Expression of Smo in pancreatic cancer $CD44^+CD24^+$cells and construction of a lentiviral expression vector to silence Smo

PENG CONG[1], CHAO YI[2] and XI-YAN WANG[2]

[1]Department of Laparoscopic and Liver Surgery, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054; [2]Department of Hepato-Pancreato-Biliary Surgery, Cancer Hospital Affiliated to Xinjiang Medical University, Urumqi, Xinjiang 830000, P.R. China

Received November 23, 2015; Accepted May 25, 2017

DOI: 10.3892/ol.2018.9315

Abstract. The present study focused on the roles of members of the Hedgehog (Hh) signaling pathway in the maintenance of malignant biological characteristics, such as tumorigenesis, similar to that of pancreatic tumor cells. Cluster of differentiation (CD)44^+CD24^+/$CD44^-CD24^-$ cells were isolated from three different pancreatic cancer cell lines by flow cytometry. Among the three pancreatic cancer cell lines, the SW1990 cell line exhibited the highest percentage of $CD44^+CD24^+$ cells, which accounted for 39.9% of the total. The expression of members of the Hh signaling pathway in $CD44^+CD24^+$/$CD44^-CD24^-$ cells was detected using reverse transcription-polymerase chain reaction and western blot analysis. The results demonstrated that members of the Hh signaling pathway were differentially expressed in $CD44^+CD24^+$ cells compared with $CD44^-CD24^-$, normal pancreatic duct cells and unsorted SW1990 cells. In addition, lentiviral expression vectors expressing Smoothened (Smo) small interfering RNA (siRNA) were constructed. Following transfection with the lentiviral expression vectors, Smo expression was markedly reduced in $CD44^+CD24^+$ cells. The present study represents a preliminary investigation into the biological characteristics of $CD44^+CD24^+$ pancreatic cancer cells.

Introduction

The incidence of pancreatic cancer is increasing globally (1). The latest data from the American Cancer Society for the USA reveals that the incidence of pancreatic cancer is 11 per 100,000 people, ranking it tenth of the known malignant tumors; the mortality rate has been ranked fourth out of the known malignant tumors (2). In addition, epidemiological data from China demonstrates an increasing incidence in pancreatic cancer (3-6).

Pancreatic cancer stem cells (CSCs) are considered to influence the early metastasis of pancreatic cancer, are insensitive to radiation and chemotherapy and are the main cause of rapid disease progression (7). CSCs cannot be eradicated, and as such drive invasion, metastasis and recurrence, and are the source radiation and chemotherapy resistance (8). In 2007, Lee *et al* (9) using flow cytometry to isolate cluster of differentiation (CD)44^+CD24^+, epithelial surface $antigen^+$ pancreatic cancer cells from surgically removed specimens. These cells have the abilities of self-renewal and multi-directional differentiation, and may adapt to alterations in circumstances. Lee *et al* (9) hypothesized that this group of pancreatic cancer cells were pancreatic CSCs. In recent years, one study has validated that the Hedgehog (Hh) signaling pathway exhibits a function in the process of embryonic development (10). The abnormal activation of growth and development is associated with the development of tumors (1,2). In 2003, Berman *et al* (11) and Thayer *et al* (12) demonstrated an association between the Hh signaling pathway and pancreatic cancer. Thayer *et al* (12) used 26 cancer cell lines, including pancreatic cancer (either from a human primary source or a metastatic tumor), to screen the expression of members of the Hh signaling pathway. Thayer *et al* (12) demonstrated that expression of members of the Hh signaling pathway, smoothened (Smo), zinc finger protein Gli1 (GLI-3) and Patch-1, was observed in each pancreatic cell line. The occurrence of Hh signaling pathway activation in pancreatic cancer serves an important role in its development (11,12). Smo a typical G protein coupled receptor, is a positive regulator of cellular proliferation and differentiation in insects and vertebrates (13,14). Abnormal activation of Smo leads to development of a number of types of cancer, which makes Smo an attractive therapeutic target. At present, vismodegib, an inhibitor of Smo, is approved by the food and drug administration for the treatment of cancer; however, cancer cells may acquire resistance (15,16). A number of resistance mechanisms have been demonstrated, including mutations in Smo, the negative control receiver, suppressor of fused homolog (Sufu) and the mitogen-activated protein kinase signaling pathway (13). Therefore, an improved

Correspondence to: Dr Xi-Yan Wang, Department of Hepato-Pancreato-Biliary Surgery, Cancer Hospital Affiliated to Xinjiang Medical University, 789 Suzhou Street, Urumqi, Xinjiang 830000, P.R. China
E-mail: xiyanwangdoc@163.com

Key words: pancreatic cancer, Hh signaling, lentivirus

understanding of the Smo regulating mechanism to develop effective therapies to treat cancer caused by Smo mutations is required.

Small interfering RNA (siRNA) is an RNA molecule that can efficiently and specifically degrade target gene mRNA to inhibit the excessive expression of genes. In addition, they may be used as a treatment in patients and are a useful tool for studying gene function in the future. The occurrence and development of pancreatic cancer is a multi-factor and multi-stage process (17,18). In the present study, experimental siRNA stability infection in pancreatic cancer cells of $CD44^+$ $CD24^+$ cells was used to determine whether or not RNA and protein expression was affected.

