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Original Article

Pancreatic cancer stem cells mediate drug resistance against evodiamine by activation of HIF-1α-Notch-Hes1/5 pathway

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Abstract: Pancreatic cancer is one of the most common cancers worldwide. In sharp contrast to its high occurrence, its treatments are still limited. It was partly due to the development of resistance against chemotherapy, which may be largely related to cancer stem cells (CSCs). This study aimed to investigate the contribution of pancreatic CSCs in Evodiamine (Evo) resistance. Evo was used to treat Panc-1, and the characteristics of remaining Evo-resistant cells were determined by transwell and wound-healing assays. CSC surface markers CD44, CD24 and ESA were used for sorted pancreatic CSCs, and mixing study was performed to investigate the contribution of CSCs in Evo resistance. Key genes knockdown/expression and Notch signaling pathway inhibition were also carried out, and their expression was examined by RT-PCR or western blot. The expression of CSC surface markers were up-regulated from 1.9 to 3.3 fold in the Evo-resistant pancreatic cancer cells, and Evo-resistant pancreatic cancer cells exhibited CSC-like characteristics in cell migration and invasion. It was also shown in our mixing study that CSCs were directly involved in the development of Evo resistance of pancreatic cancer cells. Moreover, we found pancreatic non-CSCs could converse into CSC-like cells under high dose of Evo induction. Our results revealed that the key signaling proteins involved in HIF-1α-Notch-Hes1/5 pathway were significantly up regulated in Evo-resistant pancreatic cancer cells. These results suggest that pancreatic CSCs play an crucial role in Evo resistance by HIF-1α-Notch-Hes1/5 pathway in pancreatic cancer, which provides some clues for designing effective treatments against Evo resistance in pancreatic cancer.

Keywords: Pancreatic cancer, evodiamine, cancer stem cells, HIF-1α, Notch, Hes1/5

Introduction

Pancreatic cancer is one of the most lethal cancers, with 5 year-survival rate less than 5%, and the median survival less than six months [1, 2]. The pancreatic cancer has strong resistance against traditional chemotherapy and radiotherapy, which may limit clinical outcomes against pancreatic cancer in a long term [3]. There is accumulating evidence suggesting that a group of distinct subpopulation of cancer cells, cancer stem cells (CSCs), greatly contribute to the drug- and radio-resistance as well as the progression of several cancers, including pancreatic cancer [4-7]. CSCs have been reported in pancreatic cancer cells with the specific cell surface markers ESA+/CD44+/CD24+, which suggests that it has the multipotency to differentiate into various types of tumor cells [8].

To overcome the drug-resistance of pancreatic cancer cells, it is crucial to understand how CSCs contribute to the development of pancreatic cancer resistance. The CSCs' properties are strongly related to specific microenvironments, like hypoxia. Many tumors are developed from hypoxic areas, which may promote its proliferation. There are increasing evidences supporting that hypoxia-inducible factor-1α (HIF-1α) plays an important role in tumor generation [9-11]. HIF-1α, one of hypoxia-inducible factors (HIFs), is a key transcriptional factor that contributes to the cellular adaptive res-

ponses to hypoxia. It was reported in previous research that HIF-1α might induce the conversion of non-CSCs to CSCs [12, 13]. Notch signaling pathway is a highly conserved signaling pathway regulated by HIF-1α, which usually includes receptors, ligands and intracellular effectors. After binding with various ligands, the conformation of Notch receptors changes, which induces a series of cleavages and then releases the Notch intracellular domain (NICD) into the cytoplasm [14-16]. Subsequently, NICD transports into the nucleus and form NICD/CSL complex with DNA-binding transcription factor CSL to activate target genes, like Hes1 and Hes5 [17-19]. Notch signaling pathway is involved in many aspects of cell fate control during development [20], such as self-renewal of stem cells, differentiation of pancreas [21-23], neural stem cells activation and proliferation [24]. The upregulation of Notch pathway often occurs in caners and promotes the survival and self-renewal of CSCs. It was reported that Notch signaling pathway was positively correlated with the ratio between multi-potent and undifferentiated pancreatic progenitor cells in pancreatic epithelial differentiation [21-23]. Similarly, up-regulation of the Notch signaling pathway was identified in breast and brain CSCs and was involved in their self-renewal. In these cases, the inhibition on Notch signaling pathway significantly impaired CSCs self-renewal and suppressed tumorigenesis [25, 26]. Hes1 and Hes5 are target genes of the Notch signaling pathway, which are involved in cell fate decision. Activation of Hes1 and Hes5 can promote CSCs' proliferation and inhibit their differentiation. Previous studies reported that Hes1 and Hes5 contributed to the maintenance of neural stem cells population, hematopoietic stem cells as well as cancer stem cells in lung cancer, glioblastoma, cholangiocarcinoma, cervical carcinoma and murine leukemia [27-30].

