Decreased miR- 31 Suppress Cell Migration and Proliferation By Targeting Syncytin-1 in Pancreatic Carcinoma

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Abstract

Background: Pancreatic cancer(PC) is seriously harmful to human health, and the pathogenesis is not clear. The present study aimed to explore the functional role of syncytin-1 in PC.

Methods: Syncytin-1 and miR-31 expression was analyzed by qRT-PCR and Western blot analysis in both human PC cell lines and tissuse. The prognostic significance of syncytin-1 was investigated using the immunohistochemistry(IHC) and Kaplan-Meier survival. The CCK-8 assay and transwell assays were used to determine the role of syncytin-1 and miR-31 in cell proliferation, migration and invasion. Luciferase reporter assays was used to identify possible miRNA targets in tumorigenesis.

Results: The results showed that the syncytin-1 level was significantly decreased in PC cell lines and tissues than normal($P < 0.05$), while miR-31 was markedly higher than normal($P < 0.05$), and low expression of syncytin-1 have a poor prognosis than high expression($P < 0.05$). Overexpression of syncytin-1 significantly reduced the PC cell proliferation and invasion ability in PANC-1 and BxPC-3 cells($P < 0.05$), and miR-31showed contrary results. The Dual-Luciferase reporter gene assay demonstrated that miR-31 binded directly to 3'UTR of syncytin-1 and resulting in the inhibition of syncytin-1. The overexpression of miR-31 promoted migration and proliferation of PC cells through down-regulating the expression of syncytin-1.

Conclusion: We verified that syncytin-1 can inhibit proliferation and invasion of PC cell lines by targeting miR-31.

Introduction

PC is a disease that is insidious, rapid, and malignant high, therapeutic and prognosis of digestive tract malignancies [1, 2]. Although, new breakthrough in treatment, only 10% ~ 20% of PC patients can be resected surgically, and their 5-year survival rate is only 4% [3]. So, it is very important to further improve the mechanism of PC. In recent years, more and more evidences showed that the expression of syncytin-1 was related to the occurrence of tumor.

Syncytin-1 is a base located on chromosome 7 (7q21.2), which has been confirmed to be encoded by human endogenous retroviral envelope protein gene(HERVW1) [4–7]. Syncytin-1 can promotes placental trophoblast proliferation and inhibits cytotrophoblast cell apoptosis in both knockdown and overexpression models in vitro [8–12]. Moreover, syncytin-1 has shown activates the body's inflammatory response, which can trigger T lymphocyte loss in patients with multiple sclerosis (MS) [13, 14].

Studies have indicated that syncytin-1 and estrogen stimulation induction is closely related, and which is in the cytotrophoblast fusion into the process of syncytium trophoblast key action [15]. It has been confirmed that the expression level of syncytin-1 is related to clinical manifestations in organ cancer patients [16], acute myeloid leukemia [17], breast cancer[18], endometrial carcinomas [19], ovarian cancer and colorectal cancer [20, 21]. In this study, we confirmed the role of syncytin-1 in the carcinogenesis of

PC. Furthermore, we proved miR-31 is the upstream gene targeting syncytin-1. Which indicated that syncytin-1 may play an anti-cancer role in PC through miR-31 pathway, is a potential target of PC therapy.

Materials And Method

Clinical Samples

From January 2015 to January 2017, we collected the pathological tissue samples of 60 patients with PC who were operated in the Binzhou medical University Hospital. All the patients signed the informed consent form. Inclusion criteria: 1) radical surgical resection; 2) Clear pathological results; 3) Standardized completion of adjuvant therapy; 4) The survival time after operation exceeded 1 month. Exclusion criteria: 1) preoperative chemoradiotherapy history; 2) Death during operation and perioperative period; 3) Death due to other non-tumor causes; 4) Lack of complete follow-up data and incomplete pathological data; 5) Failure to complete adjuvant therapy.

Cell Culture

Human PC cell lines PANC-1, BxPC-3, AsPC-1, human normal pancreatic epithelial cell line HPC-Y5, miR-31 mimics and negative control (miR-NC) were bought from GenePharma (Shanghai, China). All cell lines were cultivated in RPMI 1640 medium (hclone, Logan, UT, USA) according to the instructions. The transfection was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative RT-PCR analysis and Western Blotting

According to the instructions, we first extract total RNA (Takara, Shiga, Japan). Primers were synthesized by Shanghai Sangen Biotechnology Co., Ltd. for Real-time PCR. The primers were as follows (5'-3'): Syncytin-1 forward primer: GGAGGAGATGTGGCACCATT, reverse primer: CCTTCCCACCACAGAAGACC. β-actin forward primer: TGCTGTCCCTGTATGCCTCT, reverse primer: AGGTCTTTACGGATGTCAACG. Data were analyzed by the method of 2-ΔΔCt.