Patients and methods

Patients. A total of 19 cases (11 males, 8 females; mean age, 59.89 years; range, between 40 and 78 years) of pancreatic cancer were included in the present study. There were 9 tumors of the head of the pancreas, 9 tumors of the body and tail of the pancreas, and 1 pancreatic neck tumor. Pathological results according to the pathology classification system of the Cancer Hospital Affiliated to Xinjiang Medical University (Urumqi, China), included the following: 2 cases of highly differentiated carcinoma, 4 cases of moderately/highly differentiated carcinoma, 8 cases of moderately differentiated carcinoma, 4 cases of moderately/poorly differentiated carcinoma and 1 case of poorly differentiated carcinoma. Local lymph node metastasis was identified in 7 cases. The patients were all treated at the Cancer Hospital Affiliated to Xinjiang Medical University between January 2014 and December 2015. The Ethics committee of the Affiliated Cancer Hospital to Xinjiang Medical University approved the present study and all patients provided written informed consent.

Materials. SDS-PAGE gel preparation kit, dNTPs and Taq DNA polymerase, 2 kb plus DNA Marker and PCR primers were obtained from Shanghai Shenggong Biology Engineering Technology Service, Ltd. (Shanghai, China). The PCR reagent, primers and dsDNA oligos were obtained from Shanghai Jikai Gene Chemical Technology Co., Ltd. (Shanghai, China). Taq polymerase was obtained from Clontech Laboratories, Inc. (Mountainview, CA, USA) and the Qiagen plasmid Maxi kit was obtained from Qiagen, Inc. (Valencia, CA, USA). Bovine serum albumin (BSA) was obtained from Shanghai JIEBEISI Gene Technology Co., Ltd. (Shanghai, China). Lysogeny broth (LB), super optimal broth (SOB) and super optimal broth with catabolite repression (SOC) were obtained from American Type Culture Collection (Manassas, VA, USA). T4 DNA ligase, T4 DNA ligase buffer, and the restriction enzymes *Age*I, *Eco*RI, *Hpa*I and *Xho*I were obtained from New England BioLabs, Inc. (Ipswich, MA, USA). $MgSO^4$ was obtained from Wuhua Chemical Industry Co., Ltd. (Shanghai, China). Agarose was obtained from Saibaisheng Biochemical Co., Ltd. (Beijing, China). The 250 bp DNA ladder marker was obtained from Beijing Huada Jierui Biotechnology Co., Ltd. (Beijing, China). Gene sequencing for the positive clone was performed by Shanghai Genechem Co., Ltd. (Shanghai, China).

The intermediate clone vector pUC57, pLenti6/V5-D-TOPO recombinant lentiviral vector, ViraPower™ Packaging Mix, viral quantitative primers and the TaKaRaMiniBEST Viral RNA/DNA Extraction kit were obtained from Baiao Maike Bio-Technology Co., Ltd. (Nantong, China). Lipofectamine® 2000 transfection reagent kit was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). TOP10 chemical competent cells and 293T virus packaging cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and OptiMEM medium were obtained from Gibco; Thermo Fisher Scientific, Inc.

Sorting of $CD44^+CD24^+$ cells. BXPC-3, PANC-1 and SW1990 (all from Shanghai Genechem Co., Ltd., Shanghai, China) pancreatic cancer cell lines were cultured at 37˚C in an atmosphere containing 5% CO_2 in RMPI-1640 (Hyclone; Thermo Fisher Scientific, Inc.) or DMEM, supplemented with 10% FBS. Subsequently, $CD44^+CD24^+$ cells were sorted from the three cell lines using flow cytometry as previously described (19,20).

RT-PCR. TRIzol® (Thermo Fisher Scientific, Inc.) was used to extract total RNA from SW1990 cellsof each experimental group, according to the manufacturer's protocol, and the RNA concentration and purity were detected using a spectrophotometer. The RNA was reverse transcribed into complementary DNA according to the manufacturer's protocol of the two-step M-MuLV First Strand cDNA Synthesis kit (cat no. B532435; Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol. For the PCR reaction, sonic hedgehog protein (Shh), Smo, protein patched homolog 1 (Ptch1), zinc finger protein Gli1 (Gli1), zinc finger protein Gli2 (Gli2), Sufu and apoptosis regulator Bcl-2 (Bcl-2) primers (Table I) were designed and synthesized according to the sequences in GeneBank, as previously described (21). Primer sequences and their annealing temperatures, including for the reference gene β-actin, are provided in Table I. PCR was performed with the Bio-Rad MyiQ™2 two-color real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR Green I as the fluorophore. The components and their volumes in the polymerase chain reaction are shown in Table II. The reaction conditions used were: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec. Quantitative analysis of Shh, Smo, Ptch1, Gli1 and Gli2, Sufu and Bcl2 mRNA expression was performed with the $2^{-\Delta\Delta Cq}$ method (22). Data analysis was performed using Microsoft Excel 2016.

Western blot analysis. A total protein extraction kit (Trizma® base Vetec reagent grade ≥99%; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to extract the total protein of the cells and the quantity of protein was determined using a BCA protein concentration detection kit. A total of 125 μg of protein was used for 12% SDS-PAGE electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes and blocked with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. The membranes were washed three

Table I. Primers used for reverse transcription-polymerase chain reaction.

Gene	Primer sequences	Amplification fragment size, bp	Annealing temperature, ˚C
Sonic hedgehog	Forward: 5'-GTCTCCTCGCTGCTGGTATG-3' Reverse: 5'-TTGGGGATAAACTGCTTGTAGG-3'	150	56
Protein patched homolog 1	Forward: 5'-CTCCTTTGCGGTGGACAA-3' Reverse: 5'-CCTCAGCCTTATTCAGCATTTC-3'	109	54
Smoothened	Forward: 5'-CTCCTACTTCCACCTGCTCAC-3' Reverse: 5'-CAAAACAAATCCCACTCACAGA-3'	105	57
Zinc finger protein GLI1	Forward: 5'-ATCCTTACCTCCCAACCTCTGT-3' Reverse: 5'-AACTTCTGGCTCTTCCTGTAGC-3'	84	55
Zinc finger protein GLI2	Forward: 5'-GCGGAATTCGCAACGGAATG-3' Reverse: 5'-GCTGGATCCTTAGTCACA-3'	472	55
Suppressor of fused homolog	Forward: 5'-CGGACCCAC CAGAAGCGG-3' Reverse: 5'-GGAGGCGTCCTTCCGAC-3'	398	52
B-cell lymphoma	Forward: 5'-ACCTTAGCCCCATGCATTCTG-3' Reverse: 5'-CTAATCGGCTAGCTTCGAAAT-3'	287	54
β-actin	Forward: 5'-GGGACCTGACTGACTACCTC-3' Reverse: 5'-CGTCATACTCCTGCTTGCTG-3'	543	56

Table II. Polymerase chain reaction components and volumes.

