Original Article

shRNA-mediated *Slc38a1* silencing inhibits migration, but not invasiveness of human pancreatic cancer cells

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Objective: Early metastasis is a major biological feature of pancreatic cancer. The current study examined whether silencing *Slc38a1*, a gene involved in energy metabolism, using short hairpin RNA (shRNA) could inhibit the growth, migration, and invasiveness of pancreatic cancer cells.

Methods: A series of *Slc38a1* shRNAs were designed and cloned into the pGPU6/GFP/Neo vectors. An shRNA with the most efficacious inhibitory action on *SCL38A1* expression (65% inhibition) upon screening in DH5α bacteria was used to transfect SW1990 human pancreatic cancer cells. Cell growth, migration, and invasiveness were examined using cell counting kit-8, Boyden chamber without and with Matrigel, respectively.

Results: Transfection of SW1990 cells with the *SLCs38A1* shRNA significantly decreased the proliferation ($P<0.0001$) and migratory potential (by 46.7%, $P=0.0399$) of the cancer cells. Invasiveness, however, was not affected.

Conclusions: Inhibiting *Slc38a1* using shRNA technology could decrease the growth and migration of representative pancreatic cancer cells. However, the fact that invasiveness was not affected suggested that SLC38A1 is unlikely to be responsible for early metastasis.

Keywords: *Slc38a1*; shRNA; pancreatic cancer

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Introduction

Transportation of small neutral amino acids and N-methyl amino acids by Type A amino acid transporters (ATA) plays an important role in cell physiology. Three subtypes of ATA (ATA1, ATA2 and ATA3) have been identified. ATA1 is also referred to as SNAT1 or SLC38A1. SLC38A1 is over-expressed in hilar cholangiocarcinoma (1) and primary hepatic carcinoma (2), and thought to promote cancinogenesis and cancer metastasis.

In the current study, we constructed a eukaryotic vector that carried a siRNA to inhibit the expression of *Slc38a1* gene and transfected SW1990 cells (a representative human pancreatic adenocarcinoma cell line). The results indicated that selective inhibition of *Slc38a1* gene could inhibit the growth and migration, but not the invasiveness of SW1990 cells.

Materials and methods

Materials

The eukaryotic expression vector pGPU6/GFP/Neo was from Zimmer Company (Shanghai, China). DH5α *Escherichia coli* (*E. coli*) were from the Cancer Research Institute, China Medical University (Shenyang, China). The restriction endonucleases *Bbs* I and *Bam*H I, T4 ligase, and Taq enzyme were from TaKaRa (Dalian, China). RPMI-1640 culture

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Table 1 The target sites and target sequences of *Slc38a1* shRNA plasmids

Plasmid	Target sequence
sh1	5'-CACCGGATGATAACATTAGCAATTTCAAGAGAATTGCTAATGTTATCATCCTTTTTTG-3'
	5'-GATCCAAAAAAGGATGATAACATTAGCAATTCTCTTGAAATTGCTAATGTTATCATCC-3'
sh2	5'-CACCGCAATGACTCCAATGATTTCATTCAAGAGATGAAATCATTGGAGTCATTGCTTTTTTG-3'
	5'-GATCCAAAAAAGCAATGACTCCAATGATTTCATCTCTTGAATGAAATCATTGGAGTCATTGC-3'
sh3	5'-CACCGCATTGTTCCAGAGCTAAATTTCAAGAGAATTTAGCTCTGGAACAATGCTTTTTTG-3'
	5'-GATCCAAAAAAGCATTGTTCCAGAGCTAAATTCTCTTGAAATTTAGCTCTGGAACAATGC-3'
sh4	5'-CACCGCATACTCTTGGTTGTTATCATTCAAGAGATGATAACAACCAAGAGTATGCTTTTTTG-3'
	5'-GATCCAAAAAAGCATACTCTTGGTTGTTATCATCTCTTGAATGATAACAACCAAGAGTATGC-3'
shNC	5'-CACC GTTCTCCGAACGTGTCACGT *CAAGAGATT* ACGTGACACGTTCGGAGAA TTTTTT G-3'
	5'-GATC CAAAAAA TTCTCCGAACGTGTCACGT *AATCTCTTG* ACGTGACACGTTCGGAGAAC-3'
shPC	5'-CACC GTATGACAACAGCCTCAAG TTCAAGAGA CTTGAGGCTGTTGTCATAC TTTTTT G-3'
	5'-GATC CAAAAAA GTATGACAACAGCCTCAAG TCTCTTGAA CTTGAGGCTGTTGTCATAC-3'