Evodiamine (Evo) is the effective constituent of Evodia rutaecarpa, atraditional herbal medicine that exhibits various properties, such as anti-tumor, anti-nociception and vasorelaxation, etc. [31]. In pancreatic cancer, Evo has been used to enhance the therapeutic effects of gemcitabine by inhibition of PI3K/Akt pathway [32]. However, using only Evo to inhibit pancreatic cancer achieved an unsatisfactory outcome. Thus, we hypothesize it may be due to the cancer stem cells that mediated the drug-resistance in pancreatic cancer.

Drug resistance is a complex phenomenon highly related with CSCs and involved the activation or inhibition of several signaling pathways. In this study, we investigated the CSCs-like characteristics of Evo-resistant pancreatic cancer cells, and the change of molecular mechanism and signaling pathways responsible for the metastasis and drug-resistance mediated by CSCs in pancreatic cancer. The results of our study may provide novel insights into the reversal of drug resistance in pancreatic carcinoma treatment.

Materials and methods

Cell lines and cell culture

Panc-1 cells purchased from Chinese Academy of Sciences Cell Bank (CAS, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham, USA) containing 10% fetal bovine serum (FBS, Biological Industries, Cromwell, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, USA) and placed at 37°C with 5% CO_2. Panc-1 cells were cultured in serum-free medium, and cultured tumor balls were made into individual cells, incubated with magnetic beads coated with mouse anti-human CD133 antibody, and then positive CD133 cells were separated out, and continuously cultured in serum-free medium. Positive CD133 cells in CSCs were detected by a flow cytometer, and CD44 and CD24 were detected using a Western blot assay.

Cell viability assay

Panc-1 cells were seeded in 96-well cell plates and various concentrations (0, 1, 2, 4, 8, 16 μM) of Evodiamine (Sigma, St. Louis, USA) were used to treat Panc-1 cells for 24 h. Then, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, Madison, USA) was employed for determining the cytotoxicity of Evodiamine to Panc-1 cells according to manufacturer's instructions.

Wound-healing and transwell assays

Several cell lines (pancreatic CSCs, non-CSCs, R1 and R2) were seeded in 6-well cell plates and the physical gap was created by a 10 μl pipette tip. Scratch width was assessed 24 hours later. For transwell invasion assay, cells in logarithmic growth phase were adjusted to 3

× 10^4 cells/well in 200 μL medium (without serum) and seeded into the upper chamber. The lower chamber was added with 500 μL medium (with 10% FBS), and then the chamber was incubated at 37°C for 48 h. Then the migrated cells were visualized by the crystal violet and inverted microscope.

Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen, CA, USA) was used to isolate total RNA. Detections of HIF-1α, Notch 1, Hes1 and Hes5 were carried out using primers as follows: Hes1, forward 5'-CAGCCAGTGTCAACACGACAC-3' and reverse 5'-TCGTTCATGCACTCGCTGAG-3'; Hes5, forward 5'-CGCATCAACAGCAGCATAGAG-3' and reverse 5'-TGGAAGTGGTAAAGCAGCTTC-3'; HIF-1α, forward 5'-AAGCCCAGAGTCACTGGGACT-3' and reverse 5'-GTACTCACTGGGACTGTTAGGCTC-3'; Notch1, forward 5'-AGGGTTTCAAAGTGTCAGAGGC-3' and reverse 5'-CCGGTGGTCAGTCTGATCATC-3'; β-actin was used as internal control, forward 5'-CG TTGACATCCGTAAAGACCTC-3' and reverse 5'-TAGGAGCCAGGGCAGTAATCT-3'. The expression of each gene was quantified by the comparative CT method and normalized to internal control gene.

