. The RhoT1 mRNA expression in blank plasmid group was 0.897± 0.01443, while siRNA groups were obviously decreased (siRNA-1, siRNA-2, siRNA-3 were 0.1327±0.03022, 0.49±0.09184, 0.2654± 0.05327 respectively), with statistically significant difference from the blank control group (all P<0.001) (**Figure 2A**). RhoT1 protein expression in the siRNA groups were obviously decreased also (siRNA-1, siRNA-2, siRNA-3 were 0.0807±0.0227, 0.2101±0.05918 and 0.1170±0.03295 respectively), significantly decreased as compared with that of the blank plasmid group (P<0.05) (**Figure 2B**).

RhoT1 promotes the proliferation of Bxpc3 cells

To evaluate the influence of RhoT1 in regulating the proliferation of BxPC-3 cells, CCK-8 cell proliferation assay were performed at 74 h post-transfection. As shown in **Figure 3A**, at 1 day after transfection, cell proliferation was significantly inhibited in the RhoT1 siRAN-group when compared with the blank control group and blank plasmid group (P<0.05). The inhibition rate peaked at 72 hours. There was no significant difference between blank control group and blank plasmid group.

RhoT1 down-expression promotes apoptosis

The ability of RhoT1 to impact the apoptosis was analyzed by flow cytometry 24 h after transfected. The results of flow cytometry showed that RhoT1 siRAN induced early apoptosis in the BxPC-3 cells, and the early apoptosis rate was 20.27%±1.8%, which was significantly different from the blank control group and blank plasmid group (P<0.05) (**Figure 3B**).

RhoT1 down-expression inhibits cell mobility, migration and invasion

To evaluate the role of RhoT1 in pancreatic cancer progression, effects of siRNA-mediated downregulation of RhoT1 on pancreatic cancer BxPC-3 cell migration and invasion were studied. The wound healing rates (%) of control group, negative control (NC) group and siRNA group were 15.9±2.3, 13.8±2.2, 21.3± 1.6 respectively after treatment with 24 h, and were 2.9±0.36, 1.7±0.27, 14.6±0.07 after treatment with 48 h. The wound healing assay showed that RhoT1 silence significantly inhibited BxPC-3 cell mobility (P<0.05) (**Figure 4**). Transwell assay were performed after transfection of RhoT1, the result showed that the number of invaded cells in blank control group, blank plasmid group and siRNA group were