Western blot was carried out on the BioRad microgel system (Biosharp, USA). The protein of syncytin-1 and GAPDH in the primary antibody was detected by diluting anti syncytin-1 and anti GAPDH antibody (Beyotime, China) overnight at 4 ℃. The strip was detected by colorimetry and exposed to autoradiographic film.

Plasmid construct and transfection

We purchased pcDNA3.1- Syncytin-1 expression vector from geneframa company in Shanghai. Cells lines (5×10^5 cells) were vaccinated in 6-well plates. Then, cells were transfected with pcDNA3.1 and pcDNA3.1- Syncytin-1 plasmid using Lipofectamine 2000 reagent (Beyotime, China).

Cell Counting Kit (CCK) − 8 assay and Transwell assay

PANC-1 and BxPC-3 cell lines, sow in 96 well plates for 24 h. Then, at different time points, each well is added with analytical reagent (beyotime, China), and then incubated. The absorbance value at 450 nm was recorded, and the results at four time points were averaged. The cell survival rate were calculated as the percentage of untreated control. Each test was conducted in three wells and repeated three times.

Detection of cell migration ability: after digestion and cultivation of logarithmic cells, the concentration was adjusted to 2×10^5/ml. Cells were incubated at 37 °C for 24 hours. The cells were washed twice, fixed for 30 minutes and stained for 15 minutes. After drying, the number of cells migrating or invading was analyzed under microscope from 5 randomly selected fields at a magnification of × 100.

DNA constructs and luciferase reporter assays

In order to construct miR-31-luc report plasmid, the 3'-UTR fragment of syncytin-1 containing miR-31 binding site was cloned into the modified pGL3 promoter vector (Beijing transgen biotech). Using the following primers, PCR was used to amplify the full-length 3 '- UTR of syncytin-1. All luciferase data were expressed as the normalized luciferase / renin ratio.

Syncytin-1 Forward/SpeI: 5'-TCACTAGTCTTTATATAAAGTTAGCACTTT-3'

Syncytin-1 Reverse/ SphI: 5'-TAGCATGCCAAAGTGCCCTCATAGGA-3'

Statistical Analysis

Use prism 5.0 (graphpad software, USA) for statistical analysis. All results were expressed as means ± SD. $P < 0.05$ was considered statistically significant.

Results

Correlation Analysis of Syncytin-1 Expression and Clinicopathological Parameters and Overall Survival of PC

We analyzed the expression of syncytin-1 in PC and its relationship with clinicopathological factors by IHC. The results showed that syncytin-1 was mainly distributed in the cytoplasm (Fig. 1A, B). Syncytin-1 was highly expressed in 21 out of 60 (35.00%) cases of adjacent tissues and in 11 out of 60 (18.33%) cases of PC with significant difference ($\chi 2 = 4.261$, $P = 0.039$). Statistical analyses were performed to explore the correlation between syncytin-1 expression and the clinical characteristics of PC as detected by immunohistochemical staining. The result of Chi-square test showed that syncytin-1 expression was positively correlated with grade ($P = 0.041$) and tumor T stages ($P = 0.021$)(Table 1), thereby indicating that these clinical features are correlated with syncytin-1 expression.

	variables	syncytin-1 expression		total	χ^2	*P* value
		low	high			
Age(year)					1.558	0.212
	≤65	21	7	28		
	65	28	4	32		
Sex					2.262	0.133
	Female	15	6	21		
	male	34	5	39		
Grade					4.184	0.041*
	I/II	19	8	27		
	III	30	3	33		
T stage					5.353	0.021*
	T1/T2	10	6	16		
	T3	39	5	44		
N stage					0.363	0.562
	N0	27	5	32		
	N1	22	6	28		
M stage					0.127	0.721
	M0	46	10	56		
	M1	3	1	4		
TNM stage					3.763	0.052
	I/II	43	7	50		
	III	6	4	10		
* Statistically significant(*P*<0.05)						

Table 1
Correlation between syncytin-1 expression and clinicopathological characteristics