Mixing study and flow cytometry analysis

First, pancreatic CSCs and non-CSCs were sorted by CSCs surface markers CD44, CD24 and ESA in Panc-1 cells. Then, CSCs were labeled with PKH67 (Sigma, St. Louis, USA), and the labeled cells were isolated by flow cytometer for mixing study. After mixing stem cells with non-stem cells at several ratios, the mixing populations were treated with 16 μM Evodiamine for 24 h. Finally, cell viability was measured by MTS (Promega, Madison, USA) and the percentage of cells with fluorescence was analyzed by flow cytometer. In addition, the collected cells were trypsinized using EDTA-free trypsin, and washed with PBS twice. Then 5 × 10^5 cells were added with 500 μl binding buffer, and resuspended, and then added with 5 μl Annexin V-FITC and 5 μl PI, mixed, and incubated in the dark for 15 min. The cell apoptosis was determined using a flow cytometer.

Gene knockdown/expression and notch signaling pathway inhibition

Cells were seeded in a 12-well cell plate and cultured to 90% density before transfection or treatments. SiRNAs and overexpression vectors for HIF-1α or Hes1/5 were purchased from Suzhou Ribo Life Science (Suzhou, China) and transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to manufacturer's instruction. 2 μmol/L gamma secretase inhibitor (GSI) and N-[N-(3,5-difluorophenacetyl-l-alanyl)]-S-phenylglycine t-butyl ester (DAPT) (Sigma, St. Louis, USA) were used for inhibiting Notch signaling pathway.

Western blotting

Anti-CD44, Anti-CD24, Anti-ESA, Anti-HIF-1α, Anti-Notch-1, Anti-Hes1, Anti-Hes5, Anti-β-actin and goat anti-rabbit IgG antibodies were purchased from Abcam (Cambridge, UK). Cells were collected and total protein was prepared using RIPA lysis buffer. Proteins were separated by SDS-PAGE before being transferred to a PVDF membrane (Milipore, USA). After being blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween (TBST) and washed in TBST for three times, the membrane was incubated with primary antibody (1:1000) overnight at 4°C and then in the corresponding secondary antibody (1:2000) in TBST with three washing. ECL regents were used to visualize the protein. The density of the protein bands was analyzed by Image J software.

Statistical analysis

In this study, GraphPad 7 was employed to draw required figures and analyze data. Quantitative data were expressed as the mean ± standard deviation (mean ± SD), compared between groups using the independent-samples T test, and compared among multiple groups using the one-way ANOVA, expressed as F. Post hoc pairwise comparison was carried out using the LSD-t test, and comparison in expression at multiple time points was performed using the repeated measures analysis of variance, expressed by F, and Bonferroni post hoc test was applied. $P < 0.05$ implies a significant difference.

Results

Pancreatic cancer cells survive in high concentrations of Evo

To examine the chemoresistance of pancreatic cancer against Evo, cellular viability was measured in pancreatic cancer cell line Panc-1, with various concentrations of Evo concentration treatment (from 0 μM to 16 μM). As expected,

the viability of Panc-1 cells decreased as the concentration of Evo increased from 0 μM to 4 μM (**Figure 1A**). However, when the concentration of Evo increased to 8 μM or 16 μM, cellular viability did not further decreased, suggesting that the high concentration of Evo might induce the chemoresistance of pancreatic cancer cells (**Figure 1A**).

Evo-resistant pancreatic cancer cells exhibit CSC-like characteristics

Since the viabilities of Panc-1 did not further decrease under high concentrations of Evo treatment, we hypothesized that the Evo-resistant subpopulation arose in Panc-1 cells, which was likely to be related to pancreatic CSCs. To determine the molecular profiles of Evo-resistant pancreatic cancer cells, we examined the expression of pancreatic CSCs' surface markers, CD44, CD24 and ESA, in Evo-resistant cell lines R1 and R2, which survived 8 μM and 16 μM Evo treatments, respectively. As a result, the expression of CD44, CD24 and ESA increased significantly in both R1 (2.2-, 1.9- and 1.9-fold, respectively) and R2 (3.3-, 2.2- and 2.5-fold, respectively), compared to the Panc-1 control cells (**Figure 1B**).

