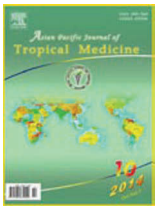
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60136-X

Lentivirus vectors construction of SiRNA targeting interference *GPC3* gene and its biological effects on liver cancer cell lines Huh-7

Chang-Jiang Lei[1#], Chun Yao[2#], Qing-Yun Pan[3], Hao-Cheng Long[1], Lei Li[1], Shu-Ping Zheng[4*], Cheng Zeng[1], Jian-Bin Huang[1]

[1]*Department of General Surgery, the Second Affiliated Hospital of Jianghan University (Wuhan City Fifth Hospital), Wuhan, Hubei, China*

[2]*Wuhan Institute of Hematology, the Second Affiliated Hospital of Jianghan University (Wuhan City Fifth Hospital), Wuhan, Hubei, China*

[3]*Integrated Department, Branch of Jianqiao, Hanyang Affiliated Hospital of Wuhan University of Science and Technology, Wuhan, Hubei, China*

[4]*Department of Pediatrics, the Second Affiliated Hospital of Jianghan University (Wuhan City Fifth Hospital), Wuhan, Hubei, China*

ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form 15 August 2014
Accepted 15 September 2014
Available online 20 October 2014

Keywords:
GPC3 gene
Slow virus carrier
Liver cancer cell lines
RNA interference

ABSTRACT

Objective: To build *GPC3* gene short hairpin interference RNA (shRNA) slow virus vector, observe expression of Huh-7 *GPC3* gene in human liver cell line proliferation apoptosis and the effect of *GPC3* gene influencing on liver cancer cell growth, and provide theoretical basis for gene therapy of liver cancer. **Methods:** Hepatocellular carcinoma cell line Huh-7 was transfected by a RNA interference technique. *GPC3* gene expression in a variety of liver cancer cell lines was detected by fluorescence quantitative PCR. Targeted *GPC3* gene sequences of small interfering RNA (siRNA) PGC-shRNA-GPC3 were restructured. Stable expression cell lines of siRNA were screened and established with the help of liposomes (lipofectamine^{TM2000}) as carrier transfection of human liver cell lines. In order to validate siRNA interference efficiency, GPC3 siRNA mRNA expression was detected after transfection by using RT-PCR and Western blot. The absorbance value of the cells of blank group, untransfection group and transfection group, the cell cycle and cell apoptosis were calculated, and effects of *GPC3* gene on Huh-7 cell proliferation and apoptosis were observed. **Results:** In the liver cancer cell lines Huh-7, *GPC3* gene showed high expression. PGC-shRNA-GPC3 recombinant plasmid was constructed successfully via sequencing validation. Stable recombinant plasmid transfected into liver cancer cell lines Huh-7 can obviously inhibit GPC3 mRNA expression level. **Conclusions:** The targeted GPC3 siRNA can effectively inhibit the expression of GPC3.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumor. More than 500 000 people are diagnosed with liver cancer worldwide each year. The pathogenesis of liver cancer is concealed, its progress is rapid, its mortality rate is high, and its treatment is difficult. Patients diagnosed with liver cancer are confirmed in its advanced stage and lost the best time for treatment[1-3]. Therefore, it is critical to understand the mechanism of HCC for targeted therapy. The formation of tumor is a multi-step, multi-stage pathological process, including the activation of oncogene and inactivation of tumor suppressor gene, the formation of capillaries, and immune escape of the monitor, *etc*. Studies confirm that there is a link between the balance relationship of cell proliferation and apoptosis and tumor occurrence and development[4-6].

Phosphatidyl inositol proteoglycan- 3 (GPC-3), a member of phosphatidyl inositol proteoglycan family, is an carcinoma embryo protein. Connected to the cell membrane through glycosyl phosphatidyl inositol, it has the effect of promoting cell growth and differentiation in

*Corresponding author: Shu-Ping Zheng, Department of Pediatrics, the Second Affiliated Hospital of Jianghan University (Wuhan City Fifth Hospital), Xianzheng Street No. 122, Hanyang District, Wuhan, Hubei, China.
Tel: 18271483056
E-mail: asdasq555@126.com
#These two authors contributed equally to this work.

Foundation project: It is supported by Wuhan Municipal Science and Technology Bureau of applied basic research project (No. 2013062301010823); Wuhan City health planning medical research project (No. WX14A11).

the process of the development of tumor. In recent years, the biological characteristics of tumor related cytokines research become a hot spot of clinical researches[7-10]. But whether the expression of GPC-3 has inhibitory effect on liver cancer cell proliferation is not yet clearly defined. This study aims to build GPC-3 plasmid by RNA interference technique and explore the transcription effects of silent *GPC*-3 gene on human liver cancer proliferation and migration.

