Original Article

Human chorionic gonadotropin-β promotes pancreatic cancer progression via the epithelial mesenchymal transition signaling pathway

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Background: The human chorionic gonadotropin free beta-subunit (hCGβ) is ectopically produced in various epithelial cancers and is associated with poor prognoses. However, its molecular mechanism remains unclear. In this study, we examined the biological role of hCGβ in pancreatic cancer progression.

Methods: Tissue specimens of 30 patients with pancreatic cancer were examined immunohistochemically to investigate the relationship between hCGβ expression and clinicopathological features. We also evaluated the molecular effects of hCGβ-downregulated pancreatic cell lines.

Results: Total of 21 cases were positive for immunostaining, and 17 of 25 metastatic lymph nodes were positive. hCGβ expression levels were correlated with pancreatic cancer T and N factors. hCGβ expression was significantly associated with poor overall and recurrence-free survival (P<0.001). In a multivariate analysis, hCGβ expression was independently associated with overall survival (HR 14.0; 95% CI: 1.5–130; P=0.019). The proliferative, invasive, and migratory abilities of hCGβ-downregulated cell lines were reduced compared with the control cell lines. Moreover, downregulation of hCGβ reduced vimentin, slug, and α-smooth muscle actin expression and increased E-cadherin expression.

Conclusions: hCGβ expression is related to cancer progression and poor prognoses via epithelial mesenchymal transition. hCGβ is a potential prognostic marker and molecular target in pancreatic cancer.

Keywords: Human chorionic gonadotropin free beta; pancreatic cancer; epithelial mesenchymal transition; transforming growth factor; pancreatic cell lines

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Introduction

Pancreatic cancer is one of the most common causes of cancer-related deaths in the world, with 458,918 new pancreatic cancer patients in 2018, causing 432,242 deaths (1). Pancreatic cancer is often detected at an advanced stage and has a poor prognosis despite new chemotherapy and improved surgical techniques (2). In advanced pancreatic cancer, the 5-year survival rate is less than 5% even with

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chemotherapy (3). A better understanding of the molecular mechanisms involved in tumor progression and the migration of pancreatic cancer is crucial for the development of targeted treatments to improve the prognosis of patients with pancreatic cancer.

Human chorionic gonadotropin (hCG) is a heterodimeric placental glycoprotein hormone with α and β subunits, of which hCGβ is structurally homologous to the cysteine knot family of growth factors composed of transforming growth factor (TGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF) (4). Although hCGβ stabilizes the production of progesterone, which is important for pregnancy, it is also highly expressed in various cancers (5). Although hCGβ is often elevated in germ cell tumors such as placental-derived tumors and testicular tumors, hCGβ can also be overexpressed in undifferentiated gastrointestinal cancers, and its expression has been reported as a possible factor associated with poor prognoses (6,7). As mentioned earlier, hCGβ is structurally homologous to TGFβ, which is strongly involved in epithelial to mesenchymal transition (EMT), suggesting that they could have similar receptor-mediated effects in cancer cells (8). Although hCGβ is involved in cancer metastasis and invasion via EMT in colorectal and ovarian cancers (9,10), the molecular mechanisms remain unclear. In pancreatic cancer, hCGβ is also ectopically expressed (11,12). However, its association with prognoses and its molecular role have not been elucidated. We speculated that hCGβ may be a poor prognostic factor in pancreatic, colon and breast cancers. Elucidating the molecular role of hCGβ in pancreatic cancer progression may lead to hCGβ becoming a new molecular target for pancreatic cancer therapy.

In the present study, we analyzed the correlation between hCGβ expression and clinicopathological features in pancreatic cancer. We also investigated the molecular roles of hCGβ expression in pancreatic cancer cell lines, especially in the proliferative, migratory, and invasive abilities of cells. We present the following article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-907/rc).

Methods

Patients

In the present study, we included 35 patients with pancreatic ductal carcinoma who underwent pancreatectomy at the University of the Ryukyus Hospital (Okinawa, Japan) between January 2011 and May 2020. We excluded 5 patients because of the presence of cancer in other organs, leaving a final cohort of 30 patients. We followed up the patients at 1- to 6-month intervals until death or September 30, 2020, whichever occurred earlier. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Internal Review Board on Ethical Issues of the University of the Ryukyus (Approval No. 1600) and informed consent was obtained from all patients. Tumor staging was classified according to the Tumor Node Metastasis Classification of Malignant Tumors defined by the Union for International Cancer Control (Eighth edition).