Reagent	Volume per reaction/μl
2X ES Tap Master Mix	12.5
Upstream primer (10 μmol/l)	1
Downstream primer (10 μmol/l)	1
RNase-free water	8.5
Complementary DNA template	2
Total volume	25

times with TBS-Tween-20 (TBST) and were incubated with the following primary antibodies overnight at 4˚C with gentle agitation: Bcl-2 (1:1,000; cat no. ab32124), Gli1 (1:500; cat no. ab92611), SMO (1:1,000; cat no. ab38686), PTCH1 (1:500; cat no. ab53715), Gli2 (1:1,000; cat no. ab26056) and SHH (1:2,000; cat no. ab53281; Abcam, Cambridge, MA, USA). The membranes were washed three times with TBST prior incubation with the alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:2,000; cat no. WB7105; WesternBreeze; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Membranes were washed three times with TBST and the blots were developed using enhanced chemiluminescence kit (Western Breeze Chemiluminescence Reagent kit; cat. no. WB7105; Thermo Fisher Scientific, Inc.). Using β-actin as a reference, the relative protein expression was calculated. The Gel Doc XR+ system (Bio-Rad Laboratories, Inc.) was used to capture and analyze results of gels.

Construction of the lentiviral expression vector expressing Smo siRNA. For the design and synthesis of Smo siRNA fragments, the information and the full-length nucleotide sequence of the human Smo gene were obtained by searching the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/; no. NM_005631) and used to design three siRNA targets (Table III). Three siRNAs were used in the current study: siRNA1 (TGATGGACACAGAACTCAT); siRNA2 (GGAGAAGATCAACCTGTTT) and siRNA3 (TGACTGTGAGATCAAGAAT) (Shanghai Genechem CO., Ltd.).

293T cells were cultured in DMEM, containing 10% FBS and seeded into 24-well culture plates ($3.5x10^{-4}$ cells/ml) one day prior to transfection using carrier GV plasmid (20 μg;), PHelper1.0 carrier plasmid (15 μg) and PHelper 2.0 carrier plasmid, 10 μg) (Shanghai Genechem, Co., Ltd.), with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) After 24 h, the expression of the intracellular fluorescent-labeled gene (GFP) was observed under a fluorescence microscope, and then the cells were collected for western blot analysis 36 h following transfection. The viral titer was determined by Virus drops degree detection; fluorescence/absolute quantitative method (23).

The $CD44^+CD24^+$ cells that were sorted as previously mentioned were cultured to the logarithmic growth phase and then digested into single cell suspension with trypsin. 293T cells were cultured for 24 h to 90% confluence, washed three times, then incubated in a 5% CO_2 incubator at 37˚C for 20 min. Cells were transfected with the Lipofectamine® 2000 transfection kit, according to the manufacturer's protocol. Following incubation at 37˚C for 72 h, the SMO gene expression in the cells of each group was determined by the RT-PCR method, the Smo siRNA with the highest transfection efficiency was screened for the follow-up study.

Statistical analysis. Analysis of expression of the genes was performed using the SASJMP10.0 software (SAS Institute,

Table III. siRNA targets designed using the NCBI GenBank database.

siRNA target	Target sequence information	GC content, %	Start position
SMO-RNAi(37303-1)[a]	TGATGGACACAGAACTCAT	42.1	2604
SMO-RNAi(37304-1)[b]	GGAGAAGATCAACCTGTTT	42.1	1831
SMO-RNAi(37305-1)[c]	TGACTGTGAGATCAAGAAT	36.8	1795

[a]siRNA1; [b]siRNA2; [c]siRNA3. siRNA, small interfering RNA.

