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Knockdown of EIF3H inhibits the development and progression of pancreatic cancer by regulating cell proliferation and apoptosis in vitro

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ABSTRACT

Nowadays, pancreatic cancer has been recognized as one of the most fatal malignancies worldwide, the molecular mechanism of which is still not fully understood. In this study, we aimed to uncover the fundamental functions of the eukaryotic translation initiation factor 3H subunit (EIF3H) in the development and progression of pancreatic cancer. Firstly, the results of immunohistochemical (IHC) staining revealed that EIF3H was highly expressed in pancreatic cancer. Moreover, lentiviruses were used to deliver shRNAs into pancreatic cancer cells for silencing EIF3H. Furthermore, the loss-of-function assays demonstrated that knockdown of EIF3H could inhibit the progression of pancreatic cancer cells by reducing proliferation capacity, promoting apoptosis, arresting cell cycle in G2 and suppressing cell migration. In summary, EIF3H may play a critical role in the development and progression of pancreatic cancer, which possesses the potential to act as a therapeutic target for pancreatic cancer treatment.

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Introduction

Pancreatic cancer is a malignant disease of the digestive tract with high morbidity and mortality, and the overall 5-year survival rate is extremely low (1). The typical characteristic of poor prognosis in patients with pancreatic cancer is due to the rapid development of the disease and the lack of effective treatment at an advanced stage (2, 3). The majority of patients with pancreatic cancer are diagnosed with advanced stages and are unable to undergo surgery, and the disease is less sensitive to radiation (2, 4). In addition, chemical resistance is often observed, which greatly reduces the efficacy of chemotherapy (5). Up to now, gemcitabine (GEM) is a first-line drug for the treatment of pancreatic cancer (6). Compared with traditional chemotherapy drugs, gemcitabine (GEM) can improve the treatment effect and the quality of life of patients (6). Unfortunately, with the emergence of GEM resistance, the therapeutic efficacy of GEM in pancreatic cancer is declining (7, 8). Therefore, it is of great significance to further explore the molecular mechanism of pancreatic cancer in order to find key molecular targets to improve the therapeutic effects.

Eukaryotic translation initiation factor 3 (EIF3) is a large multi-protein complex that was originally thought to be a factor that binds to the 40S ribosomal subunit and prevents it from binding to the 60S subunit (9). EIF3 initiates protein translation by forming large complexes with ribosomes and mRNA (10). Abnormal protein synthesis can cause a variety of diseases, including cancer. To date, some of the 13 subunits that have been identified, namely EIF3A-EIF3M, are abnormally expressed in tumors (11). The H subunit (EIF3H) is overexpressed in several malignancies, including breast cancer, prostate cancer, and hepatocellular carcinoma (12-15). In addition, it has been found that overexpression of EIF3H promotes the proliferation and invasion of colorectal cancer cells and immortalizes fibroblasts (11, 16), while EIF3H silencing can inhibit the proliferation and colony formation of breast, gastric and hepatocellular carcinoma cells(14, 15, 17). However,

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the effects of EIF3H in pancreatic cancer cells have not been reported.

This study first proposed that EIF3H is highly expressed in pancreatic cancer tissues and cells. Furthermore, EIF3H is positively correlated with the clinicopathological grade and prognosis of pancreatic cancer. We also demonstrated that EIF3H has a vital role in the growth, migration, and tumor development of pancreatic cancer cells, and may be a new candidate therapeutic target in the future.

Materials and methods

Immunohistochemical (IHC) Staining

The 99 pairs of pancreatic cancer tissue and matched non-cancer normal tissue were purchased from Shanghai Outdo Biotech Company. First, the specimen was deparaffinized and antigen-blocked with citric acid. Subsequently, the EIF3H antibody was added and incubated at 4°C overnight. After elution with PBS, several times, secondary antibody IgG (1: 400, Abcam, USA, Cat. # ab6721) was added and incubated at room temperature. After multiple elutions with PBS again, the tissue sections were stained with DAB and hematoxylin. Finally, images were taken under a microscope and evaluated by the German immune response score.