Kaplan-Meier analysis was used to assess the overall survival according to syncytin-1 expression and to further explore the correlation between syncytin-1 expression and PC survival. The 1-year, 3-year cumulative survival rate was 63.6%, 27.3% in patients with PC and high syncytin-1 expression; and 38.8%, 18.4% in those with low expression of syncytin-1. The patients with PC and low expression of syncytin-1

have a poor prognosis (Fig. 1C, $\chi^2 = 4.079$, $P = 0.043$). Therefore, syncytin-1 expression is associated with survival and prognosis. Further, univariate and multivariate Cox regression analyses were performed to determine whether the expression of syncytin-1was an independent prognostic factor of patients' outcomes. The results revealed that the M stage and TNM stage was identified as independent prognostic factor, the expression of syncytin-1was not identified as independent prognostic factor (Table 2). Our results suggested that syncytin-1 might represent a novel and potentially useful biomarker for the progression and prognosis of patients with PC, but not independent.

variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Syncytin-1	0.521	0.269-1.010	0.053	0.788	0.351-1.768	0.563
sex	1.198	0.664-2.161	0.548			
Grade	3.113	1.764-5.494	0.000	2.873	1.354-6.094	0.006
Age	1.302	0.733-2.315	0.368			
T stage	1.205	0.552-2.628	0.640			
N stage	1.455	0.796-2.662	0.223			
M stage	3.201	1.103-9.291	0.032	0.366	0.014-9.537	0.545
TNM stage	1.878	1.167-3.022	0.009	2.172	0.467-10.099	0.322
* Statistically significant(P<0.05)						

Table 2
Univariate and multivariate analyses of the factors correlated with Overall survival of Pancreatic carcinoma patients

The Expression of Syncytin-1 in PC Tissues

The expression levels of syncytin-1 was evaluated in human PC samples by qPCR and western blot analysis. The data from qPCR analysis showed that the expression of syncytin-1 was expressed at low levels in PC samples compared with normal tissues *(*P < 0.01; Fig. 1D). In addition, western blot analysis in 3 pairs of PC samples also revealed low syncytin-1 expression level in PC tumors (P < 0.01; Fig. 1E,F).

The Expression of Syncytin-1 in PC cell lines

We evaluated the expression of syncytin-1 in PC cell lines PANC-1, BxPC-3, AsPC-1 and normal pancreatic epithelial cell line HPC-Y5. In the present study, the expression detection of syncytin-1 was subjected to western blot analysis. We found that the expression of syncytin-1 was significantly decreased in PANC-1, BxPC-3, and AsPC-1 cells, while the expression of syncytin-1 was obviously increased in normal HPC-Y5 cells, and the expression of endogenous syncytin-1 in PANC-1 cells was significantly higher than that in other cancer cells, while BxPC-3 cells is the lowest(Fig. 2A, B). This result provides a basis and premise for the subsequent exogenously highly expressed adenovirus syncytin-1 or sh- Syncytin-1 as a tool to study the effects of syncytin-1 on the function of both cells PANC-1 and BxPC-3.

Syncytin-1 inhibits PC cell proliferation, migration and invasion in vitro

To investigate whether syncytin-1 is essential for PC cell proliferation, We generated stable syncytin-1-overexpression BxPC-3 cell lines, syncytin-1-knockdown PANC-1 cell line, as well as corresponding control cell lines. Compared with the vector control group using a CCK8 assay and Plate cloning experiment overexpression of syncytin-1 significantly suppressed BxPC-3 tumor cell growth ($P < 0.01$, Fig. 2C-E). Compared with the shNC group using a CCK8 assay and Plate cloning experiment, shsyncyntin-1 significantly promoted proliferation in PANC-1 tumor cell growth ($P < 0.01$, Fig. 2F-H).

Overexpression of syncytin-1 inhibits PC cell migration and invasion

Because we found that overexpression of syncytin-1 inhibited PC cell proliferation, we further assessed the effect of syncytin-1 on migration and invasion, the data showed that overexpression of syncytin-1 markedly suppressed the migration and invasion capacities of BxPC-3 ($P < 0.01$, Fig. 3A-D). While the shsyncytin-1 in PANC-1 cells showed that markedly promoted the migration and invasion capacities ($P < 0.01$, Fig. 3E-H).

The 3'-UTR of syncytin-1 is a possible target of miR-31

We would like to know if miRNAs are involved in syncytin-1-induced PC generation. In order to solve this problem, we used Diana, targetscan, and pita database to analyze bioinformatics, and constructed the ceRNA network of syncytin-1. We found that there were three miRNAs (including miR-31, mir-202 and mir-108) in the network. In addition, Starbase program was used to predict the potential miRNA targets of syncytin-1. Among these miRNAs, only the expression of miR-31 was negatively correlated with the expression of syncytin-1 in PC samples ($P < 0.01$), miR-31 binds to the syncytin-1 mRNA 3'UTR and down-regulates the synthesis of syncytin-1 protein to potentially regulate the biological processes of cancers (Fig. 4A). Luciferase report analysis showed that miR-31 mimic significantly inhibited the activity of syncytin-1 wild-type reporter plasmid, indicating that syncytin-1 directly affected miR-31(Fig. 4B).