2. Materials and methods

2.1. Material

Liver cancer cell lines Huh-7 (Shanghai Cell Bank, Chinese Academy of Sciences), TRIzol (Invitrogen Corporation), PCR kit (TaKaRa, Japan), reverse transcription kits (ABI Applied Biosystems, USA), Annexin V/PI double staining streaming method apoptosis detection kit (Bender Company), fetal bovine serum (Gibco BRL, USA), high glucose DMEM medium (Gibco BRL, USA), RNA electrophoresis liquid (Kang, China), trypsin (Invitrogen Corporation), the slow virus carrier system (Shanghai Jikai Gene Chemical Technology Co. Ltd.), restriction enzyme, T4DNA ligase (New England Biolabs), flow cytometry instrument, automatic enzyme standard instrument (BIO-RAD550), plasmid extraction kit (Qiagen), lipofectamine^{TM2000} and Opti-MEM medium (Invitrogen Corporation) were used in the present study.

2.2. Method

2.2.1. Cell culture

The mixture containing 15% fetal bovine serum, DMEM medium and 1% PS nutrient solution was prepared. Huh-7 cryopreserved tubes were removed from liquid nitrogen tank, quickly put into the 37 ℃ water bath for rewarming and thawing. About 10 mL culture was drawn from super clean bench and added to centrifuge tube. After the thaw of Huh-7, defrost liquid was drawn and added to the centrifugal tube, beated until blended, sealed, and centrifugated at 800 r/min for 5 min. The supernatant was abandoned, then it was added with nutrient solution for dilution, moved into a culture flask and cultured at 37 ℃ in 5% CO_2 incubator. The next day the liquid was changed. When Huh-7 grew and fused into a single, it was washed with phosphate buffer solution (PBS) for three times, digested and subcultured by 0.25% trypsin and 0.02% ethylene diamine tetraacetic acid.

2.2.2. GPC short hairpin RNA (shRNA) expression vector construction

According to the gene information of GPC in GenBank (NM_001130145), from the backward position (75th-100th base pair) of the initiation codon "ATG" of the open reading frame in the target gene, 21 base sequences after AA sequence was searched as as a potential small interfering RNA (siRNA) targeted points. Target gene sequence with the GC content between 40% and 55% was selected as a potential optimization. BLAST retrieval was used in expressed sequence tag database in Gen-Bank. The selected sequence and the corresponding genome database were compared to rule out homologous sequence of other coding sequences and determine its specificity.

2.2.3. Construction of GPC3 gene expression vector

An ABI PRISM 7500 (ABI Applied Biosystems, USA) was used to amplify cDNA, with length of 121 bp. Gene sequence was designed based on the analysis software of ABI company (ABI Prism based analyzer). PCR products and overexpression plasmid vector of pEGFP-GPC-3 FLAG were separately performed with *Xho* Ⅰ and *Kpn* Ⅰ double enzyme digestion.

2.2.4. Screening of effective targets for GPC3 RNA interference (RNAi)

GPC overexpression plasmid was mixed with RNAi virus plasmid vector of different interference targeted sites of GPC as experimental group, while mixed with pCMV-Neo-Bam as the blank plasmid group. Well growing Huh-7 cells with the fusion of 80% to 90% were taken to co-transfect compounds of each group into Huh-7 cells. After 24 h, expression of GFP for virus RNAi plasmid report gene (green fluorescence) was observed under the fluorescence microscope. Visible infection efficiency should be higher than 80%, otherwise the infection was done again.

2.2.5. Slow virus infection and screening stable expression

A day before virus infection of liver cancer cell lines Huh-7 of PGC-shRNA-GPC3, Huh-7 cells in good growth condition was put into 6 holes culture plate, with PGC-shRNA-GPC3 as the experimental group, at the same time empty carrier group and blank control group were set up. A total of 50 μL OPTI DMEM + 2 μL lipofectamine^{TM2000} and 50 μL OPTI DMEM + 1 μg PGC-shRNA-GPC3 were mixed for 20 min at room temperature, the liquid was dropped evenly on the cell surface, and placed in 5% CO_2 incubator for 6 h. The transfection liquid was discarded and the medium was changed. Then selective medium with

blasticidin (1.25 μg/mL) was added for selection. Visible resistant clone grew after 2 weeks. Single cell clone was taken into 24 orifice plate and continued to expand, and stable transfection of cloned cells could be obtained about 1 month later.