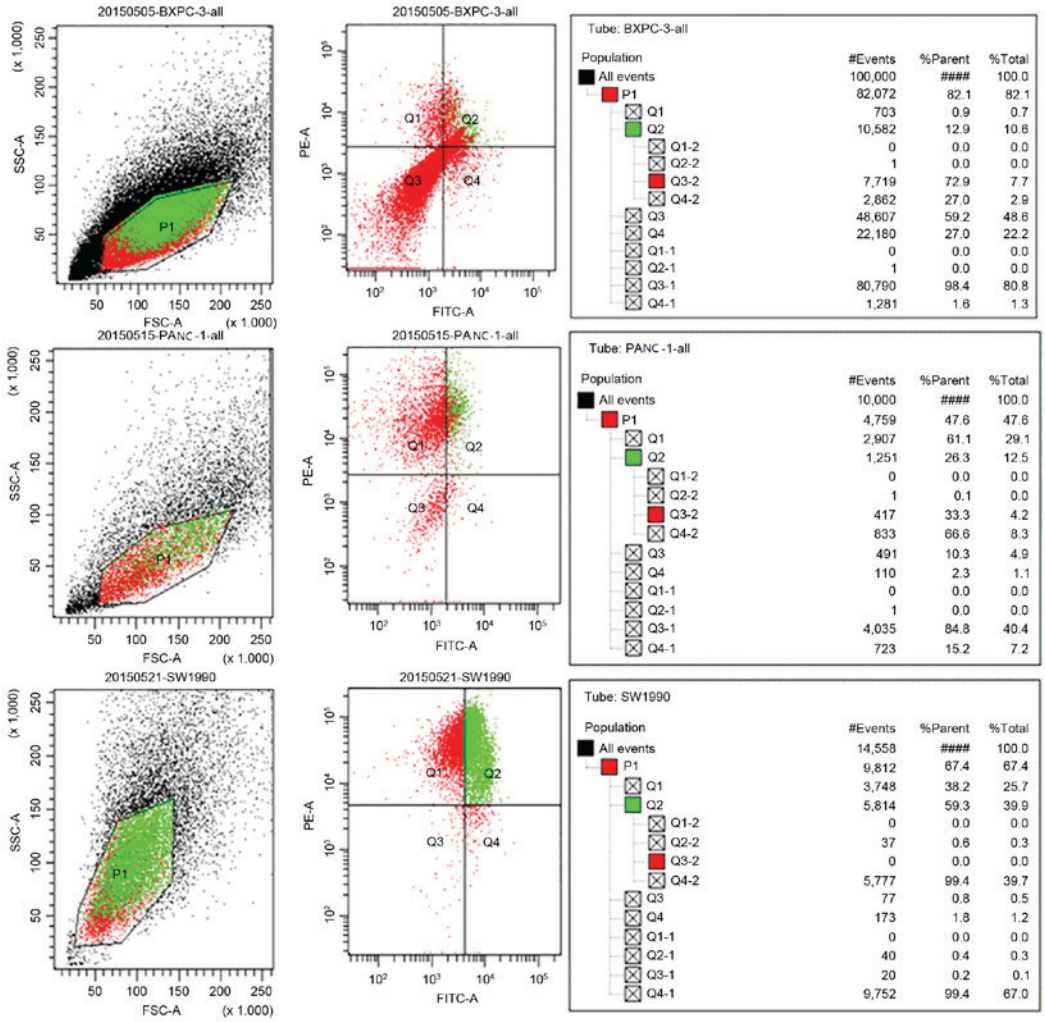

Figure 1. CD44$^+$CD24$^+$ cell proportion in three pancreatic cancer cell lines. Flow cytometry analysis of the proportion of CD44$^+$CD24$^+$ cells in BXPC-3, PANC-1 and SW1990 cells. CD, cluster of differentiation.

Cary, NC, USA). Data are expressed as the mean ± standard deviation. Differences among >2 groups were assessed using one-way analysis of variance with Bonferroni post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sorting of CD44$^+$CD24$^+$ cells. Three lines of CD44$^+$CD24$^+$pancreatic cancer cells were isolated using flow cytometry (BXPC-3, 10.6% CD44$^+$CD24$^+$ cells; PANC-1, 12.5% CD44$^+$CD24$^+$ cells; SW1990, 39.9% CD44$^+$CD24$^+$ cells; Fig. 1). CD44$^+$CD24$^+$ SW1990 pancreatic cancer cells were selected for further experiments.

Expression of Hedgehog (Hh) signaling pathway members in pancreatic cancer cells. The expression of the Hh signaling pathway members Shh, Smo, Ptch1, Gli1, Gli2, Sufu and Bcl2 (24) was investigated in unsorted SW1990 pancreatic cancer cells, sorted CD44$^+$CD24$^+$ cells, sorted CD44$^-$CD24$^-$ cells and normal pancreatic duct cells obtained from patients using RT-PCR (Fig. 2) and western blot analysis (Fig. 3). The expression of Gli1 and Bcl2 in pancreatic ductal cells and cancer cells was increased compared with that in SW1990 pancreatic

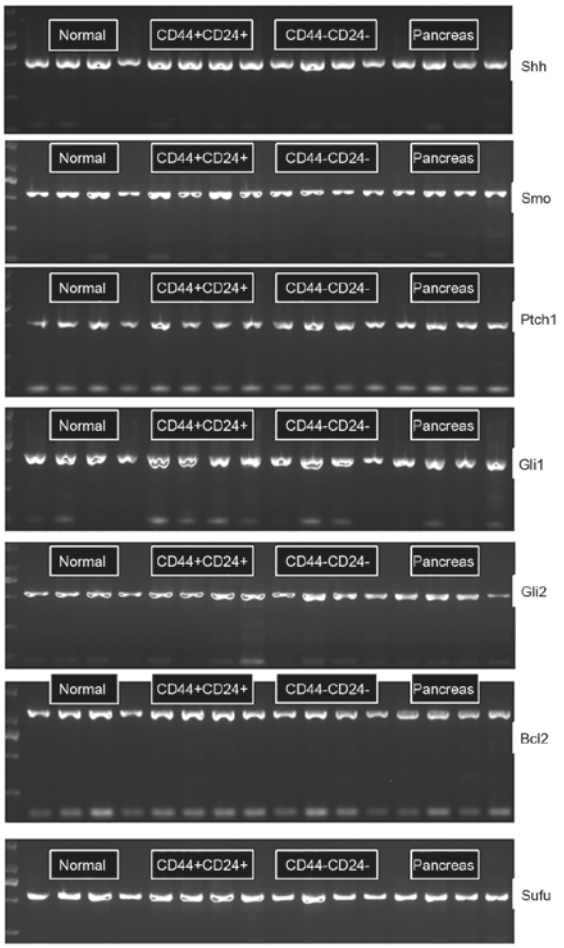

Figure 2. The electrophoresis maps of reverse transcription-polymerase chain reaction products of members in the Hedgehog signaling pathway in pancreatic cancer tissue, CD44+CD24+ SW1990 cells, CD44-CD24- SW1990 cells, normal pancreatic tissue adjacent to carcinoma cell search group. Shh, sonic hedgehog; Smo, smoothened; Ptch1, protein patched homolog 1; Gli1, zinc finger protein GLI1; Gli2, zinc finger protein GLI2; Bcl-2, B-cell lymphoma 2; Sufu, suppressor of fused homolog; CD, cluster of differentiation.