medium, fetal bovine serum (FBS), trypsin, lipofectamine 2000 and Trizol reagent were from Gibico (Invitrogen, Carlsbad, CA, USA). Plasmid DNA extraction kit was from Qiagen (Shanghai, China). Reverse transcription kit was from Promega (Fitchburg, WI, USA). All oligonucleotides, including PCR primers, were synthesized by Shanghai Ying Jun Bio-technology. Cell counting kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). Boyden chamber for migration assay was from Corning Costar (Rochester, NY, USA). Matrigel (0.3 mg/mL) was from BD Biosciences (Bedford, MA, USA).

Cell culture

SW1990 cells were obtained from Shanghai Cell Institute, Chinese Academy of Sciences, and expressed a high level of SLC38A1. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin in a humidified atmosphere containing 5% CO_2 at 37 ℃.

shRNA synthesis and vector construction/verification

The *SCL38A1* siRNAs, and a negative control (with scrambled sequence with no match to any known gene) were selected based on the full-length cDNA of human *Slc38a1* mRNA (gene bank number: NM_001077484.1) using an siRNA design software by Ambion (*Table 1*). An siRNA against *GAPDH* was included as a positive control (shPC) to verify transfection reliability, RNA extraction and gene expression quantification. To avoid premature termination, TTCAAGAGA was used in the loop of all constructs. The PCR products were annealed at the following condition: 95 ℃ 5 min, 85 ℃ 5 min, 75 ℃ 5 min, 70 ℃ 5 min, 4 ℃ preservation. The annealing process yielded 10 μmol/L shRNA template, which was diluted to 20 nmol/L for ligation.

After agarose electrophoresis purification, shRNA was inserted to the pGPU6/GFP/Neo vector at the *Bam*H I and *Bbs* sites. The resulting vector was inoculated to competent DH5α *E. coli*, selected using kanamycin resistance, and verified with sequencing by Shanghai Ying Jun Bio-technology. A total of four constructs, referred to as pGPU6/GFP/Neo-shRNA-1-4 (sh1-4), were tested in cultured SW1990 cells. The experiment also included pGPU6/GFP/Neo-shNC (shNC) as a negative control, and pGPU6/GFP/Neo-shPC (shPC) as a positive control.

Experiments in SW1990 cells

One day after the seeding in 6-well plates, the same amount of SW1990 cells (2×10^5/well) were transfected with sh1-4, shNC, or shPC using Lipofectamine 2000. A mock transfection (transfection reagents only) was also included. After incubation at 37 ℃ in a CO_2 incubator for 48 h, *Slc38a1* mRNA was examined using RT-PCR. Briefly, total RNA was extracted for RT-PCR amplification of *Slc38a1* cDNA. The PCR products were separated using a 1.5% agarose gel, and analyzed using an imaging system from BioRad (Hercules, CA, USA).

Cell number/growth was examined by counting the cell number at 48, 72 and 96 h after the transfection using