Up-regulation of miR-31 in human PC

The expression levels of miR-31 were evaluated in human PC samples. Our results show that the expression of miR-31 was (3.72 ± 0.51) in PC tissues of patients, markedly higher than (2.09 ± 0.27) in adjacent tissues, with a significant difference (t = 17.632, *P* = 0.012; Fig. 4C). Indicating miR-31 might be involved in the pathological development of human PC.

Overexpression of miR-31 result in promotion of cell migration and proliferation, reduce the protein levels of the syncytin-1

To explore the role of miR-31 involved in PC, we transfected BxPC-3 and PANC-1 cell lines with miR-31 mimics to upregulate their expression. The transwell assay and CCK8 assay showed that the migration and proliferation rate of BxPC-3 and PANC-1 cells were significant increased after overexpression of miR-31(Fig. 4D). The downregulation of the syncytin-1 protein levels were also detected by western blot assay after the transfection (Fig. 4E).

Discussion

More and more studies show that the critical roles of syncytin-1 in human diseases [22–28]. However, the functional role of syncytin-1 is complicated. The integrin of syncytin-1 vary significantly among different cancers [29–35]. For example, in colorectal cancer, the methylation level of syncytin-1 promoter is higher than the normal tissues[36], but is lower in the testis and endometrial cancer [37, 38], which is related to tissue types and their signaling pathways. Nevertheless, the signal pathway of syncytin-1 in PC is not clear. In order to clarify the role and pathway of syncytin-1, we analyzed the effect of syncytin-1 on cell proliferation and cell transfer, as well as the relationship between syncytin-1 and upstream miRNA through PC cells.

On the basis of our experimental data in vitro, the expression of syncytin-1 was downregulated either in PC tissues and cell lines, compared with normal tissues and cells. Further study showed that the expression of syncytin-1 was related to T stage and pathological grade. In CCK8 and Transwell experiments, it could significantly inhibit the proliferation and invasion of PC cell lines, indicating that syncytin-1 may play an anti-cancer role in the process of PC development.

MiRNAs are a kind of noncoding RNAs, which have been proved to play a role in the post-transcriptional level by targeting the 3'-untranslated region (3'-UTR) of the downstream gene[39–43]. We use variety of online tools to predict the potential RNAs for syncytin-1, including DIANA, TargetScan, and PITA. Among these miRNAs, only the expression of miR-31 was negatively correlated with the expression of syncytin-1 in PC samples ($P < 0.01$), miR-31 binds to the syncytin-1 mRNA 3'-UTR and down-regulates the synthesis of syncytin-1 protein to potentially regulate the biological processes of cancers, and miR-31 mimic significantly inhibited the activity of syncytin-1 wild-type reporter plasmid, indicating that syncytin-1 directly affected miR-31.

Conclusion

To sum up, we confirmed the antitumor effect of syncytin-1 in the development of PC by its expression in tissues and cell lines. In addition, miR-31 can play a regulatory role by targeting the 3'-UTR of syncytin-1 mRNA, further clarifying the mechanism of syncytin-1.

Declarations

Ethics approval and consent to participate:

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Consent for publication:

Authors grant World Journal of Surgical Oncology to publish the article.

Availability of data and materials:

The author states that the data and materials are authentic.

Competing interests:

The authors have no conflicts of interest to declare.

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Authors' contributions:

Conception and design: Changmin Liu, Jing Yang; Administrative support: Judong Luo; Provision of study materials or patients: Feng Wang; Collection and assembly of data: Zhiwen Cheng, Xia Han; Data analysis and interpretation: ZhenboWang; Manuscript writing: All authors; Final approval of manuscript: All authors.