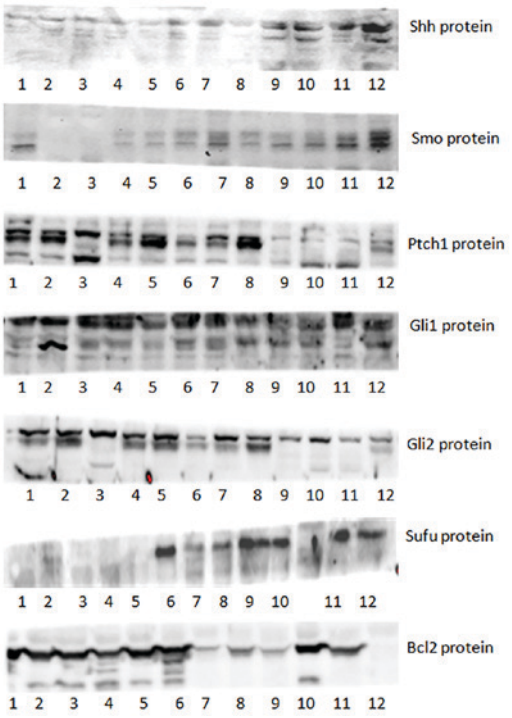

Figure 3. Western blot analysis of members of the Hedgehog signaling pathway in pancreatic cancer tissue cells, CD44+CD24+ SW1990 cells, CD44-CD24- SW1990 cells, normal pancreatic tissue adjacent to carcinoma cells. Shh, sonic hedgehog; Smo, smoothened; Ptch1, protein patched homolog 1; Gli1, zinc finger protein GLI1; Gli2, zinc finger protein GLI2; Bcl-2, B-cell lymphoma 2; Sufu, suppressor of fused homolog.

cancer cells (Fig. 4); however, no significant differences were observed between the expression of the other proteins in pancreatic cancer cells compared with that in the pancreatic duct cells. The expression of Shh and Smo in $CD44^+CD24^+$ cells was markedly increased compared with that in pancreatic cancer cells; however, no significant differences were observed between the expression of the other proteins in the two groups. In addition, no significant differences in expression were observed between the $CD44^-CD24^-$ cells and pancreatic cancer cells.

Construction of the siRNA expression vector carrying Smo. Sequencing of the KL15570-Smo siRNA sequencing demonstrated that the sequence was consistent with the designed fragment (data not shown). The electrophoresis map of the PCR products of the KL15570-Smo siRNA vector digested by *Sac*II B restriction endonucleases confirmed that the fragment had been inserted (Fig. 5). Fluorescence microscopy confirmed that Three groups of interfering lentivirus cells had been successfully transfected with the plasmid (Fig. 6). The viral titers for the siRNA1, siRNA2 and siRNA3 plasmids were $5x10^8$, $6x10^8$ and $5x10^8$ viral TU/ml, respectively.

Detection of Smo gene expression in $CD44^+CD24^+$ cells infected with three Smo- siRNA lentiviral expression vectors. RT-PCR analysis demonstrated that Smo expression was markedly decreased in $CD44^+CD24^+$ cells following infection with the constructed Smo siRNA lentiviral expression vectors (Fig. 7). The blocking efficiency of the three siRNAs were 54.293, 32.188 and 62.531%, respectively (data not shown). The efficiency of siRNA3 was the highest; therefore the lentiviral expression vector carrying Smo-siRNA3 was used in the follow-up study to block the Hh signaling pathway.

Discussion

The Hh signaling pathway serves key roles in the proliferation of epidermal stem cells, the development of embryonic cells with stem cell characteristics, and the development of mouse cerebral cortex cells following birth (25). Its abnormal activation is associated with the occurrence and development of a number of types of malignant tumor, multiple drug resistance and other characteristics. The presence of $CD44^+$ $CD24^-$ cells was detected by Tao *et al* (8) using the RT-PCR method; the results demonstrated that the expression of Smo and GLI-1 genes was significantly increased between $CD44^+$ $CD24^-$ cells and non $CD44^+$ $CD24^-$ cells, and indicated that associated factors of the Hh signal pathway were overexpressed in breast cancer stem cells (CSCs). The Hh signal pathway serves important roles in the proliferation, differentiation and maintenance of malignant biological characteristics of CSCs (26). Therefore, multiple studies have focused on the association between the Hh signaling pathway and the development of pancreatic cancer.