hCGβ immunostaining of surgical specimens

We obtained formalin-fixed, paraffin-embedded blocks from the 30 patients and selected the blocks containing the cancerous areas. We obtained a total of 30 cancerous lesions, 1 each from 30 patients. We performed hCGβ immunohistochemistry on the 30 selected blocks. Briefly, after deparaffinization and rehydration, we incubated the tissue sections with a rabbit polyclonal antibody against hCGβ (1:350; A0231; Dako, Tokyo, Japan), followed by reaction with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision/HRP; Dako). We performed the assessment of hCGβ expression using an immunoreactive score (IRS) for each cancerous lesion, calculated by multiplying the staining range (%) by the intensity (0, negative; 1, weak; 2, moderate; 3, strong) of the stained cells. We defined cancer lesions with detected hCGβ staining at ×40, ×100, and ×200 magnification as strong, moderate, and weak, respectively. Two independent pathologists (K.T. and H.K) who were blinded to clinical outcomes performed the hCGβ staining evaluation. We defined cases with an intensity ≥1 as hCGβ expression-positive, divided the 30 cases into 2 groups, positive and negative, and compared their clinicopathological characteristics.

Cell culture

We employed two human pancreatic cancer cell lines, culturing AsPC1 and PK45H cell lines in Roswell Park Memorial Institute-1640 medium with 10% fetal bovine serum (FBS). The cell lines were maintained at 37 ℃ under 5% CO_2.

hCGβ knockdown with small hairpin RNA

We transfected the AsPC1 and PK45H cells with hCGβ small hairpin RNA (shRNA) (MISSION® shRNA Library, TRCN0000082825, Sigma-Aldrich, St. Louis, MO, USA) using X-tremeGENE™ HP DNA Transfection Reagent (Sigma-Aldrich) according to the manufacturer's instructions. As a negative control (scramble), we employed MISSION pLKO.1-puro-Non-Mammalian shRNA Control (SHC002, Sigma-Aldrich). We harvested the cells at 48 hours post-transfection for future experiments. Successful downregulation of hCGβ expression in the ASPC1 and PK45H cells was verified by western blotting.

Western blotting

We lysed the samples with radioimmunoprecipitation assay lysis buffer, centrifuged them for 1 minute at 10,000×*g*, and performed a Bradford protein assay to measure the amounts of protein. After we added sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, bromphenol blue, and 0.125 M Tris) to cytoplasmic protein obtained from the cell lines, the mixtures were incubated at 95 ℃ for 3 minutes. We performed the SDS-PAGE using 12% acrylamide gel, transferring the proteins (15 μg) onto polyvinylidene difluoride membrane with the Trans-Blot® Turbo™ Transfer Kit (BIO-RAD, Hercules, CA, USA). We blocked the membrane proteins in 5% bovine serum albumin with Tris-buffered saline and Tween-20 for 20 minutes at room temperature. We incubated the membranes with primary antibody at 4 ℃ overnight, and then incubated them with m-IgGk BP-HRP (1:10000; sc-516102; Santa Cruz Biotechnology, Dallas, TX, USA) as a secondary antibody. We employed the following primary antibodies: hCGβ (1:500; sc-271062), E-cadherin (1:500, sc-8426), vimentin (1:500, sc-6260), slug (1:500, sc-166476), and α-smooth muscle actin (SMA) (1:500, sc-53142), (all Santa Cruz Biotechnology) and β-actin (1:500, #12620, Cell Signaling Technology). After we washed the membranes with phosphate-buffered saline and Tween-20 3 times, we added peroxidase luminescent matrix to the membrane. We visualized the membrane using a luminescent image analyzer (LAS4000; Cytiva, Marlborough, MA, USA).

Cell proliferation assay

We measured the cell proliferation capacity employing the Premix WST-1 Cell Proliferation Assay System (TAKARA BIO INC, Otsu, JAPAN). We incubated the AsPC1 and PK45H scramble and knockdown cell lines for 24, 48, and 96 hours at 37 ℃ under 5% CO_2, then added 10 μL of Premix WST-1 and incubated them again at 37 ℃ under 5% CO_2 for 1 hour. We measured the absorbance of the formazan products using a microplate reader (SH-1000 Lab, Corona Electric Co, Hitachi, Japan). We performed all tests 3 times each.

Invasion assay

The cultured AsPC1 and PK45H scramble and knockdown cell lines were trypsinized and centrifuged to remove dead cells and cell debris. We then added serum-free medium and added a total of 500 μL to each Corning Matrigel Invasion Chamber 24-Well Plate 8.0 micron culture insert (Corning Life Sciences, Durham, NC, USA). We placed the inserts in individual wells prepared with 750 μL of medium with 10% FBS and incubated them for 24 hours at 37 ℃ under 5% CO_2. After removing the inserts from the wells and wiping off the medium, we wiped the inside of the inserts with a cotton swab. This operation was performed carefully to avoid touching the outside of the inserts. We stained the cells attached to the outside of the insert using Diff-Quik stain™ (Sysmex Corporation, Kobe, Japan), and their numbers were measured by two blinded researchers using a light microscope. The number of invaded cells was taken as the average of the numbers measured in multiple fields of view, and we calculated the invasion rate (%) (number of invaded cells in the sample/number of invaded cells in the control × 100. We performed all tests 3 times each.