2.3. Detection of GPC3 expression after steady interference by fluorescence quantitative PCR

2.3.1. Total RNA extraction

Total RNA was extracted by Trizol reagent kit. Cracking liquid was scraped and transfered to 1.5 mL sterilised Eppendorf tube at room temperature for 5 min. About 0.2 mL chloroform was added and shocked for 15 seconds, kept for 2 min after thoroughly incorporated, and centrifuged for 15 min. Supernatant (0.4 to 0.6 mL/1 mL) was drawn and 0.5 mL isopropyl alcohol was added, blended, then kept for 10 min at room temperature, centrifuged and the supernatant was abandoned. About 1 mL of 75% alcohol was added to wash the sediment, and then dried at room temperature. Suitable amount of DEPC H_2O was added for dissolution, and quantified by UV spectrophotometer.

2.3.2. Retrovirus synthetic cDNA

PGC-shRNA-GPC3 lentivirus stable transfection cell, RNA of transfected empty granulocyte and untransfected cell were extracted respectively, and RNA concentration was measured by spectrophotometry and recorded. Operation was strictly followed the instructions of High Capacity cDNA RT Kit (ABI Applied Biosystems, USA). The samples of 0.5 μg RNA, 1 μL 10× reverse transcriptase buffer, 0.4 μL deoxyribonucleotide triphosphates, 0.5 μL reverse transcriptautomotive service engineers, and 1 μL primers were added into a 20 μL centrifuge tube, and added double steaming water up to 10 μL for conventional reverse transcription into cDNA.

2.3.3. Real-time PCR

With Primer 5.0 software design, internal gene primer was sent to Shanghai Yingjun Synthetic Biological Technology Co., Ltd. GPC upstream primer: 5′-CGA GAT AAG CAC CTT TCA CAA CC-3′, GPC-3 downstream primers: 5′-AGA AGA AGC ACA CCA CCG AGA-3′. The PCR reaction conditions were 95 ℃ for 30 seconds, 95 ℃ for 5 seconds, 45 ℃ for 60 seconds for a total of 40 cycles. Mapping ΔCT value based on cDNA log of the concentration gradient, the linear slope absolute value close to zero showed that amplification of internal standard and target genes were at the same efficiency. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression of GPC3 relative strength (CT value defined as the cycle number when fluorescent signal of each reaction tube reached its set threshold). The experiment was repeated for three times to obtain the average value.

2.4. Detection of cell invasive ability by Transwell

A layer of fibronectin was coated on the surface of Transwell PVPF film (10 μg/mL, 50 μL) at 37 ℃ for 2 h, washed with PBS again, moved into 24 orifice plate with serum of 600 μL containing 10% culture medium, added cells (100 μL, diluted with culture medium containing 0.1% serum by 1:3), moved into the incubator, then took out after 12 to 18 h, and wiped cells close to the inner chamber PVPF membrane; the other sides of cells were with fixed with formaldehyde for 30 min at room temperature, stained with crystal violet staining for 20 min, washed three times, and then observed under microscope to record the cell counts.

2.5. Scratch test for cell migration

The day before transfection, the cells were spreaded with a density of 70%-80% in 6 cm Petri dish. After 16 h, cells were transfected according to the transfection reagent instruction (8 μg plasmid, 20 μL). After 24 h of transfection, the plate was respreaded with density of 30%-40%. After cells completely adherent to the wall, the line was scratched gently with 200 μL tip in 90 degree vertically, which was forced evenly to make sure there was no cells left. Fresh medium was refreshed every 3 d. Inverted microscope was used for a few days (100×) to record the cell migration until the cells scratches were full. Both sides of the lines were the average gaps. Image-Pro Plus was used in analysis AGs of each cell.

2.6. Detection of cell apoptosis by flow cytometry

Cells were vaccinated in 6 orifice plate for incubation and scavenged the original medium 48, 72 and 96 h after transfection, respectively. Then they were washed with PBS twice, the orginal cells were digested with the pancreatin which did not contain ethylene diamine tetraacetic acid. They were collected in 1.5 mL Eppendorf tube for centrifugation for 5 min at 2 000 r/min. The supernatant was then removed. According to the operating instructions of double dye apoptosis kit of Annexin V-FITC/Propidium Iodide (PI), 500 μL binding buffer was added respectively to suspend cells. About 2 μL Annexin V-FITC was added,

and then 5 μL PI was mixed at room temperature, and the reaction time was 5 min away from light for flow cytometry instrument detection.

2.7. Statistical analysis

SPSS11.5 software was used for statistical data analysis. Measurement data were calculated with mean±SD.

3. Results

3.1. Expressions of GPC3 mRNA in HCC cell lines

Fluorescence quantitative PCR results showed that GPC3 mRNA was highly expressed in liver cancer cells. Huh-7 cells with highest expression was screened for subsequent experiments (Figure 1).