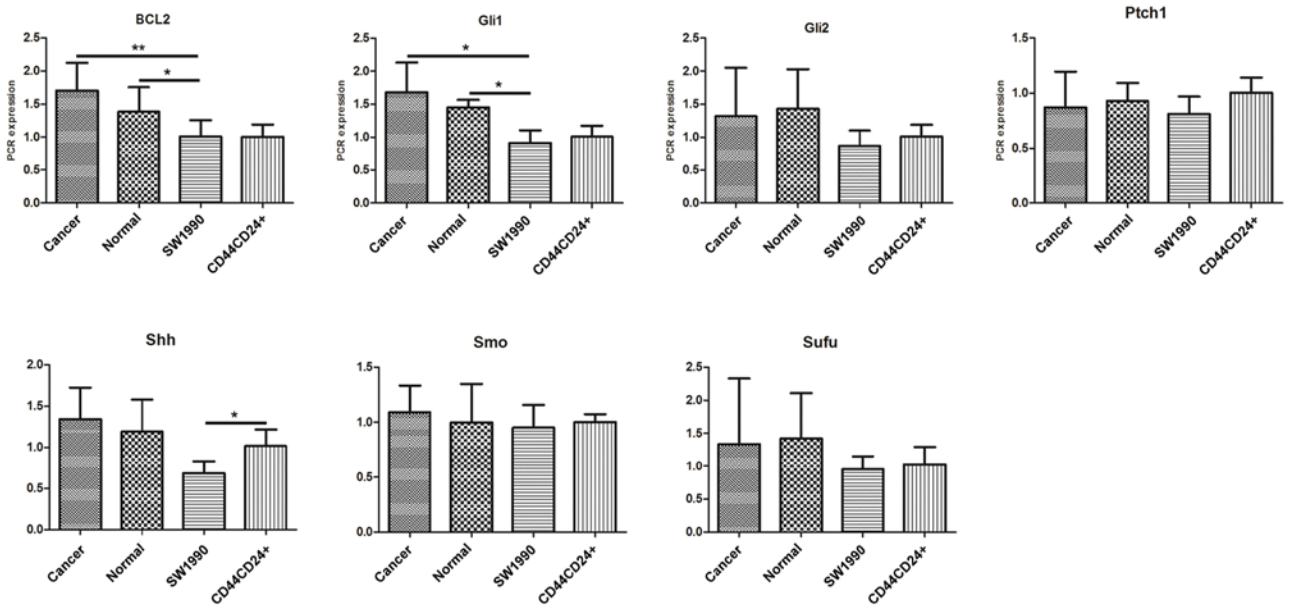

Figure 4. Relative expression level of members of the Hedgehog signaling pathway in the cells of each group. The polymerase chain reaction Shh, sonic hedgehog; Smo, smoothened; Ptch1, protein patched homolog 1; Gli1, zinc finger protein GLI1; Gli2, zinc finger protein GLI2; Bcl-2, B-cell lymphoma 2; Sufu, suppressor of fused homolog; CD, cluster of differentiation. *P<0.05, **P<0.01.

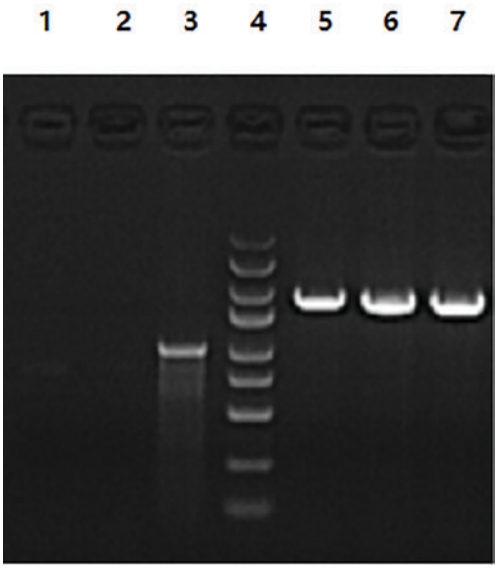

Figure 5. Electrophoresis maps of digestion products of the smoothened siRNA plasmid. Lane 1, blank; 2, control; 3, overexpression; 5-7, siRNA. SiRNA, small interfering RNA.

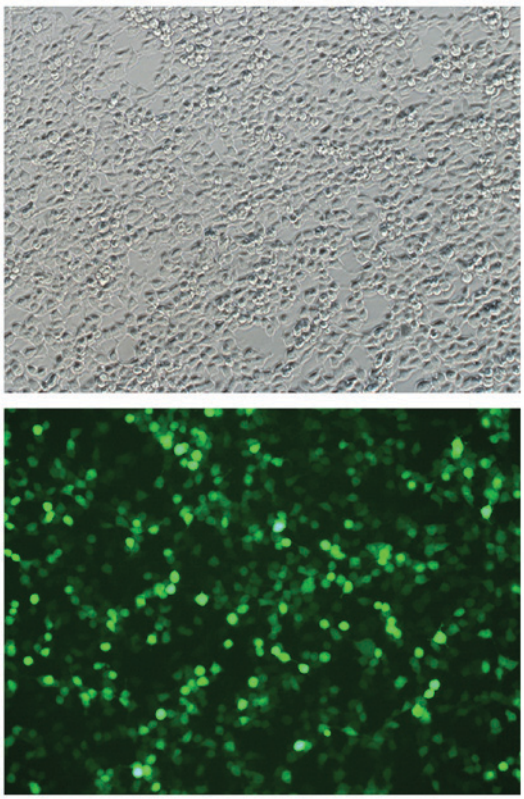

Figure 6. Images of HEK-293T cells 72 h following transfection with lentiviral vector plasmid (SMO-RNAi 37304-1). Magnification, x200.

Onishi *et al* (27) confirmed *in vivo* and *in vitro* that cyclopamine, a specific blocking agent for the Hh signaling pathway, can significantly improve the sensitivity of pancreatic cancer cells to chemotherapeutic medicines, including fluorouracil and gemcitabine. Hao *et al* (28) demonstrated that the invasion and metastasis of pancreatic cancer cells could be significantly inhibited by blocking the Hh signaling pathway. In addition, gene expression studies have demonstrated that expression of Hh signaling pathway-associated molecules are significantly higher in pancreatic cancer tissues compared with normal tissues (29).

Gene therapy has been developed using DNA recombination technology to correct mutated or defective genes by introducing normal genes or therapeutic genes into cells, including tumor and immune cells, in order to modify the biological behavior of tumor cells (30).