We further investigated the characteristics of R1 and R2 by wound-healing and transwell assays. The pancreatic CSCs were isolated according to surface markers CD44, CD24 and ESA in Panc-1 cells, and the remaining cells were considered as non-stem cells. Both Panc-1 CSCs and Panc-1 non-CSCs were used for evaluating the migration and invasion abilities of R1 and R2. In the wound-healing assay, the scratch widths of Panc-1 CSCs, R1 and R2 were obviously smaller than that of Panc-1 non-CSCs, showing stronger migration abilities (**Figure 1C**). Similarly, Panc-1 CSCs, R1 and R2 were also more invasive than Panc-1 non-CSCss in transwell assays (**Figure 1D**). According to our result, the migration and invasion abilities among Panc-1 CSCs, R1 and R2, is about the same. In addition, it was found through flow cytometer that there was no significant difference in apoptosis among Panc-1 CSCs, R1, and R2, which indicate similar characteristics between pancreatic CSCs and survived Evo-resistant cells (**Figure 1E**).

Pancreatic CSCs mediate the resistance against Evo

Considering the similarity between pancreatic CSCs and Evo-resistant cells, we speculated that CSCs mediated Evo resistance in pancreatic cancer. To confirm that, subpopulation mixing study was carried out. We mixed Panc-1 CSCs (CD44+/CD24+/ESA+) and non-CSCs (the remaining cells) in different ratios and examined Evo resistance under high concentration of Evo (16 μM). It was shown in our results that the cell viability in the mixing populations was increased as the percentage of Panc-1 stem cells increased, and the Evo resistance of Panc-1 CSCs was 2.6-fold stronger than that of Panc-1 non-CSCs (**Figure 2**).

High concentrations of Evo induce the conversion of non-CSCs into CSCs in pancreatic cancer

To examine the conversion of Panc-1 non-CSCs to CSCs, we fluorescently labeled Panc-1 CSCs and sorted the labeled CSCs by flow cytometry. After 1:1 mixing CSCs and non-CSCs, they were cultured with or without Evo (16 μM) for 24 h. By flow cytometry, we found the labeled CSC subpopulation remained stable (50.4% in the mixing population) when without Evo, while its percentage was elevated to 70.1% in the mixing population cultured in 16 μM Evo containing media (**Figure 3A**), which suggested that high dose of Evo has a strong screening effect on pancreatic cancer cells.

To investigate whether non-CSCs can be converted to CSCs under high dose of Evo treatment, we first sorted CSCs from the cultured mixing populations by surface markers CD44, CD24 and ESA. Then, ratio change of the labeled cells was analyzed. According to the result, at the beginning, the labeled cells dominated the CSCs population (99.2%). However, after 16 μM of Evo treatment, the percentage of labeled cells was drastically reduced to 81.3% (**Figure 3B**), which might suggest that the conversion from non-CSCs into CSCs existed in pancreatic cancer under high dose of Evo treatment.

Evo resistance of pancreatic cancer cells is acquired by activating HIF-1α-Notch-Hes1/5 pathway

The Notch signaling pathway plays a key role in maintaining CSCs population in pancreatic cancer, which may be activated by the transcription factor HIF-1α. It has been reported that HIF-1α contributes to the conversion of non-CSCs into CSCs in pancreatic cancer by inducing autophagy [33]. Therefore, we first detected the tran-