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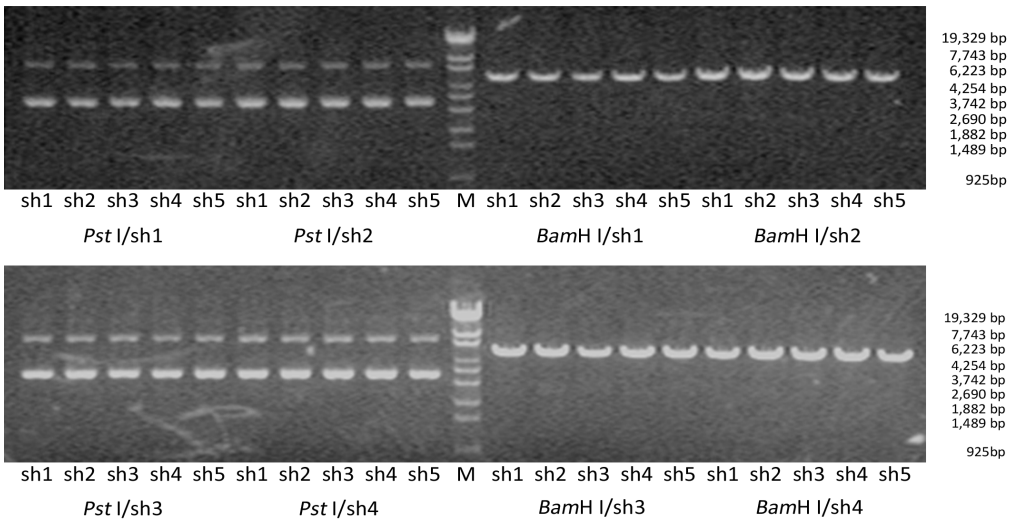

Figure 1 Identification of the recombinant vectors. *BamH* I and *Pst* I were used to digest and identify the extraction plasmid. Positive recombinant vector can be cut apart by *BamH* I but not *Pst* I. M, Lambda/Eco130I.

a colorimetric CCK-8 assay at 450 nm. Migration was examined using a modified Boyden chamber containing a gelatin-coated polycarbonate membrane filter (8-μm pore size). Briefly, RPMI-1640 containing 10% FBS was placed in the lower chamber. SW1990 (at a final concentration of 5×10^4 cells/mL) was suspended in serum-free RPMI-1640 in the upper chamber, and incubated at 37 ℃ under 5% CO_2 for 8 h. Cells migration was quantified by counting the cells that migrated to the lower chamber using crystal violet staining and an optical microscope, and expressed as the mean number of cells per field (average of 10 random fields). The invasion was examined after 24 h culture in Boyden chamber, with the upper surface of the filter coated with 20 μL Matrigel. All experiments were conducted in triplicate.

Statistical analysis

All data are expressed as $\bar{x}\pm s$, and analyzed using an one-way analysis of variance (ANOVA) with the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

Verification of shRNA vector

Electrophoresis after digestion with *Pst* I and *Bam*H I confirmed that the shRNA size conformed to the design (*Figure 1*). Two clones for each construct were further verified with sequencing. The sequence analysis conformed to the design in all cases.

Expression of the pGPU6/GFP/Neo vector that included a cassette of green fluorescent protein revealed >70% transfection efficiency (*Figure 2*).

Effects on Slc38a1 expression

At the 48th hour after the transfection, *Slc38a1* mRNA was decreased by all four shRNAs (P<0.05 *vs*. the negative control; *Figure 3*). The effect of sh4 on *Slc38a1* expression was strongest among the four shRNAs (at 65.28%±3.54%), and was hence used for further experiments.

Effects on proliferation, migration, and invasion of SW1990 cells

Sh4 decreased the proliferation (P<0.0001, *Figure 4*), migration (by 46.7%, P=0.0399; *Figure 5*), but not invasiveness (*Figure 6*) of SW1990 cells as reflected by Matrigel assay.

Discussion

Three types (L, A and B) of amino acid carriers were proposed in 1960s (3). In addition to a critical role in transporting amino acids to organs with significant barrier between the blood and tissue, much more data implicated amino acid transporters involved malignant transformation