Cell Culture

Human pancreatic cancer cell lines, PANC-1, SW1990 and normal pancreatic cells HPDE6-C7 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were incubated at 37°C under a 5% CO_2 atmosphere, supplemented with Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Gibco BRL). It should be noted that the medium was changed every 72 h, and 0.05% trypsin and 0.02% EDTA were passaged at a concentration of 80%.

Lentiviral shRNA Vector Construction and Cell Transfection

First, BR-V112 linear vector (Shanghai Biological Science Co., Ltd., Shanghai, China) was obtained by digestion with restriction enzyme AgeI (NEB, Cat. # R3552L) and EcoRI (NEB, Cat. # R3101L). At the same time, three RNA interference target sequences were designed using EIF3H as a template (shEIF3H-1: 5'- TGGCAATACATCAGGTGTTAA-3', shEIF3H-2: 5'-CTGGAATTGTCAAAGTCTCAA-3', shEIF3H-3: 5'-AGGCAGCTGGAATCTTTGATT-3'). The sequence with the highest knockdown efficiency was selected and ligated to the linearized vector BR-V112. The clones on the medium were selected for PCR identification, and the positive clones were sequenced and analyzed. Positive clones were cultured to facilitate extraction of high-purity plasmids, and plasmid extraction according to the kit instructions (Endo Free midi Plasmid Kit, TIANGEN, Cat. # DP118-2). After that, 293T cells were co-transfected with three plasmids (BRV-112, Helper 1.0 and Helper 2.0), 72 h after transfection lentivirus (unpurified cell supernatant) was harvested. Subsequently, according to quality standards, various indicators of the lentivirus were determined, such as sterility, viscosity, color, and titer. The lentivirus titer = the number of fluorescent cells/amounts of virus stock. Finally, the negative control group was transfected with negative lentivirus shCtrl, and the shEIF3H group was transfected with shEIF3H. After 72 h, the expression of the green fluorescent protein was observed with a fluorescence microscope to evaluate the transfection efficiency.

Quantitative Real-Time -PCR (qPCR)

First, human pancreatic cancer cell lines PANC-1 and SW1990 were collected and their RNA was extracted using Trizol (Thermo Fisher Scientific, Cat. # 204211) according to the manufacturer's instructions. The Nanodrop 2000/2000C spectrophotometer was used to determine the concentration and quality of the extracted RNA. Furthermore, the Promega M-MLV kit was used to obtain cDNA by methods of reverse transcription. The cDNA as a template and primers as follows (EIF3H: 5'-GGATGGACTCGCTGCTCATT-3', 5'-GGCCATGAAGAGCTTGCCTA-3'), GAPDH was chosen as a reference control (GAPDH: 5'-TGACTTCAACAGCGACACCCA-3', 5'-CACCCTGTTGCTGTAGCCAAA-3'). Finally, qPCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) and the fusion curve was drawn.

Western Blot

First, total cell proteins of human pancreatic cancer cell lines PANC-1and SW1990 were extracted and quantified using BCA protein assay kit (Thermo Fisher Scientific, Cat. # A53227). Afterward, the proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the sample was transferred to a polyvinylidene fluoride (PVDF) membrane at 4°C. After blocking, the membrane was first incubated with primary antibody, EIF3H (1:500, abcam, Cat. # ab171419), cleaved PARP (1:1000, CST, Cat. #5625), Caspase 8 (1:1000, abcam, Cat. # 13423-1-AP), Caspase 8 (1:1000, abcam, Cat. # ab52298), Caspase 3 (1:1000, CST, Cat. #9662), N-cadherin (1:2000, abcam, Cat. # ab76011) SNAIL+SLUG (1:2000, abcam, Cat. # ab180714) and GAPDH (1:3000, Bioworld, Cat. # AP0063) as an internal reference. It was then incubated with secondary antibody, goat anti-rabbit (1: 3000, Beyotime, Cat. # A0208). Finally, the immune response was visualized using the Amersham ECL + plusTM Western Blot system, and the blots were imaged using a luminescent image analyzer.