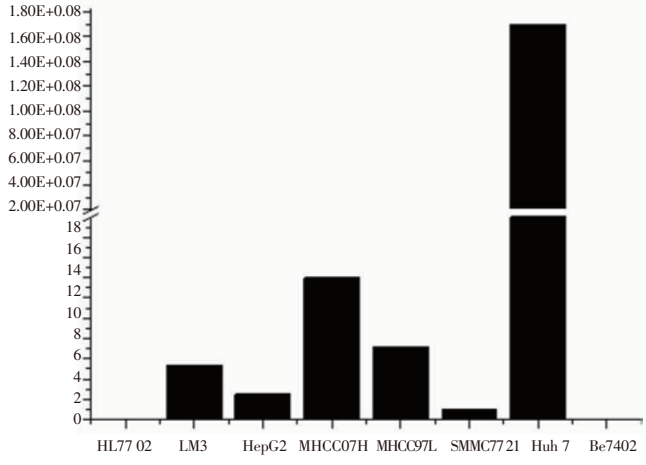


Figure 1. Expressions of GPC3 mRNA in HCC cell lines by RT-PCR.

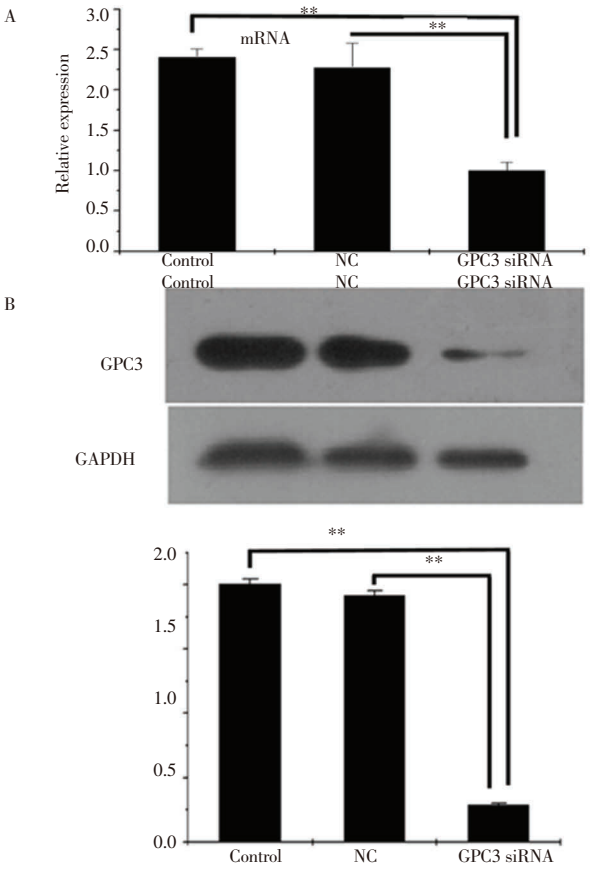


Figure 3. Inhibition effect of siRNA against GPC3 by fluorogenic quantitative PCR.

3.2. Construction and identification of PGC-shRNA-GPC3 lentiviral vector

After connecting the carrier with the shRNA section in *GPC3* gene, PCR electrophoresis was performed. The size of the PCR positive clone sections that was combined