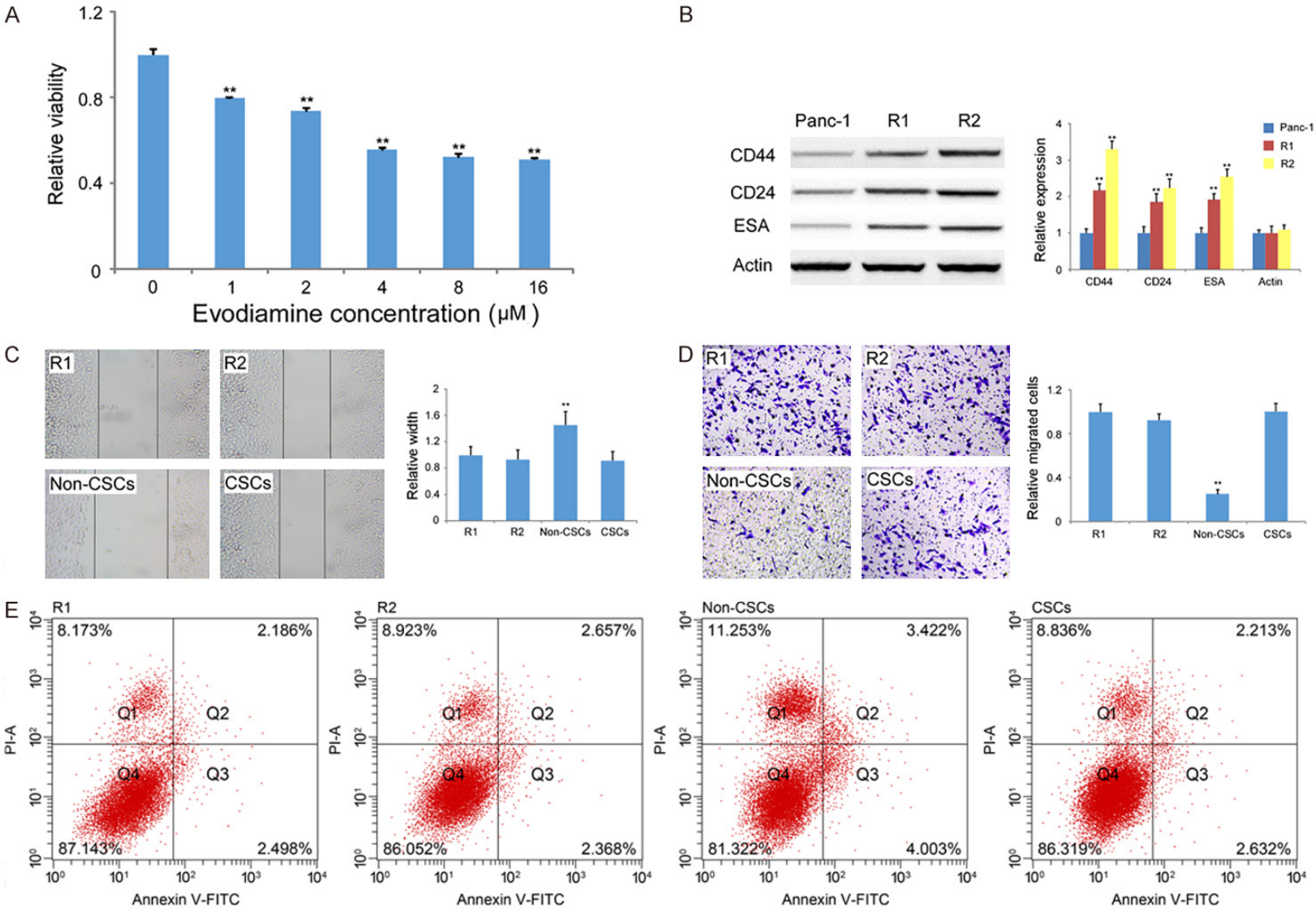

Figure 1. The CSC-like characteristics of Evo-resistant pancreatic cancer cells. (A) Cell viability after Evodiamine treatment. (B) Expression of CSC surface markers. Relative expression was normalized to Panc-1, and actin was used as internal control. (C) Would-healing and (D) Transwell assays. Data were normalized to R1. (E) Flow cytometry analysis of apoptosis. The experiment was performed with at least 3 repeats. Data were presented with mean ± standard deviation. **, $P < 0.01$.

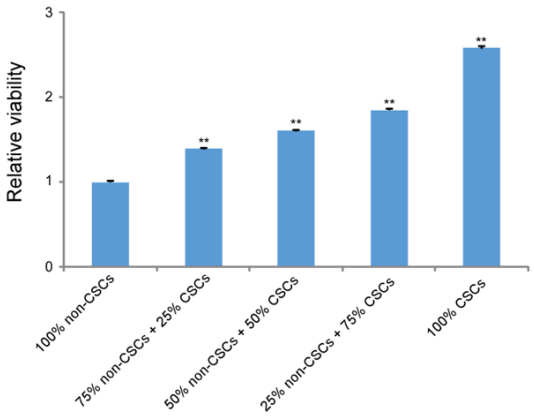

Figure 2. Mixing study to investigate the contribution of pancreatic CSCs to Evo resistance. CSCs and non-CSCs were mixed in different ratios. Relative viabilities were normalized to non-CSCs. The experiment was performed with at least 3 repeats. Data were presented with mean ± standard deviation. **, $P < 0.01$.