MTT Assay

Human pancreatic cancer cell lines PANC-1and SW1990 cells (2×10^3/well) were seeded in a 96-well plate in an amount of 100 μL/well. After trypsinization, the medium was completely resuspended. Subsequently, 20 mg (5 mg/mL) of 3 (4,5dimethylthiazole 2yl) 2, 5 (diphenyltetrazole bromide) (MTT) (Genview, Beijing, China; Cat. # JT343) was added. After 4 h, the medium was completely removed and 100 μL of dimethyl sulfoxide (DMSO) was added. After shaking the mixed solution for 5 min, a Microplate reader was used to detect the OD value (the value of OD_{490} reflects the number of viable cells), and record the data for analysis.

Flow Cytometry Apoptotic Assay

PANC-1 and SW1990 cells with EIF3H knockdown were seeded in 6-well plates (2 mL/well). After the cells were continuously cultured for 5 days, they were trypsinized and suspended. Annexin V-APC was added and stained in the dark for 15 min. The percentage of cell phase was determined by FACScan to evaluate the apoptotic rate, and the results were analyzed.

Flow Cytometry Cell Cycle Assay

PANC-1 and SW1990 cells with EIF3H knockdown were cultured at 5 mL/well in a 6 cm dish. After 5 days, PBS with 0.1% BSA was added, and the cell suspension was centrifuged at 200 g for 5 min. Cells were fixed with ethanol and then stained with propidium iodide (PI). This method revealed the distribution of cells in the three main stages of the cycle (G1 vs S vs G2).

Transwell Assay

The chamber was placed in an empty 24-well plate, and 100 μL of the serum-free medium was added to the chamber. After EIF3H knockdown, PANC-1 and SW1990 cells were cultured in 24-well plates for 24 h. The cells in the upper chamber were separated by a polyester membrane with a diameter of 8μm and a matrigel was placed. The number of seeded cells was 5×10^4 cells/well, of which the inner chamber was 100 μL/well and the outer chamber was 600 μL/well. These cells were trypsinized and resuspended in a low serum medium. Subsequently, the Transwell chamber was removed and washed with PBS. After 24 h of cell incubation, cells invaded under the membrane were fixed and stained with 20% GIEMSA. The filter membrane was fixed by 85% ethanol and stained with 0.1% crystal violet for 20 min. Finally, the cells were observed and photographed by fluorescence microscope.

Wound-healing Assay

PANC-1 and SW1990 cells with EIF3H knockdown at 1×10^5 cells/well were seeded in a 96-well plate (100 μL/well). The sample cells were continuously cultured until the cell fusion rate reached more than 90%, and then serum medium with a low concentration was replaced. The scraper was placed on the lower part of the 96-hole plate and gently pushed up over time to track the distance of the gap. Pictures were taken at preset time points (0 h and 48 h for PANC-1; 0 h and 8 h for SW1990), and cell migration rates were then calculated for each group.

Statistical Analysis

The data were expressed as mean ± SD ($n \geq 3$) and analyzed using GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). The qPCR was analyzed by $2^{-\Delta\Delta CT}$ method. T-test was used

to compare the difference. P values less than 0.05 were considered statistically significant.