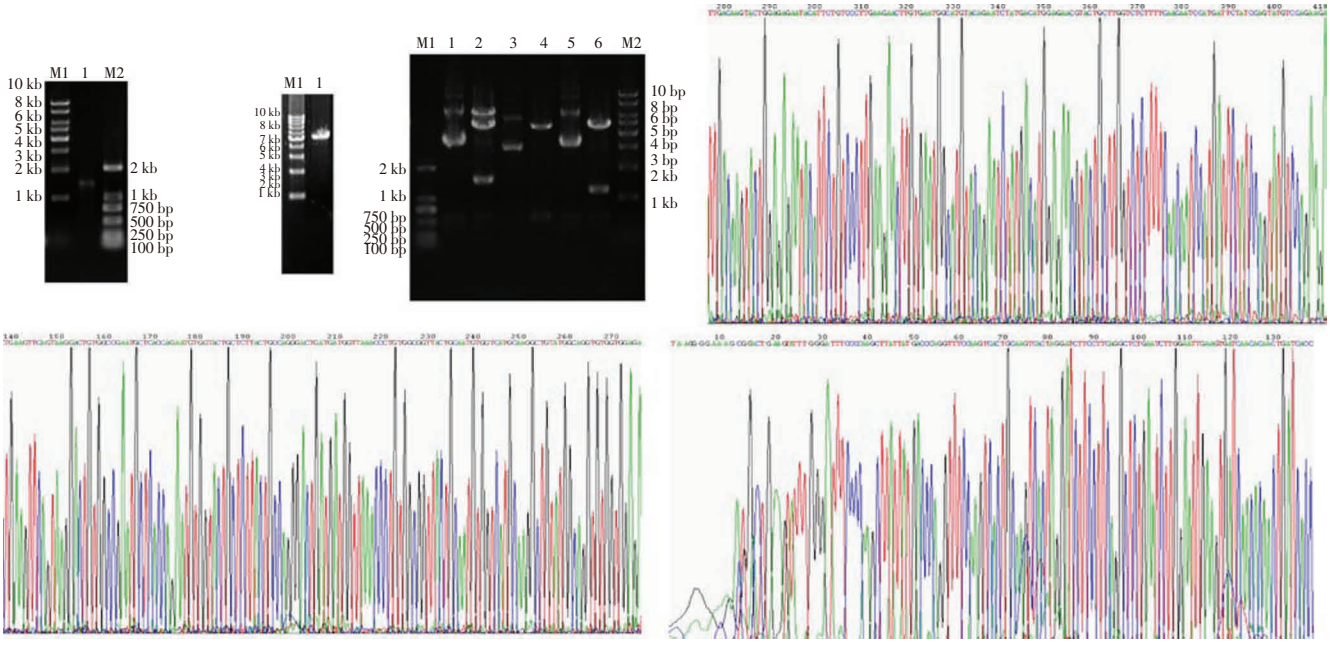


Figure 2. Construction and identification of GPC3 expressed plasmid vector.

into shRNA fragments was 343 bp, and the empty carrier cloning PCR fragment had size of 306 bp, consistenting with the theoretical value. The recombinant positive clone fragment was undergone sequencing and the results showed that the four groups of restructured RNAi lentiviral vector fragments were all consistent with the synthesized targeted chain, confirmed that synthesized DNAoligo has been successfully inserted into the carrier for the construction of RANi lentiviral vector (Figure 2).

3.3. mRNA expression changes of GPC3 gene

The $2^{-\Delta\Delta Ct}$ method was used to analyse and compare the relative expressive abundance of RT-PCR results after interfered with *GPC3* gene. 24 h after transfection, there was no significant difference of GPC3 expression between control and transfection group; 48 h and 72 h after transfection, GPC3 protein level in interference group was significantly lower than that of control group ($P<0.05$); 96 h after instantaneous interference, GPC3 protein levels were up-regulated in interference group and there was no statistically significant difference compared with control group ($P>0.05$). According to the analysis of qPCR, GPC3 mRNA expression level at 48 h after interference was significantly reduced by 66% compared to that at 24 h after interference with GPC3/ β -actin value of 0.18±0.04 ($P<0.05$). And GPC3 mRNA expression level after 72 h was reduced by 48% compared with that of 24 h, with GPC3/ β value of 0.36±0.07 ($P<0.05$). While GPC3 mRNA level after 96 h showed no significant differences compared with that of 24 h (Figure 3).

3.4. Effect of targeted GPC3siRNA on biology behavior of Huh-7 cell

After transfected Huh-7 cells with the siRNA of GPC3 gene by liposome and silenced the expression of GPC3. The proliferation (Figure 4), invasion (Figure 5) and transfer (Figure 6) of Huh-7 cells could be obviously inhibited and its apoptosis was promoted (Figure 7) without influencing blank carrier transfection and blank control groups.

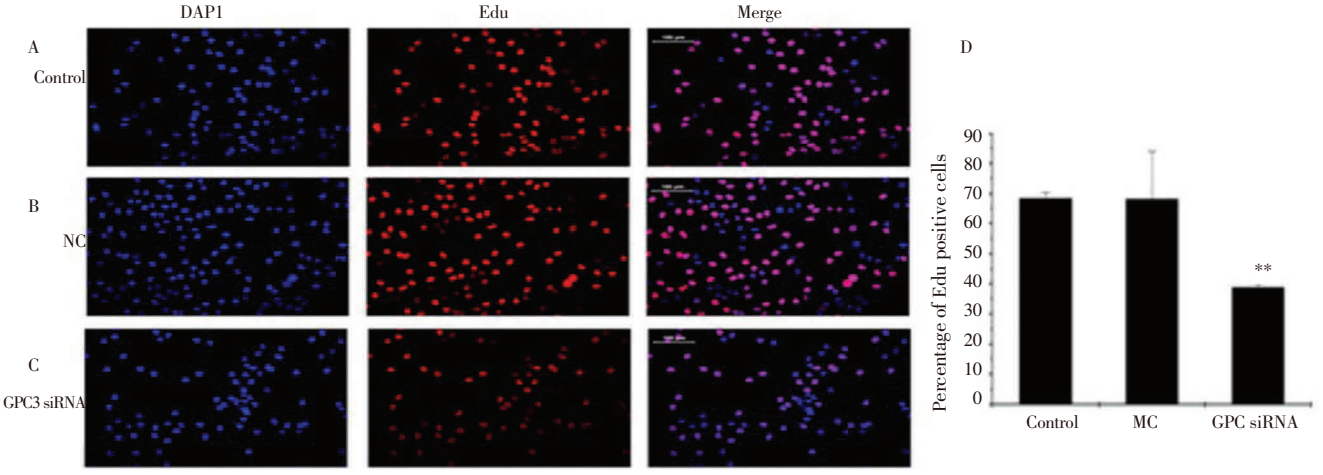


Figure 4. Proliferation of cells.