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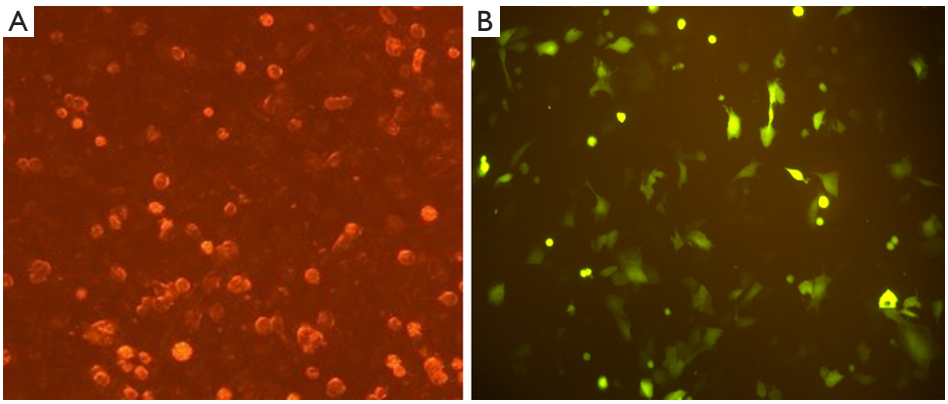

Figure 2 Transfection of SW1990 cells with sh4. (A) Cells transfected with recombinant plasmid sh4 under a light microscope, 200×; (B) Cells transfected with recombinant plasmid sh4 containing a *GFP* gene under a fluorescent microscope, 200×.

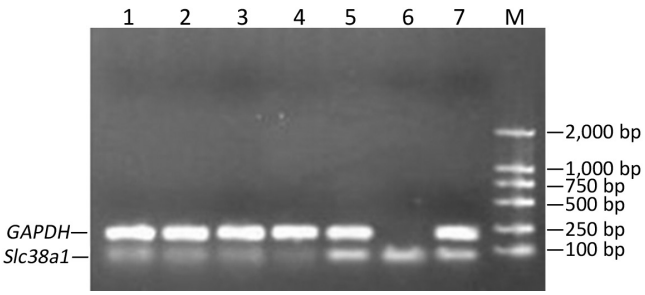

Figure 3 *Slc38a1* mRNA in SW1990 cells after transfection of *Slc38a1* shRNAs. Lanes l-4, cells transfected with sh1-4; lane 5, cells transfected with shNC; lane 6, cells transfected with shPC; lane 7, mock transfection; M, molecular ladder.

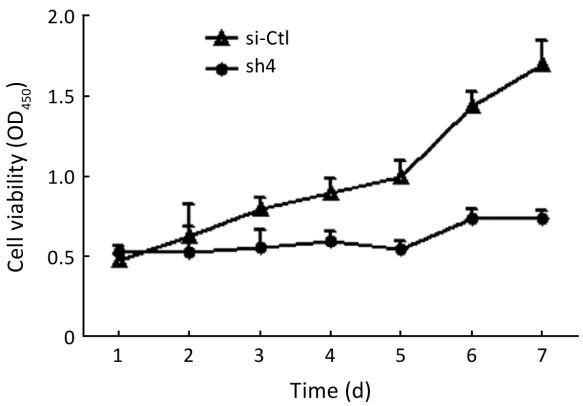

Figure 4 sh4 significantly inhibited the growth of SW1990 cells.