Results and discussion

EIF3H is abundantly expressed in pancreatic cancer

According to the analysis of IHC (Table 1) (Fig. 1A), the expression of EIF3H in pancreatic cancer tissues or cells was significantly higher than normal tissues or cells ($P < 0.001$). Subsequently, Mann-Whitney U analysis (Table 2) indicated that the expression of EIF3H was significantly correlated in pathological data, such as pathological grade. Pearson Correlation analysis (Table 3) also confirmed this conclusion. In detail, the expression of EIF3H increases as the malignancy of the patient's tumor deepens. Kaplan-Meier survival analysis (Fig. 1B) revealed that EIF3H expression was significantly correlated with the overall survival of pancreatic cancer. In other words, as EIF3H expression increases, patient survival decreases. In addition, protein expression of EIF3H in normal pancreatic cells HPDE6-C7 and cancer cells PANC-1, SW1990 was detected by WB (Fig. 1C), indicating that EIF3H was abundantly expressed in the SW1990 and PANC-1. To sum up, EIF3H may be related to the occurrence, development and prognosis of pancreatic cancer.

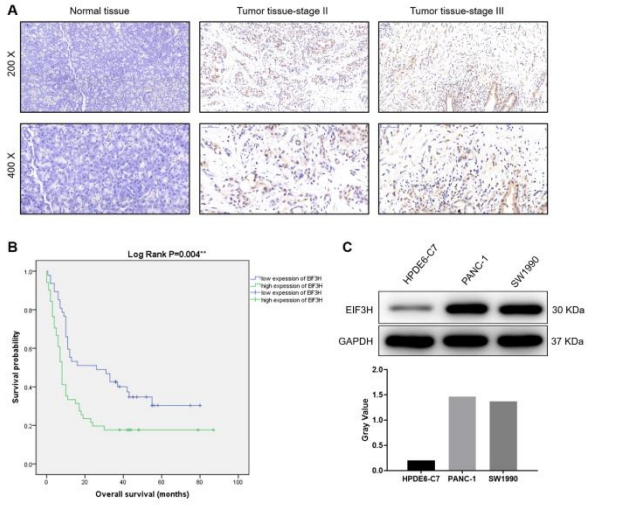


Figure 1. EIF3H is highly expressed in pancreatic cancer: (A) The expression of EIF3H in the normal and tumor samples detected by IHC. (B) Kaplan-Meier survival analysis of overall survival for pancreatic cancer. (C) Protein expression of EIF3H in normal pancreatic cells HPDE6-C7 and cancer cells PANC-1, SW1990 was detected by Western Blot. The data are presented as the mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.

EIF3H is downregulated in shRNA mediated knockdown of PANC-1 and SW1990 cells

First, the results of screening effective interference targets by qPCR (Fig. 2A) showed that among PANC-1 cells transfected with lentivirus, the highest knockdown efficiency of EIF3H was 91.2% in the shEIF3H-3 group ($P < 0.01$), so this group was selected for downstream experiments. After transfecting cells with shCtrl or shEIF3H for 72 h, the fluorescence of the cells was observed under the microscope, and the transfection efficiency was found to exceed 80% (Fig. 2B). In addition, knockdown efficiencies of EIF3H in PANC-1 and SW1990 cells were 86.8% ($P < 0.001$) and 75.9% ($P < 0.001$) in shEIF3H group, compared to shCtrl group as indicated by qPCR (Fig. 2C). Western Blot (Fig. 2D) results showed similar trends, the EIF3H protein level in the shEIF3H group was down-regulated compared with the shCtrl group. The above experiments fully demonstrated that the knockdown model of EIF3H in PANC-1 and SW1990 cells were successfully constructed.