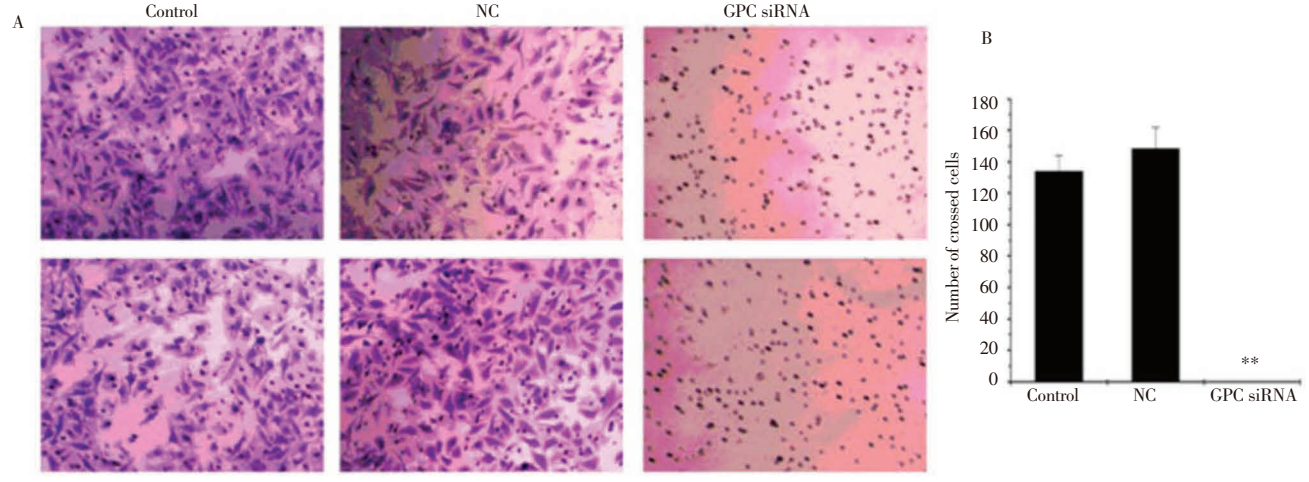


Figure 5. Invasion capacity of cells.

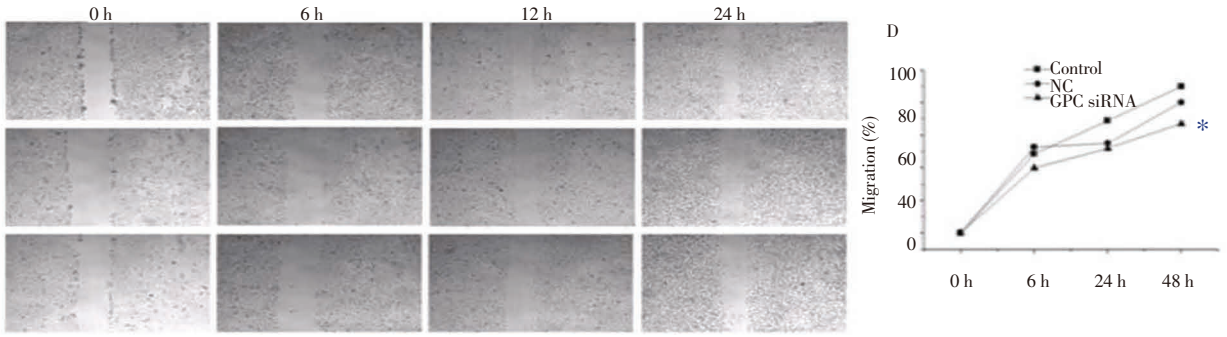


Figure 6. Migration of cells.