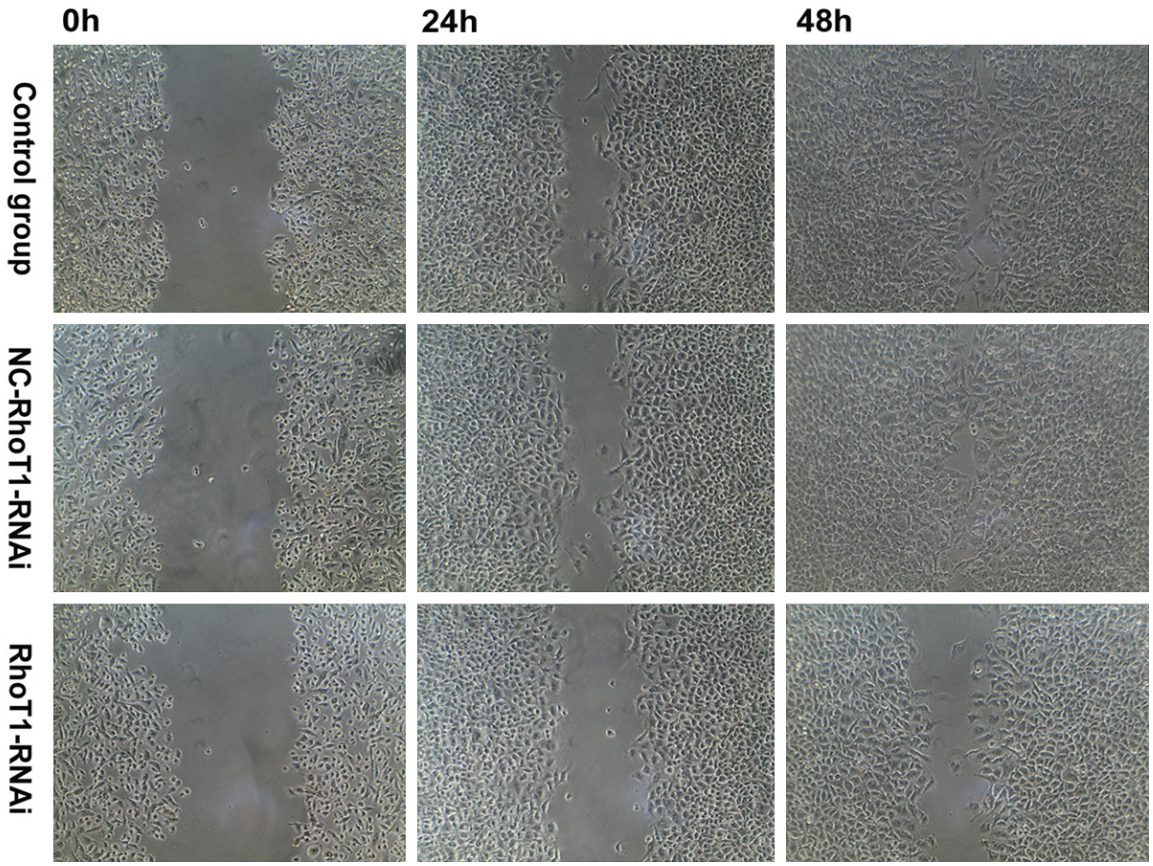

Figure 4. Cell migration was assessed by a wound healing assay, the wound healing rates (%) of control group, negative control (NC) group and siRNA group were 15.9±2.3, 13.8±2.2, 21.3±1.6 respectively after treatment with 24 h, and were 2.9±0.36, 1.7±0.27, 14.6±0.07 after treatment with 48 h. There were all significantly different between siRNA group and control groups (P<0.05). Wound healing assay showed that RhoT1 silence significantly inhibited BxPC-3 cell mobility.

761±8.39, 769±7.13, and 432±8.15, respectively. The numbers of migrated cells through the membranes in the siRNA group were significantly lower than those in blank control group and blank plasmid group (all P<0.01) (**Figure 5**). The results showed that RNAi silencing RhoT1 could inhibit migration and invasion of the BxPC-3 cells.

Discussion

In present study, we used lentivirus-mediated RNAi to inhibit RhoT1 expression in 293T cells and investigated the effect of RhoT1 down-expression in pancreatic cancer cell lines Bxpc3. The expression level of RhoT1 gene by lentiviruses transfection was detected by RT-PCR and western blot. The results showed that lentiviral RNAi vector of RhoT1 had been successfully constructed and identified, which cloud lay a foundation for the further research on the function of RhoT1 in the development of pancreatic cancer. Subsequently, CCK-8 cell proliferation assay, flow cytometry, wound healing assay and transwell assay were performed, our data indicated siRNA-mediated down-regulation of RhoT1 could induce cell apoptosis and inhibit cell proliferation, migration and invasion of Bxpc3 cell *in vitro*.

RhoT1 is a member of the family of Ras-homologous (Rho) GTPases. Rho GTPases have been reported to regulate cell polarity and motility through their effects on the cytoskeleton, membrane trafficking and cell adhesion, and contribute to most steps of cancer initiation and progression [11-14]. The Rho family of GTPases contains 20 members. Most of what is known regarding the role of Rho GTPases in cancer cell invasion comes from the study of