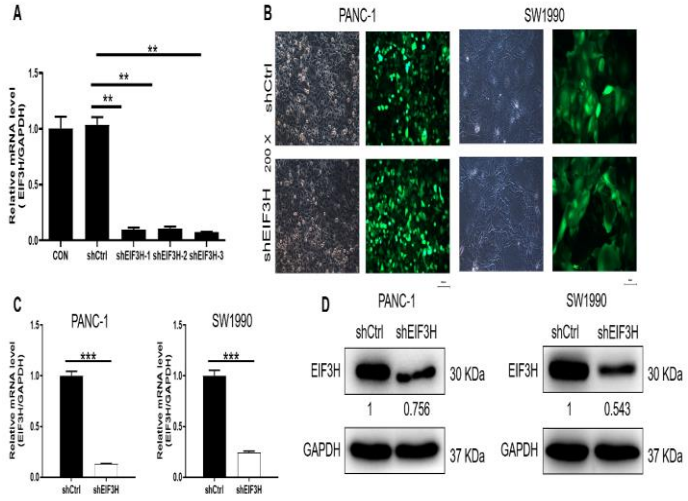


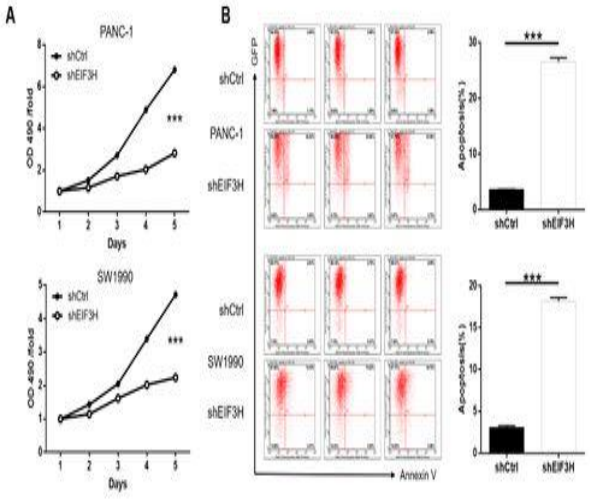
Figure 2. The construction of EIF3H knockdown pancreatic cancer cell model: (A) Effective interference targets were screened by qPCR. (B) Transfection efficiency for PANC-1 and SW1990 cells are evaluated by expression of green fluorescent protein 72 h post-infection. (C, D) The specificity and validity of the lentivirus-mediated shRNA knockdown of EIF3H expression are verified by qPCR (C) and Western Blot (D). The data are presented as the mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.

Knockdown of EIF3H inhibits cell proliferation of pancreatic cancer cells

Knocking down EIF3H or normal PANC-1 and SW1990 cells for 5 consecutive days of culture, the effect of EIF3H on the growth of human pancreatic cancer cells was detected by MTT assay. The MTT results are shown in Fig. 3A, the proliferation rate of the PANC-1 and SW1990 cells in the shEIF3H group was both significantly slower than that in the shCtrl group ($P < 0.001$). These results revealed that EIF3H knockdown can inhibit the proliferation of human pancreatic cancer cells.

Knockdown of EIF3H induces apoptosis and arrests the cell cycle of pancreatic cancer cells

To examine the effects of EIF3H knockdown on the apoptosis and cycle of human pancreatic cancer cells, Annexin V staining was used for Flow Cytometry analysis. As shown in Fig. 3B, the percentage of PANC-1 and SW1990 apoptosis in shEIF3H increased by 7.28-fold and 5.85-fold ($P <0.001$), respectively, compared with the shCtrl group. In the same way, as shown in Fig. 3C, the proportion of cells in the G2 phase in both PANC-1 and SW1990 cells was significantly increased compared to the shCtrl group ($P < 0.01$). Furthermore, the decreased expression of EIF3H in PANC-1 cells resulted in the upregulation of the expressions of apoptosis-related proteins cleaved PARP, Caspase 8, Caspase 9, and Caspase 3 (Fig. 3D). In general, EIF3H has a certain effect on cell processes, and its knockdown can promote apoptosis and disrupt the cell cycle of human pancreatic cancer cells.