MicroRNAs are endogenous non-coding small RNA molecules, which serve important roles in tissue inflammation, cell proliferation and apoptosis, tissue differentiation, and

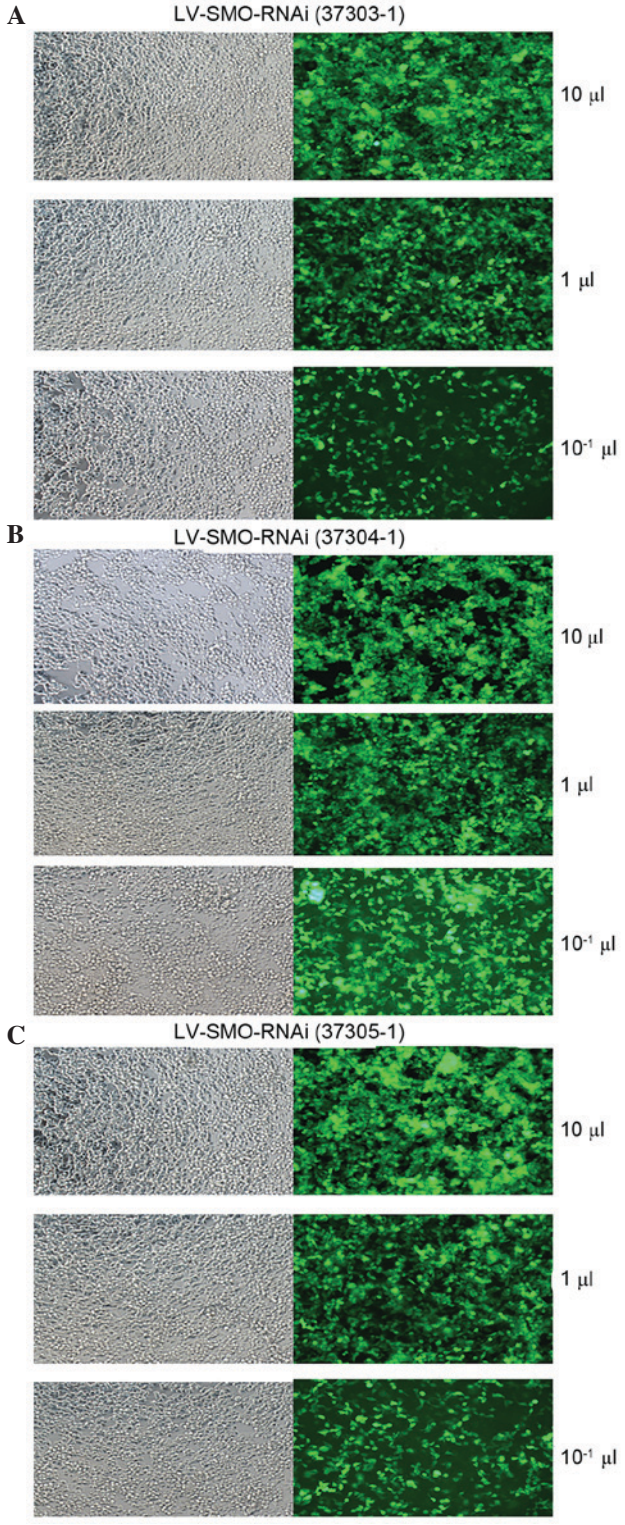

Figure 7. Expression of the Smo gene in $CD44^+CD24^+$ cells transfected with the siRNA lentiviral vector. (A) Smo gene expression in cells transfected with siRNA1; (B) Smo gene expression in cells transfected with siRNA2; (C) Smo gene expression in cells transfected with siRNA3. Smo, smoothened; siRNA, small interfering RNA.

malignant tumor occurrence and development (31). siRNA viral vector transfection is a primary method used for studying signal transduction pathways at present. In addition, it is possible to use these molecules in clinical applications for tumor-targeted therapy due to low cost and high efficiency (32).

The tumorous characteristics, including cell morphology and growth characteristics, of three pancreatic cancer cell lines (BXPC-3, PANC-1 and SW1990) and the percentage of $CD44^+CD24^+/CD44^-CD24^-$ cell subsets of pancreatic cancer cells isolated by flow cytometry.

In the present study, three siRNA fragments were designed against the Smo gene in the signal pathway, and the lentiviral expression vectors carrying the three Smo fragments were successfully constructed. Through the transfection of $CD44^+CD24^+$ cells, the Smo siRNA lentiviral expression vector that was the most suitable for the follow-up study was selected according to the inhibition rate of Smo expression. This not only laid a good experimental basis for the smooth development of the follow-up study of this research, but also provided reliable experimental evidence for the gene therapy of targeting interference of the Hh signaling pathway. The Hh signaling pathway serves an important role in the maintenance of pancreatic CSC malignant biological characteristics, but further studies are required to elucidate the underlying molecular mechanisms for this association.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81360328).

Availability of data and materials

The datasets used and/or analyzed during the current study and the and materials are available from the corresponding author on reasonable request.

Authors' contributions

PC was involved in the design of the experiment, drafted and revised the manuscript. CY was involved in the design of the experiment, collected and processed the specimens and was additionally responsible for the collection and analysis of the data. XYW was involved in the design of the experiment, revised the important technical and theoretical content and provided final approval of the version to be published. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics committee of the Affiliated Cancer Hospital to Xinjiang Medical University and all patients provided written informed consent.