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Figures

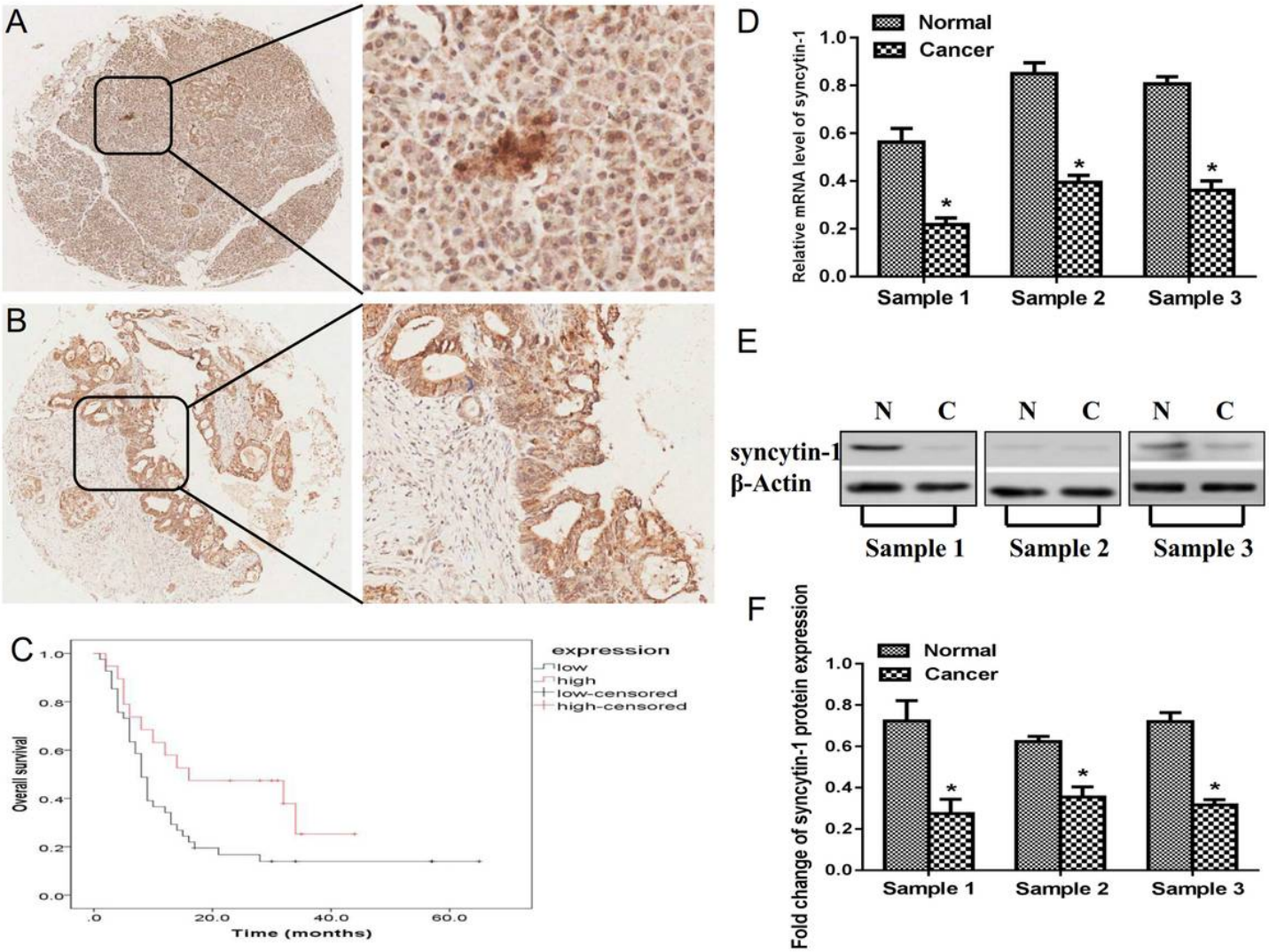

Figure 1

Downregulated expression of syncytin-1 associates with progression and prognosis in PC. (A) Immunohistochemical staining of syncytin-1 in normal pancreatic tissues. (B) Immunohistochemical staining of syncytin-1 in PC tissues. (C) Kaplan-Meier survival curves showed that PC patients with high expression of syncytin-1 have a higher survival than those with lower expression of syncytin-1 (P = 0.043). (D) qRT PCR showed that the expression of syncytin-1 was lower in PC samples than normal tissues (*P < 0.01). (E,F) Protein levels of syncytin-1 in three samples detected by western blotting showed that the expression of syncytin-1 was lower in PC samples than normal tissues (*P < 0.01).