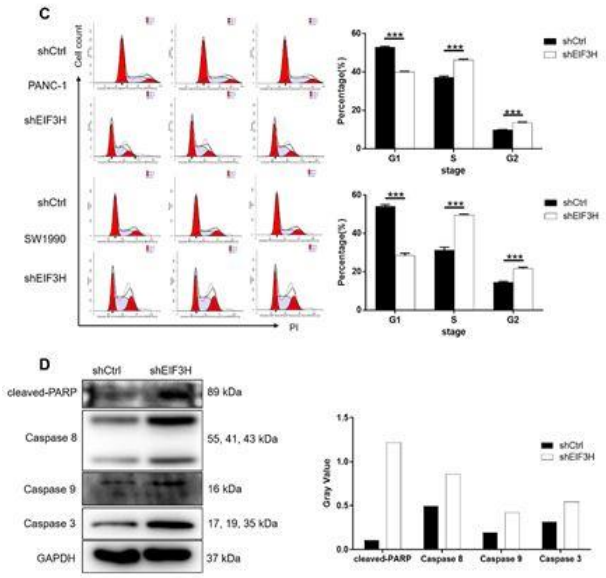


Figure 3. Knockdown of EIF3H inhibits cell proliferation and arrests cell cycle, promotes apoptosis in pancreatic cancer cells: (A) Cell proliferation of PANC-1 and SW1990 cells with or without knockdown of EIF3H is evaluated by MTT assay. (B) Flow Cytometry analysis based on Annexin V-APC staining is utilized to detect the percentage of early apoptotic cell for PANC-1 and SW1990 cells. (C) Cell cycle distribution is analyzed in PANC-1 and SW1990 by Flow Cytometry analysis. (D) Protein expression of apoptotic markers cleaved PARP, Caspase 8, Caspase 9 and Caspase 3 was detected by Western Blot. The data are expressed as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.

Knockdown of EIF3H inhibits migration of pancreatic cancer cells

In order to evaluate the effects of EIF3H on metastasis, Transwell and wound-healing assay were performed to determine the migration ability of human pancreatic cancer cells. The results of the Transwell assay showed that the migration rates of PANC-1 and SW1990 cells were reduced by 82% and 93% ($P < 0.001$), respectively (Fig. 4A). In addition, wound-healing experiments suggested that the mobility of PANC-1 decreased by 54% at 48 h ($P < 0.001$), while SW1990 decreased by 19% at 8 h in the shEIF3H group ($P < 0.05$) (Fig. 4B). Additionally, EIF3H knockdown in SW1990 cells resulted in downregulation of epithelial-mesenchymal transition (EMT) marker proteins N-Cadherin and SNAIL+SLUG (Fig. 4C). As a consequence, EIF3H knockdown impeded migration by downregulating EMT in pancreatic cancer cells.

Despite advances in the prognosis and treatment of pancreatic cancer, no significant progress has been made in improving survival. Therefore, there is an urgent need to better understand the molecular mechanisms of pancreatic cancer. In recent years, the importance of various small molecules related to RNA transcription and protein translation in the occurrence and development of cancer has been verified, especially pancreatic cancer. For example, Shibin et al., proposed that circular RNA circRHOT1 is upregulated and promotes cell proliferation and invasion in pancreatic cancer(18). Shi et al., pointed that long non-coding RNA LINC00346 promotes pancreatic cancer growth and gemcitabine resistance by sponging miR-188-3p to derepress BRD4 expression(19). Yang et al., clarified that ZIP4 promotes muscle atrophy and cachexia in mice with orthotopic pancreatic tumors by stimulating RAB27B(20). Furthermore, overexpressed histone acetyltransferase 1 regulates cancer immunity by increasing programmed death-ligand 1 expression in pancreatic cancer(21). Therefore, understanding the basics of pancreatic cancer in terms of RNA transcription and protein translation will provide novel insights.