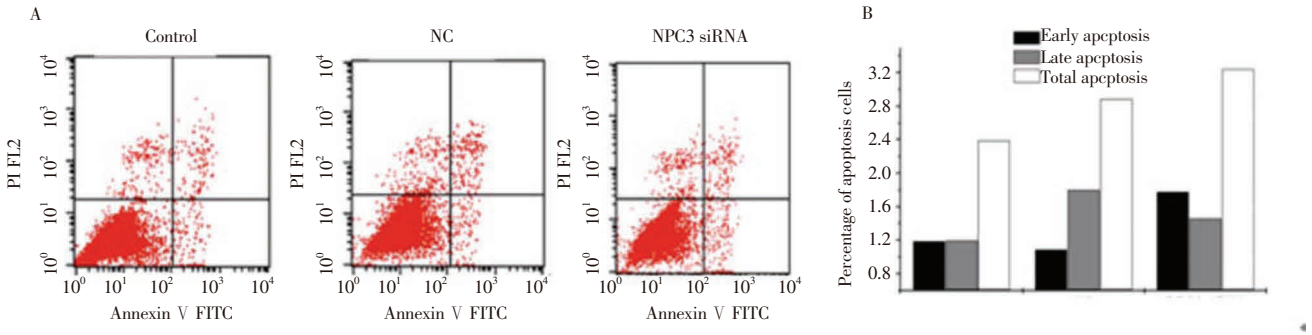


Figure 7. Apoptosis of cells.

4. Dicussion

At present, the incidence of liver cancer has exceeded gastric cancer and taken the first place among digestive tract malignant tumors, accounting for 55% of the total population around the world, with mortality rates as high as 45%. Liver cancer seriously harms to human health. Each year about 110 000 people die of liver cancer in China, accounting for 45% of liver cancer deaths in the world. Given the features of high malignant degree, rapid development, poor prognosis, and high mortality of HCC[11,12], developing the molecular targets becomes an urgent need for new treatments.

Tumor is caused by abnormal monoclonal hyperplasia of cells out of control at the gene level due to various factors. The occurrence and development of tumor involves a variety of complex processes, including the abnormal activation of oncogenes and inactivation of tumor suppressor genes[13]. The occurrence of liver cancer is related to abnormal biological behavior of cellular proliferation and apoptosis. Among them, abnormal gene expression is related to the regulation of cell proliferation and apoptosis is one of the crucial factors in the process of tumor formation.

Gene therapy is termed as using DNA transfer to treat and prevent human disease, of which the viral vector mediated gene transfer has become the most common method in tumor gene therapy, with its high transfection rate and good targeting capacity. On the one hand, it overcomes disadvantages in traditional tumor gene therapy such as low transfection efficiency of traditional tumor gene therapy, weak targetting capacity and low expressing amount of the antioncogenes. On the other hand it increases the permeability of the tumor cells, thus boosting the effects of chemotherapy drugs on tumor cell damage[14-16].

RNAi is a widely existing process in animals and plants induced by double-stranded RNA molecules gene to silence specific sequence at the mRNA level with high specificity and efficiency. RNAi is a new technology developed in recent years, it can specifically and effectively block the expression of target genes, causing a decomposition of its sequence homology mRNA molecules in cells, thus interfering with the expression of corresponding genes[17]. High efficiency of RNAi depends on the improvement of transfection efficiency and optimization of the carrier system. Plasmid mediated RNAi has certain limitations such as low transfection rate and short duration, *etc*. Adenovirus vector is unable to achieve the long-term stability of gene expression. Although retrovirus vector has the characteristics of long-term expression, it is mainly used for transfecting cells in division phase[18]. In recent years, the lentiviral vector is widely used as a kind of effective and versatile gene transfer tool. It is an ideal carrier for interference with high transfection efficiency by integrating into the host cell genome to realize the stability of the gene expression[19,20]. In this study, GPC3 siRNA can obviously inhibit *GPC3* gene expression in liver cancer cells, suggesting that by importing synthetic siRNA specifically

targeting the cancer gene into the tumor cells can specifically inhibit the expression of gene.

The occurrence and development process of the malignant tumor is often accompanied by a disequilibrium between cell proliferation and apoptosis. At the same time it also involves abnormal expression and anomaly inactivation of a variety of apoptosis-regulating genes. Cell apoptosis is a programmed cell death process induced by the changes of internal and external environment, the trigger of cell death signals or gene regulation. Therefore, further study of the relationship between proliferation and apoptosis related genes and their expression products with tumors can help to reveal the pathogenesis of tumors, which plays an important role in early diagnosis, prevention and treatment of diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Ibrahim GH, Mahmoud MA, Aly NM. Evaluation of circulating Transforming growth factor-beta1, Glypican-3 and Golgi protein-73 mRNAs expression as predictive markers for hepatocellular carcinoma in Egyptian patients. *Mol Biol Rep* 2013; **40**(12): 7069-7075.