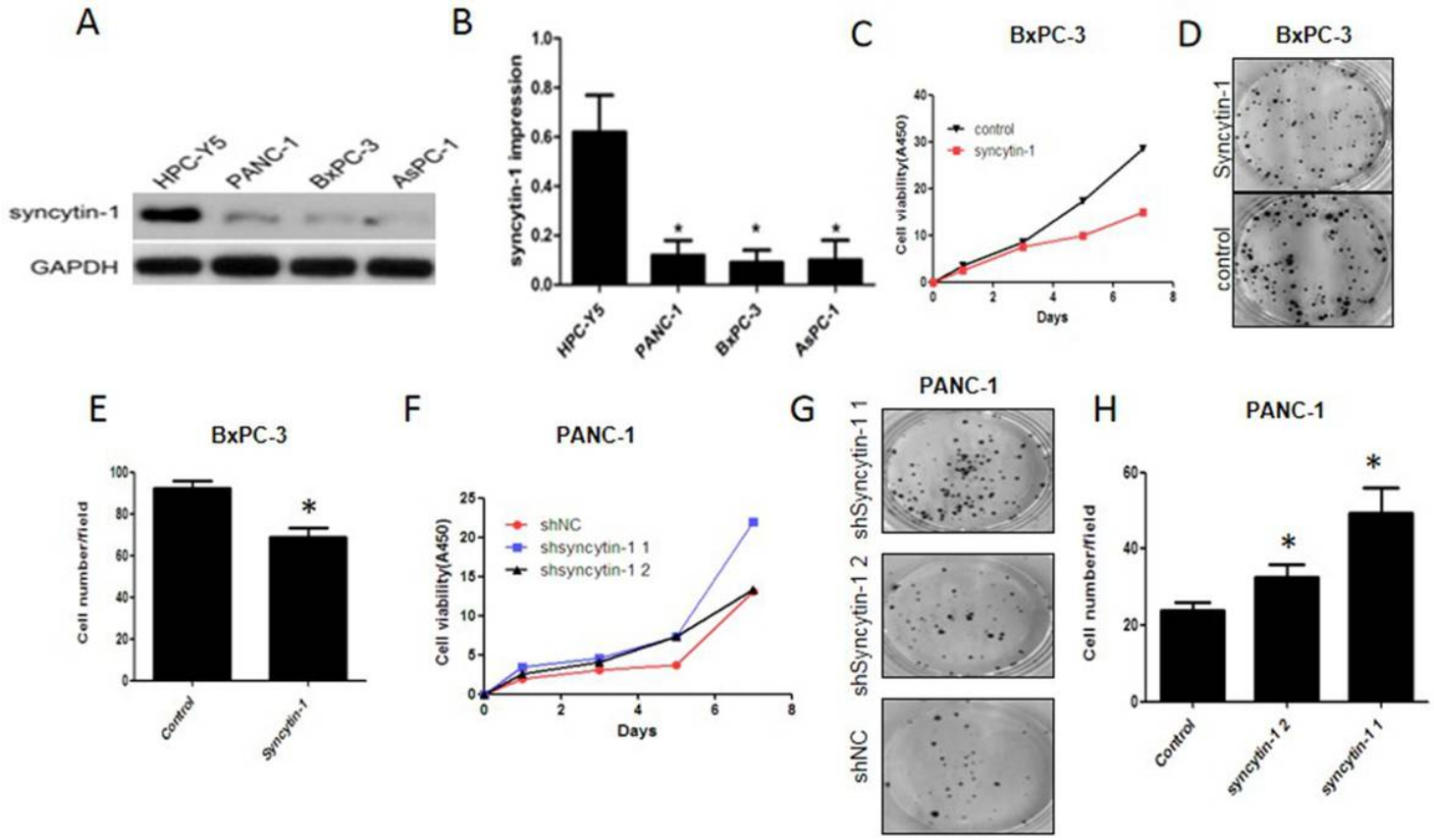

Figure 2

The expression of syncytin-1 in pancreatic cancer cells. (A,B) Syncytin-1 expression on protein level was determined in normal pancreatic epithelial cell line HPC-Y5 and three PC cell lines PANC-1, BxPC-3 and AsPC-1, the results showed that the expression of syncytin-1 was lower in PC celles than normal (*P < 0.01). (C-E) Comparison with the control group using a CCK-8 assay and late cloning experiment showed that the overexpression of syncytin-1 significantly suppresses BxPC-3 tumor cell growth (*P < 0.01). (F-H) Comparison with the shNC group using a CCK-8 assay and Plate cloning experiment confirmed that shsyncyntin-1 significantly promotes the proliferation in PANC-1 tumor cell growth (*P < 0.01).