scriptional level of HIF-1α and Notch-1 in survived Evo-resistant R1 and R2 cells. Compared with non-resistant Panc-1 cells, the transcriptional levels of HIF-1α and Notch-1 in both R1 and R2 cells were significantly up regulated, and the expression is higher in R2 cells than in R1 cells (**Figure 4A**). We then evaluated the expression of Notch target genes (Hes1 and Hes5) by RT-PCR, and the result further demonstrated that the HIF-1α-Notch-Hes pathway was activated in Evo-resistant pancreatic cancer cells (**Figure 4A**). In addition, we also detected the protein expression of HIF-1α, Notch-1, Hes1, and Hes5 in the cells by a Western blot assay, and the results were consistent with the above (**Figure 4B**, Table S1).

To determine the relationship between Evo resistance and HIF-1α-Notch-Hes pathway in pancreatic cancer, we down regulated the expression of HIF-1α by HIF-1α siRNA or blocked the Notch signaling pathway by GSI in R2 cells. It was shown that both of the two ways markedly reduced the resistance of R1 cells to Evo, and knockdown of HIF-1α was more effective (**Figure 4C**). Besides, we also knocked down the expression of Hes1 or Hes5 in R2 cells. The results showed that inhibition of Hes1 or Hes5 exhibited a weaker reduction of Evo resistance than blocking Notch signaling pathway. However, inhibition of Hes1 and Hes5 simultaneously was as effective as knockdown of HIF-1α (**Figure 4C**).

To further confirm the HIF-1α-Notch-Hes1/5 pathway mediated Evo resistance in pancreatic cancer, we elevated the expressions of HIF-1α and Hes1/5 in Panc-1 cells. Drug-resistance analysis revealed that the Evo resistance of Panc-1 cells was enhanced by overexpression of HIF-1 or Hes1/5 together (**Figure 4D**). However, overexpression of only Hes1 or Hes5 was less effective in the enhancement of Panc-1 cells Evo resistance (**Figure 4D**).

Discussion

Pancreatic cancer is one of the most lethal malignant tumors across the world, and characterized by its strong resistance to chemotherapy and radiotherapy. Recent studies indicated that CSCs play an important role in the resistance and progression of pancreatic cancer [6, 34]. Evodiamine has been employed to treat various cancers including pancreatic cancer [32, 35]. As a traditional herbal medicine, Evo has huge potential against pancreatic cancers. However, limited by its strong drug resistance, the anti-cancer effect of Evo is unsatisfactory.

The high resistance of pancreatic cancer cells to Evo was confirmed in our study. Especially under high Evo concentrations, the viability of pancreatic cancer cells remains relatively high. We therefore speculated that it was because of the rapid proliferation of Evo-resistant cells, as well as activation of Evo adaptive mechanism induced by high dosage of drugs. To investigate the resistance mechanisms of Evo-resistant cancer cells, we examined the expression of CSC markers in pancreatic cells and tested their migration and invasion characteristics by wound-healing and transwell assays. As expected, survived Evo-resistant R1 and R2 cells showed up-regulation of CSC surface markers and exhibited strong migration and invasion ability.

Since the Evo-resistant cells exhibited pancreatic CSC-like characteristics, we assumed that pancreatic CSCs mediated the development of Evo resistance in Panc-1 cells. By mixing Panc-1 CSCs (CD44+/CD24+/ESA+) with non-CSCs at different ratios, it is revealed that the Evo resistance enhanced as the percentage of CSCs increased, which suggested that CSCs mediated the Evo resistance in pancreatic cancer. To further verify the conversion from non-CSCs to CSCs under high dose of Evo treat-