[2] Pedersen ME, Snieckute G, Kagias K, Nehammer C, Multhaupt HAB, Couchman JR, et al. An epidermal microRNA regulates neuronal migration through control of the cellular glycosylation state. *Science* 2013; **341**(6152): 1404-1408.

[3] Feng M, Ho M. Glypican-3 antibodies: a new therapeutic target for liver cancer. *FEBS Lett* 2014; **588**(2): 377-382.

[4] Guo Xiao, Xiong L, Zou L, Sun T, Zhang J, Li H, et al. L1 cell adhesion molecule overexpression in hepatocellular carcinoma associates with advanced tumor progression and poor patient survival. *Diagn Pathol* 2012; **7**: 96.

[5] Cotter TG. Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer* 2009; **9**(7): 501-507.

[6] Pettigrew CA, Cotter TG. Deregulation of cell death (apoptosis): implications for tumor development. *Discov Med* 2009; **8**(41): 61-63.

[7] Pez F, Lopez A, Kim M, Wands JR, Caron de Fromentel C, Merle P. Wnt signaling and hepatocarcinogenesis: molecular targets for the development of innovative anticancer drugs. *J Hepatol* 2013; **59**(5): 1107-1117.

[8] Xu C, Yan Z, Zhou L, Wang Y. A comparison of glypican-3 with alpha-fetoprotein as a serum marker for hepatocellular carcinoma: a meta-analysis. *J Cancer Res Clin Oncol* 2013; **139**(8): 1417-1424.

[9] Huang TS, Shyu YC, Turner R, Chen HY, Chen PJ. Diagnostic performance of alpha-fetoprotein, lens culinaris agglutinin-reactive alpha-fetoprotein, des-gamma carboxyprothrombin, and glypican-3 for the detection of hepatocellular carcinoma: a systematic review and meta-analysis protocol. *Syst Rev* 2013; **2**: 37.

[10] Yao S, Zhang J, Chen H, Sheng Y, Zhang X, Liu Z, et al. Diagnostic value of immunohistochemical staining of GP73, GPC3, DCP, CD34, CD31, and reticulin staining in hepatocellular carcinoma. *J Histochem Cytochem* 2013; **61**(9): 639-648.

[11] Wang ZB, Yuan J, Chen W, Wei LX. Transcription factor ERG is a specific and sensitive diagnostic marker for hepatic angiosarcoma. *World J Gastroenterol* 2014; **13**: 3672-3679.

[12] Zheng DY, Ruan J, Liu FY, He BF, Huang N, Luo X, et al. Inhibition of GPC3 expression via RNA interference influences the apoptosis of a human hepatocellular carcinoma cell line Huh-7. *J Trop Med* 2011; **11**: 516-519.

[13] Hossain D, Bostwick DG. Significance of the *TMPRSS 2: ERG* gene fusion in prostate cancer. *BJU Int* 2013; **111**(5): 834-835.

[14] A Agaimy, H Kirsche, S Semrau, Iro H, Hartmann A. Cytokeratin-positive epithelioid angiosarcoma presenting in the tonsil: a diagnostic challenge. *Human Pathol* 2012; **43**(7): 1142-1147.

[15] McKay KM, Doyle LA, Lazar AJ, Hornick JL. Expression of ERG, an Ets family transcription factor, distinguishes cutaneous angiosarcoma from histological mimics. *Histopathology* 2012; **61**(5): 989-991.

[16] Miettinen M, Wang ZF, Paetau A, Tan SH, Dobi A, Srivastava S, et al. ERG transcription factor as an immunohistochemical marker for vascular endothelial tumors and prostatic carcinoma. *Am J Surg Pathol* 2011; **35**(3): 432-441.

[17] Saad Y, El-Serafy M, Eldin MS, Abdellatif Z, Khatab H, Elbaz T, et al. New genetic markers for diagnosis of hepatitis C related hepatocellular carcinoma in Egyptian patients. *J Gastrointestin Liver Dis* 2013; **22**(4): 419-425.

[18] Cottereau E, Moizard MP, David A, Raynaud M, Marmin N, Toutain A, et al. Duplication of exon 2 of the GPC3 gene in a case of Simpson-Golabi-Behmel syndrome. *Am J Med Genet A* 2014; **164A**(1): 282-284.

[19] Mateos ME, Beyer K, López-Laso E, et al. Simpson-Golabi-Behmel syndrome type 1 and hepatoblastoma in a patient with a novel exon 2-4 duplication of the *GPC3* gene. *Am J Med Genet A* 2013; **161A**(5): 1091-1095.

[20] Pan Z, Chen C, Long H, Lei C, Tang G, Li L, et al. Overexpression of GPC3 inhibits hepatocellular carcinoma cell proliferation and invasion through induction of apoptosis. *Mol Med Rep* 2013; **7**(3): 969-974.