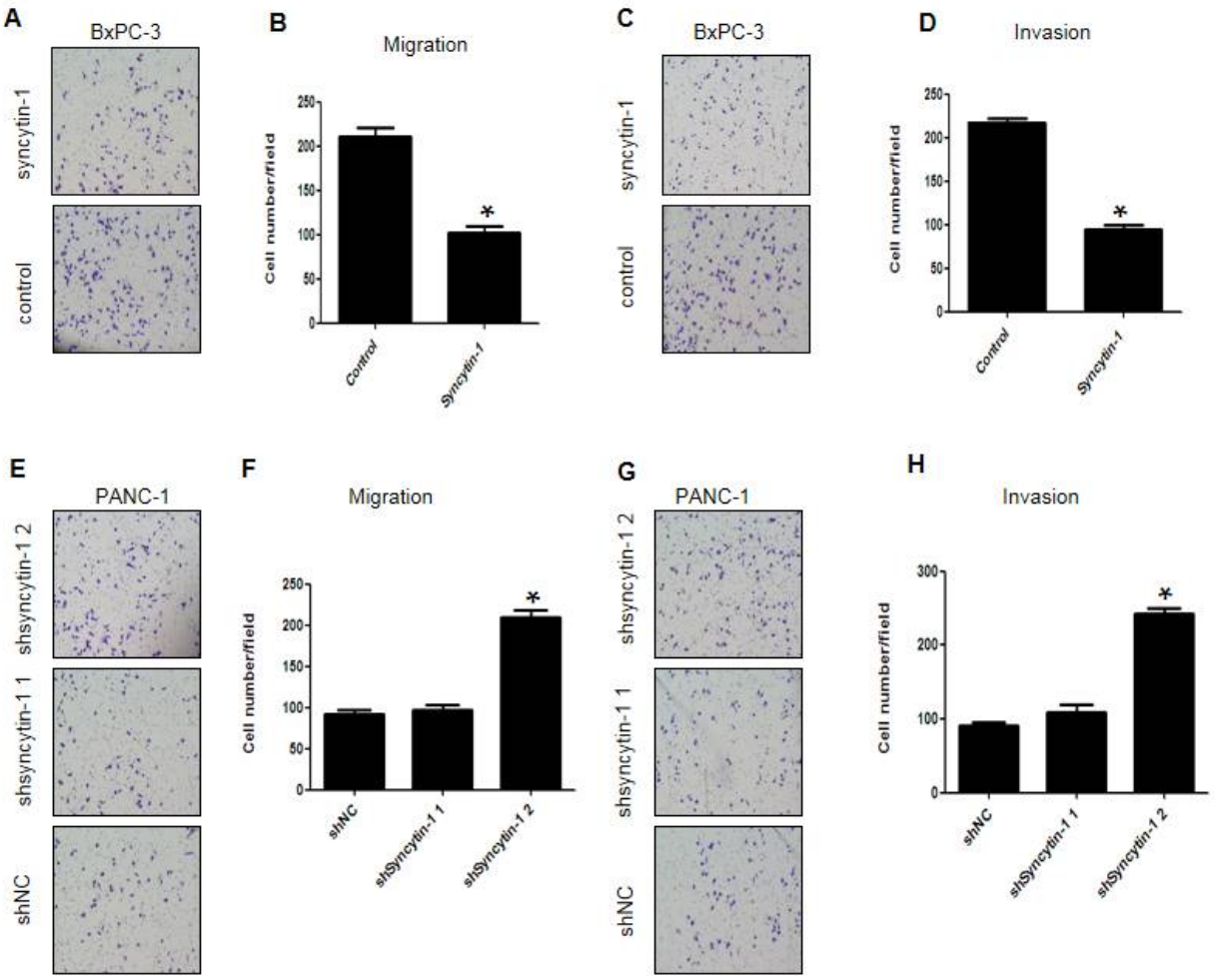

Figure 3

Syncytin-1 suppresses PC cell migration and invasion. Migration(A, B) and invasion (C, D) abilities of the overexpressed syncytin-1 in BxPC-3 tumor cells were measured by Transwell assays (*P<0.01). Migration (E, F) and invasion (G, H) abilities of shSyncytin-1 in PANC-1 cells were measured by Transwell assays (*P<0.01).