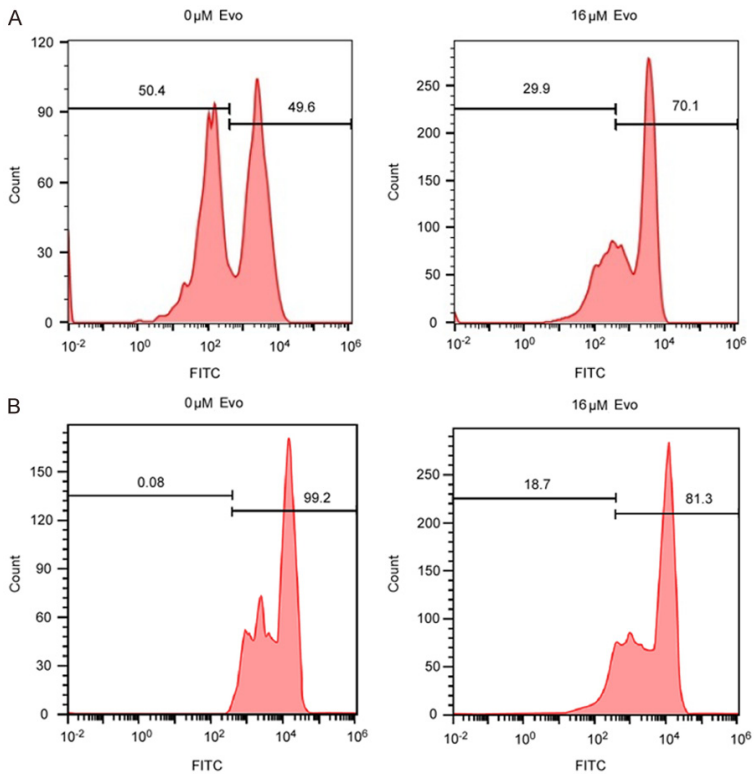

Figure 3. Conversion of pancreatic non-CSCs into CSCs induced by Evo. A. Percentage of fluorescently labeled CSCs analyzed by flow cytometry. B. Percentage of fluorescently labeled CSCs in CD44+/CD24+/ESA+subpopulation. The CD44+/CD24+/ESA+subpopulation was sorted after cultured for 24 h to determine the percentage of fluorescently labeled cells.

ment, we fluorescently labeled pancreatic CSCs before mixing cells. It was shown that percentage of labeled CSCs was elevated in the mixing population cultured in 16 μM Evo containing media. This result suggested that high dose of Evo has a strong screening effect on pancreatic cancer cells and a part of non-CSCs were converted to CSCs and survived under high dose of Evo treatment. This conclusion was supported by a previous study that pancreatic non-CSCs could convert into CSCs via HIF-1α induced autophagy [33]. To further confirm the mechanism, we examined the expression of HIF-1α, Notch-1 and its downstream target, Hes1 and Hes5, in survived Evo-resistant cell lines. It was shown that all of their expression was elevated in R1 and R2 cells compared with the non-resistant Panc-1 cells. According to previous researches, the Notch signal was very important to maintain pancreatic CSCs [36], and it can be activated by HIF-1α. Thus, the conversion may be mediated by the HIF-1α-induced activation of Notch signaling pathway.

We also investigated the role of HIF-1α-Notch-Hes1/5 pathway in inducing the Evo resistance of pancreatic cancer cells. Inhibition of HIF-1α significantly decreased the Evo resistance in R1 and R2 cells, which demonstrated that the Evo resistance was mediated by HIF-1α-activated Notch signaling pathway. Besides, reduction of Evo resistance was more significant by knockdown of HIF-1α rather than by blocking Notch signal pathway, which suggested there might be other HIF-1α-mediated pathways involved in the Evo resistance of pancreatic cancer. Inhibition of Hes1 and Hes5 also achieved a better outcome in reducing Evo resistance than blocking Notch signal pathway. This result suggested that Hes1 or Hes5 might be aviated via a Notch-independent way [37] and contribute to the Evo resistance in pancreatic cancer. We can also conclude from these data that the effect of inhibiting only Hes1 or Hes5 was weaker than blocking Notch signal pathway, which indicated that there was a synergistic effect between Hes1 and Hes5 in enhancing the Evo resistance in pancreatic cancer. However, expressing Hes1 and Hes5 in Panc-1 increased the Evo resistance only slightly more than expression single Hes1 or Hes5. Thus, we could hypothesize that functional redundancy might exist between Hes1 and Hes5 in the Evo resistance.

Conclusions

In summary, our study has revealed that CSCs is directly involved in the development of Evo resistance of pancreatic cancer cells by converting non-CSCs to CSC-like cells under high dose of Evo induction. Moreover, we have investigated the mechanism of pancreatic CSCs mediated Evo resistance by HIF-1α-Notch-Hes1/5 pathway, which may provide novel insights into the treatment of Evo resistance in pancreatic cancer.