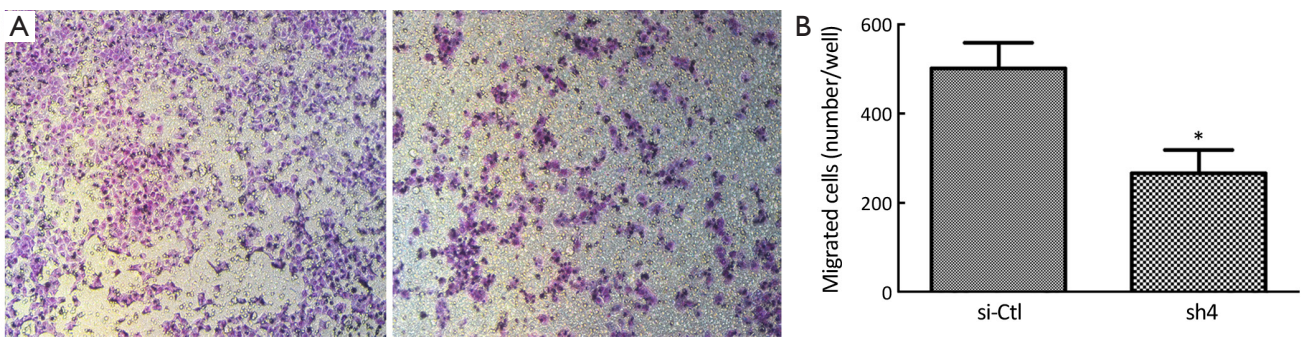

Figure 5 Migration assay of SW1990 cells. (A) sh4 decreased the migration of SW1990 cells in transwell chambers; (B) The number of migrated cells. *, P<0.01 *vs*. scrambled control.

of mammalian cells or its invasion. Among the various amino acid transporters, LAT-1 of type L system is up-regulated in a variety of tumors, including carcinoma of the urinary bladder (4), adenocarcinoma of the esophagus (5), oral squamous cell carcinoma (6), and particularly in breast cancer (7), choriocarcinoma BeWo cells (8) and hepatic

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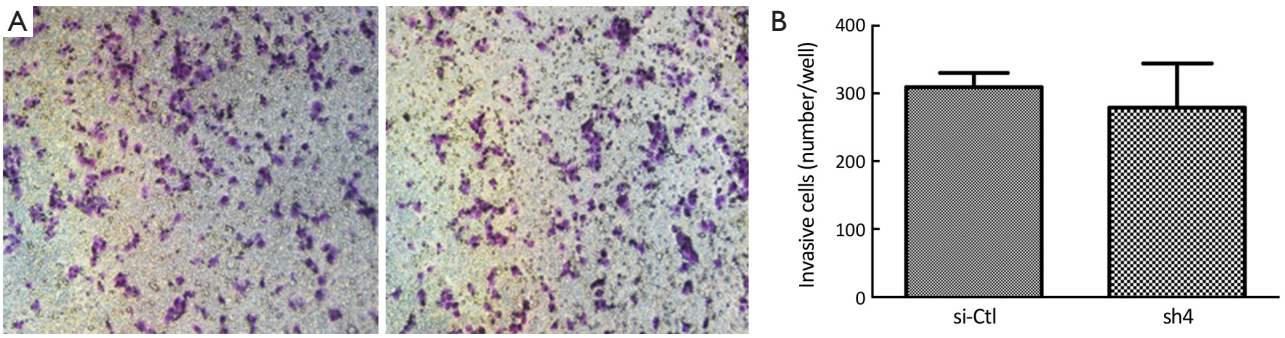

Figure 6 Invasion assay of SW1990 cells. (A) sh4 could not influence the invasiveness of SW1990 cells; (B) The number of invasive cells through the Matrigel.

cancer (9). SLC38A1 is a subtype of type A system which is Na^+-dependent and transports amino acids from extracellular space into the cells (10). Enhanced SLC38A1 expression has been observed in several other types of malignancies, including liver cancer (2), hilar cholangiocarcinoma (1) and C6 glioma (11). SLC38A1 over-expression is thought to promote cancer cell migration (2).

RNA interference (12-13) technology is widely used to investigate the function of a specific gene (14-15). shRNAs are typically small hairpin RNAs with stem loop structure, and could inhibit the expression of target genes (16). In the present study, we synthesized a series of shRNAs against *Slc38a1* using the BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). Upon transfection with the resulting vector, the shRNAs significantly decreased the expression of the target gene in SW1990 pancreatic cancer cells. Selective inhibiting the expression of *Slc38a1* profoundly reduced cell proliferation of pancreatic cancer SW1990 cells, as well as the migration. However, invasiveness (Boyden chamber experiments with Matrigel) was not affected, suggesting that SLC38A1 may promote carcinogenesis and migration of pancreatic cancer, but establishment of metastatic lesions requires additional processes/molecules.

The success in building the *Slc38a1* shRNA expression vector in the current study also provided important experimental basis for further function study.

Acknowledgements

Disclosure: The authors declare no conflict of interest.

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