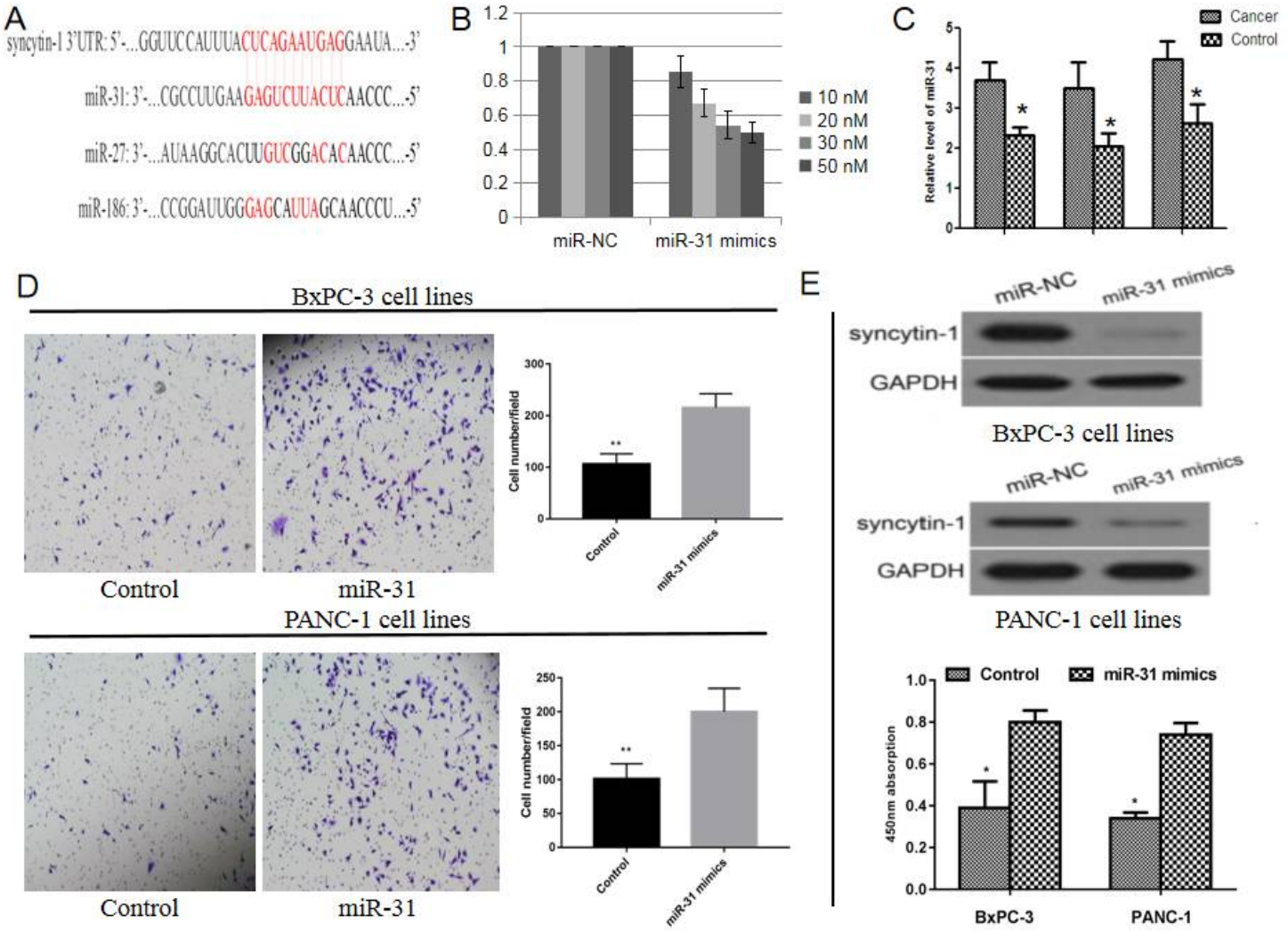

Figure 4

Overexpression of miR-31 result in promotion of cell migration and proliferation, reduce the protein levels of the syncytin-1 (A) miR-31 directly targets the 3'UTR of syncytin-1 mRNA. The predicted target site of miR-31 (middle) in the syncytin-1-3'UTR region (bottom) was detected by three software. (B) The dual-luciferase activity assay was used to analyze the relative luciferase activities, miR-31 mimics reduced the fluorescence intensity of syncytin-1-3'-UTR cells in a dose-dependent manner, while the miR-NC did not change the fluorescence intensity of syncytin-1-3'-UTR cells. Each bar represents the mean ± SEM of three independent experiments. *P < 0.001, compared with miR-NC. (C) qRT PCR showed that the expression of miR-31 was significantly higher in the PC tissue than that in the tissue adjacent to carcinoma, with a significant difference (P=0.012). (D) The transwell assay and CCK8 assay showed that the migration and proliferation rate of BxPC-3 and PANC-1 cells were significant increased after overexpression of miR-31.**P < 0.001, compared with Control. (E) Overexpression of miR-31 result in downregulation of the syncytin-1 protein levels of BxPC-3 and PANC-1 cells were detected by western blot. *P < 0.001, compared with miR-NC.

Supplementary Files

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