EIF3 initiates protein translation by forming large complexes with ribosomes and mRNA, and abnormal protein synthesis can lead to cancer(22). Considerable findings have been reported, for example, EIF3B correlates with advanced disease stages and poor prognosis, and it promotes proliferation and inhibits apoptosis in non-small cell lung cancer(23). EIF3C-enhanced exosome secretion promotes angiogenesis and tumorigenesis of human hepatocellular carcinoma(24). EIF3D silencing suppresses the development of renal cell carcinoma by downregulating the Cyclin B1/CDK1 signaling pathway and inducing G2/M block(25). In addition, Wang et al., demonstrated that EIF3H knockdown inhibits cell proliferation and colony formation in gastric cancer cell lines and leads to cell cycle arrest at the G0/G1 phase(17). Yu et al., supported that the proliferation of colorectal cancer cells is suppressed by silencing of EIF3H(16). Recently, Hu et al., put forward that EIF3H interacts with PDCD4 enhancing lung adenocarcinoma cell metastasis(26). Moreover, EIF3H promotes aggressiveness of esophageal squamous cell carcinoma by modulating Snail stability (27). Therefore, it can be basically concluded that EIF3H plays an important role in the occurrence and development of tumors. Based on this perspective, this study explored whether EIF3H is involved in the progression of pancreatic cancer.

This study first proposed that EIF3H is highly expressed in pancreatic cancer. Furthermore, EIF3H is not only significantly correlated with the clinicopathological grade of pancreatic cancer, but also positively correlated with the prognosis. It was confirmed that the RNA interference strategy can effectively reduce the mRNA and protein expression of EIF3H in human pancreatic cancer cell lines, which has been confirmed by qPCR and Western Blot. In conclusion, knockdown of EIF3H inhibited the growth and colony formation, caused cell cycle disturbances, and induced apoptosis. At the same time, it also has a certain inhibitory effect on the cell migration of pancreatic cancer cells. Overall, shRNA-mediated EIF3H knockdown inhibits pancreatic cancer cell proliferation and enhances apoptosis, and EIF3H may play an important role in pancreatic cancer cells.

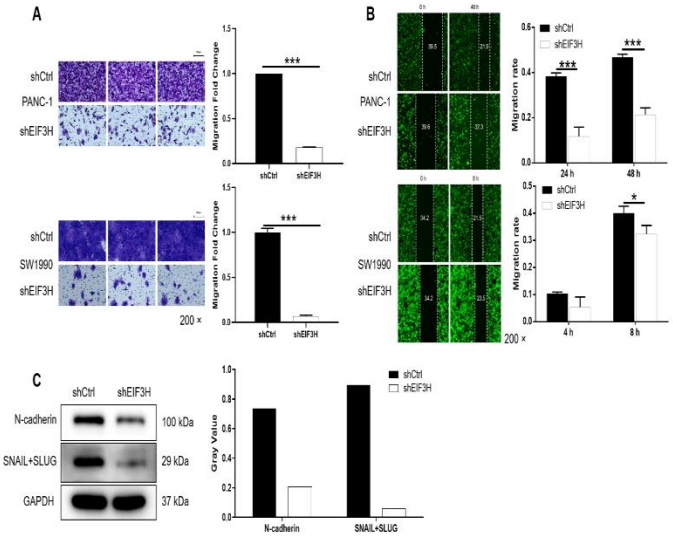


Figure 4. Knockdown of EIF3H inhibits cell migration in pancreatic cancer cells: (A) Cell migration of PANC-1 and SW1990 cells with or without knockdown of EIF3H is evaluated by Transwell assay (magnification: 200×). (B) Cell migration of PANC-1 and SW1990 cells with or without knockdown of EIF3H is evaluated by wound-healing assay (magnification: 200×). (C) Protein expression of epithelial mesenchymal transition (EMT) marker proteins N-Cadherin and SNAIL+SLUG was detected by Western Blot. The data are expressed as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.

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Author's Contribution

Weiming Zhu designed the project, Yuqiang Shan performed experiments, analyzed data and wrote the manuscript. Wencheng Kong helped with the IHC staining. Yuqiang Shan and Akao Zhu assisted in performing the cells experiments. Jiangtao Li helped to perform in vivo mouse studies. Huicheng Jin analyzed the statistical and bioinformatics data. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Ethics approval

The study is approved by the Ethics committee of Nanjing Medical University.

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