Patient consent for publication

All patients provided written informed consent for the publication of all the associated data and accompanying images in the current study.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
2. Hammerschmidt M, Brook A and McMahon AP: The world according to hedgehog. Trends Genet 13: 14-21, 1997.
3. Lee Y, Miller HL, Russell HR, Boyd K, Curran T and McKinnon PJ: Patched2 modulates tumori-geneis in patched1 heterozygous mice. Cancer Res 66: 6964-6971, 2006.
4. McMahon AP: More surprises in the Hedgehog signaling pathway. Cell 100: 185-188, 2000.
5. Jemal A, Siegel R, Xu J and Ward E: Cancer statistics, 2010. CA Cancer J Clin 60: 277-300, 2010.
6. Li GL, Chen WQ, Wang QS,Ma AW and An R: Analysis of the association between pancreas cancer and diabetes. J Cancer Control Treat 23: 132-136, 2010 (In Chinese).
7. Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. Nature 414: 105-111, 2001.
8. Tao YJ, Mao J, Zhang QQ and Li LH: Hedgehog signaling pathway is activated in breast cancer $CD44^{+}$ $CD24^{-}$ cells. Shandong Med 51: 33-35, 2011 (In Chinese).
9. Lee CJ, Dosch J and Simeone DM: Pancreatic cancer stem cells. J Clin Oncol 26: 2806-2812, 2008.
10. Mimeault M and Batra SK: Frequent deregulations in the hedgehog signaling network and cross-talks with the epidermal growth factor receptor pathway involved in cancer progression andt argeted therapies. Pharmacol Rev 62: 497-524, 2010.
11. Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, Parker AR, Shimada Y, Eshleman JR, Watkins DN and Beachy PA: Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature 425: 846-851, 2003.
12. Thayer SP, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, Qi YP, Gysin S, Fernández-del Castillo C, Yajnik V, *et al*: Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 425: 851-856, 2003.
13. Jiang K and Jia J: Smoothened regulation in response to Hedgehog stimulation. Front Biol (Beijing) 10: 475-486, 2015.
14. Yang L, Xie G, Fan Q and Xie J: Activation of the hedgehog-signaling pathway in human cancer and the clinical implications. Oncogene 29: 469-481, 2010.
15. Sekulic A, Migden MR, Oro AE, Dirix L, Lewis KD, Hainsworth JD, Solomon JA, Yoo S, Arron ST, Friedlander PA, *et al*: Efficacy and safety of vismodegib in advanced basal-cell carcinoma. N Engl J Med 366: 2171-2179, 2012.
16. Atwood SX, Sarin KY, Whitson RJ, Li JR, Kim G, Rezaee M, Ally MS, Kim J, Yao C, Chang AL, *et al*: Smoothened variants explain the majority of drug resistance in basal cell carcinoma. Cancer Cell 27: 342-353, 2015.
17. Patel R, Ede J, Collins J and David Willens: Pancreatic cancer presenting as new-onset diabetes. Case Rep Oncol 7: 171-174, 2014.
18. Tong GX, Geng QQ, Chai J, Cheng J, Chen PL, Liang H, Shen XR and Wang DB: Association between pancreatitis and subsequent risk of pancreatic cancer: A systematic review of epidemiological studies. Asian Pac J Cancer Prev 15: 5029-5034, 2014.
19. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF and Simeone DM: Identification of pancreatic cancer stem cells. Cancer Res 67: 1030-1037, 2007.
20. Farhana L, Dawson MI, Das JK, Murshed F, Xia Z, Hadden TJ, Hatfield J and Fontana JA: Adamantyl retinoid-related molecules induce apoptosis in pancreatic cancer cells by inhibiting IGF-1R and Wnt/β-catenin pathways. J Oncol 2012: 796729, 2012.
21. Yao J, An Y, Qian JJ, Bai DS, Xu ZK and Miao Y: Hedgehog signaling pathway inhibitor reverts acquired chemoresistance in human pancreatic cancer SW1990 cell line. Chin J Pancreatol 184-188, 2012.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Lizée G, Aerts JL, Gonzales MI, Chinnasamy N, Morgan RA and Topalian SL: Real-time quantitative reverse transcriptase-polymerase chain reaction as a method for determining lentiviral vector titers and measuring transgene expression. Hum Gene Ther 14: 497-507, 2003.
24. Fu J, Rodova M, Roy SK, Sharma J, Singh KP, Srivastava RK and Shankar S: GANT-61 inhibits pancreatic cancer stem cell growth in vitro and in NOD/SCID/IL2R gamma null mice xenograft. Cancer Lett 330: 22-32, 2013.
25. Clement V, Sanchez P, de Tribolet N, Radovanovic I and Ruiz i Altaba A: HEDEGHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. Curr Biol 17: 165-172, 2007.
26. Li Y, Kong D, Ahmad A, Bao B and Sarkar FH: Pancreatic cancer stem cells: Emerging target for designing novel therapy. Cancer Lett 338: 94-100, 2013.
27. Onishi H, Morifuji Y, Kai M, Suyama K, Iwasaki H and Katano M: Hedgehog inhibitor decreases chemosensitivity to 5-fluorouracil and gemcitabine under hypoxic conditions in pancreatic cancer. Cancer Sci 103: 1272-1279, 2012.
28. Hao K, Tian XD, Qin CF, Xie XH and Yang YM: Hedgehog signaling pathway regulates human pancreatic cancer cell proliferation and metastasis. Oncol Rep 29: 1124-1132, 2013.
29. Jiang H, Li Q, He C, Li F, Sheng H, Shen X, Zhang X, Zhu S, Chen H, Chen X, *et al*: Activation of the Wnt pathway through Wnt2 promotes metastasis in pancreatic cancer. Am J Cancer Res 4: 537-544, 2014.
30. Fujihira A, Suzuki T, Chang MO, Moriyama T, Kitajima M and Takaku H: Antitumor effects of baculovirus-infected dendritic cells against human pancreatic carcinoma. Gene Ther 21: 849-854, 2014.
31. Wu R, Li F, Zhu J, Tang R, Qi Q, Zhou X, Li R, Wang W, Hua D and Chen W: A functional variant at miR-132-3p, miR-212-3p, and miR-361-5p binding site in CD80 gene alterssusceptibility to gastric cancer in a Chinese Han population. Med Oncol 31: 60, 2014.
32. Tuschl T: Expanding small RNA interference. Nat Biotechnol 20: 446-448, 2002.