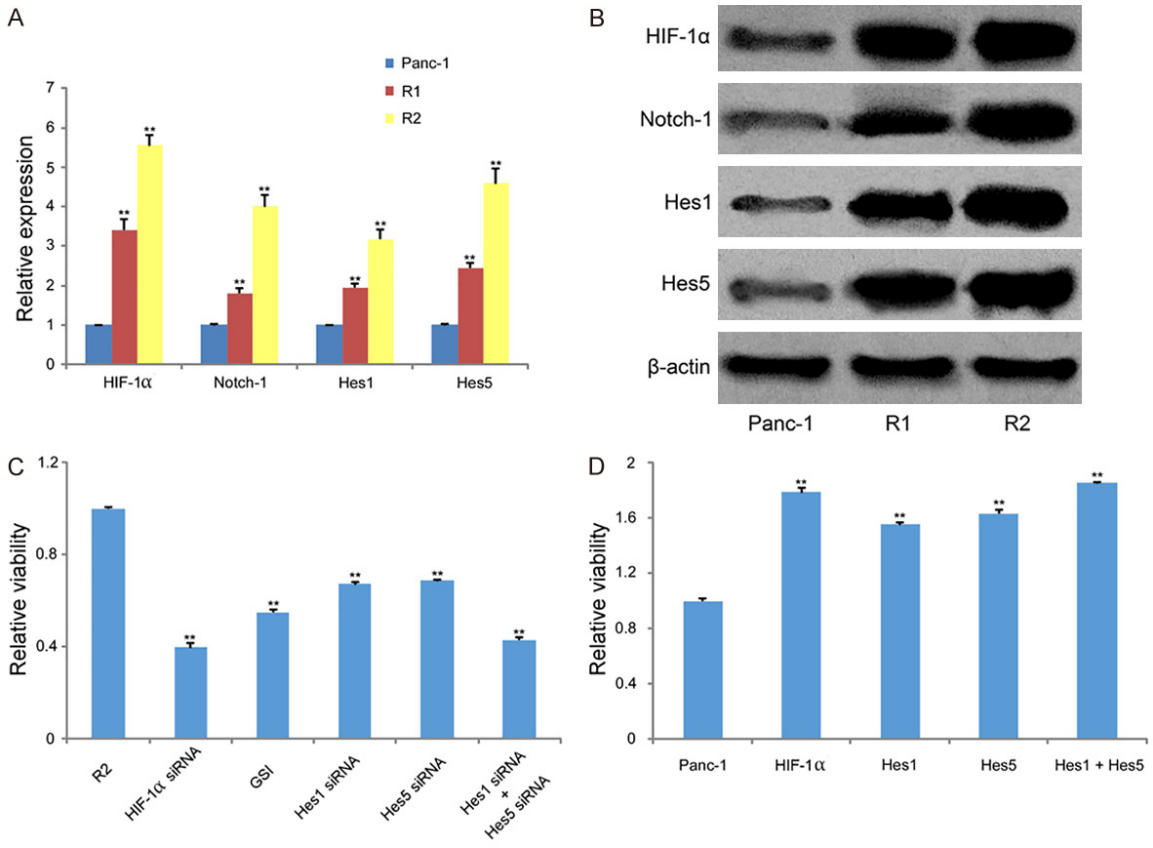

Figure 4. HIF-1α-Notch-Hes1/5 pathway mediated the Evo resistance of pancreatic cancer cells. A. Transcriptional levels of HIF-1α-Notch-Hes1/5 pathway components. Relative expressional levels were normalized to Panc-1. B. The relative protein expression of HIF-1α-notch-hes1/5 pathway components. The relative expression level was normalized to β-actin. C. Inhibition of HIF-1α-Notch-Hes1/5 pathway. Relative viabilities were normalized to R2. D. Overexpression of HIF-1 or Hes1/5 in R2. Relative viabilities were normalized to Panc-1. The experiment was performed with at least 3 repeats. The data were presented with mean ± standard deviation. **, $P < 0.01$.

Disclosure of conflict of interest

None.

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Table S1. Original data of western blot

	Panc-1	R1	R2
CD44	1.024	2.184	3.225
CD24	1.005	1.954	2.328
ESA	1.022	2.015	2.584
Actin	1.004	1.01	1.022
HIF-1α	0.684	1.722	2.484
Notch-1	0.524	1.384	1.857
Hes1	0.621	1.488	2.044
Hes5	0.548	1.548	2.225
β-actin	1	1	1