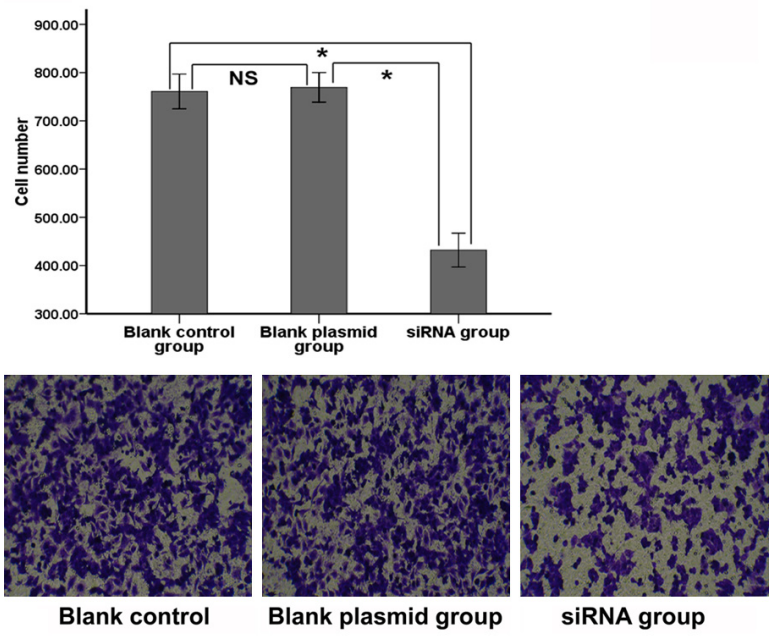

Figure 5. Transwell assay were performed after transfection of RhoT1, the result showed that the number of migrated cells in blank control group, blank plasmid group and siRNA group were 761±8.39, 769±7.13, and 432±8.15, respectively. The numbers of migrated cells through the membranes in the siRNA group were significantly lower than those in blank control group and blank plasmid group (all $P<0.01$). *$P<0.01$.

the prototypic members RhoA, RhoB and RhoC, Rac1 and Cdc42 [15]. However, little is known about the functional role of RhoT1 in human cancer tumorigenesis, metastasis and progression. RhoT1 is the known mitochondrial outer membrane protein, classified as atypical GTPases because it is not regulated as the other classical GTPases. Previous studies reported that RhoT1 played a critical role in kinesin-mediated mitochondrial movement linking mitochondria and motor proteins and trafficking along microtubules, and influences mitochondrial positioning through the regulation of mitochondrial movement.

Recently, the Rho GTPases was well-established regulators of cell migration, and had been implicated in the process of tumor cell invasion and metastasis [16-21]. Lots of pervious studies showed that over-expression of RhoA and RhoC in tumors was associated with lymph node metastasis and invasion [22, 23]. However, low-expression of RhoB was inversely correlated with tumor aggressiveness [24-27]. In this study, we have examined the role of RhoT1 on Bxpc3 pancreatic cancer cell lines by performing loss of function assays. The experimentally functional study has indicated that down-regulation of RhoT1 suppresses the proliferation, migration, and invasion and induces cell apoptosis of Bxpc3 cells, which is consistent with previous reports. A previous study used RNA interference technology to investigate CXCL12-induced migration of control and RhoT1-silenced cells in modified Boyden chemotaxis chambers. The results showed RhoT1-silenced cells migrated slowly along the chemotactic gradient [28]. Down-expression of this GTPase also disrupted the directionality and inhibited migration of T-cell in 3D collagen gels. These results suggested an important role of RhoT1 in the control of lymphocyte adhesion and migration. RhoT1 function has been examined previously in neuronal cells, where cancer cells can be distant from each other. Studies have shown that impaired mitochondrial maintenance is closely related to a range of human pathologies, including cancer. Therefore, abnormal regulation of the interaction of mitochondria with the cytoskeleton might be an essential mechanism leading to RhoT1 deficiency in the development of pancreatic cancer. Further studies are necessary to explore the underlying mechanism.

In conclusion, the result of the present study showed that recombinant lentivirus vector targeting RhoT1 was successfully constructed, which cloud provide a cell model for the further experiment. Moreover, functional study indicated that RhoT1 was associated with pancreatic cancer cell migration and invasion, suggesting that RhoT1 was implicated in development of pancreatic cancer.

Acknowledgements

This study is supported by the Tongji University youth talent training action plan project (Grant No: 2013KJ088), and supported by China Na-

tional 863 Project Foundation for Cancer Genomics (Pancreas Genomics) (Grant No: 1006-AA02A302).

Disclosure of conflict of interest

None.

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