Wound healing assay

We trypsinized and centrifuged cultured AsPC1 and PK45H scramble and knockdown cell lines to remove dead cells and cell debris, then added serum-free medium and applied a total of 70 μL to each culture dish containing ibidi culture inserts (ibidi, Martinsried, Germany). After incubation for 24 hours at 37 ℃ under 5% CO_2, we removed the culture inserts using sterile forceps to prevent the cell layer from peeling off. We then washed the wells with phosphate-buffered saline and added 2 mL of serum-free medium to prevent cell proliferation. We visualized cell migration to 500 μm, the width of the cell-free gap, under a light microscope and measured the closure rate (%) (migrated cell surface area/total surface area ×100) after 24 and 48 hours. We performed all tests 3 times each.

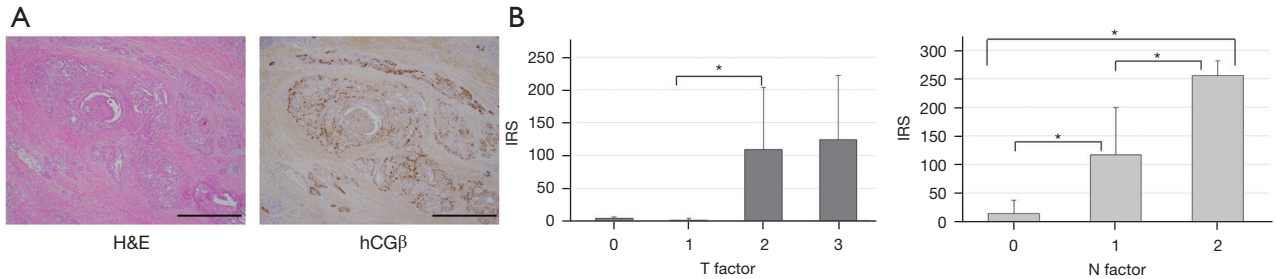

Figure 1 hCGβ expression in pancreatic cancer tissues and correlations with tumor progression. (A) Examples of hematoxylin and eosin (H&E) and hCGβ immunohistochemistry of primary lesion. The magnification is ×40. (B) Relationship between IRS and tumor T factor and N factor. IRS was calculated by multiplying the staining intensity by the staining range. *, P<0.05. Scale bars =50 μm. hCGβ, human chorionic gonadotropin free beta-subunit; IRS, immunoreactive score.

Statistical analysis

We employed chi-squared tests for comparisons of categorical variables (such as sex) and Mann-Whitney U tests for comparisons of continuous variables (such as age) between the hCGβ expression-positive and hCGβ expression-negative groups. We estimated survival using Kaplan-Meier curves. Correlations between clinical outcome and hCGβ expression were assessed with the log-rank test. We calculated overall survival (OS) from the date of surgery to the date of death from any cause. Recurrence- free survival (RFS) was calculated from the date of surgery to the date of disease recurrence or death from any cause. We used the Cox hazard regression method to identify independent risk factors for OS and RFS. For all the statistical analyses, we considered 2-tailed P values of 0.05 or less as statistically significant. We performed all statistical analyses with IBM SPSS Statistics version 24 (IBM, Armonk, NY, USA).

Results

Clinicopathological features of cases with hCGβ expression in pancreatic cancer tissue of 30 patients who underwent hCGβ immunostaining in pancreatic cancer tissue were positive in 21 (70%). Some of the immunostaining results of the primary lesions are shown in *Figure 1A*. With respect to age, sex, primary site, stage, histopathology, and with or without adjuvant chemotherapy, there were no significant differences between the hCGβ-positive and -negative groups (*Table 1*). However, the presence of recurrence (P=0.011), CEA levels (P=0.001), and CA19-9 levels (P=0.043) were significantly associated with hCGβ expression (*Table 1*).

Association between IRS and tumor progression

To clarify the association between hCGβ expression and tumor progression, we performed an association analysis of the IRS with T and N factors. There was no significant increase in the IRS between T0 and T1, but there was a significant increase between T1 and T2, and no significant increase between T2 and T3 (*Figure 1B*). For the N factor, the IRS was significantly elevated from N0 to N1 and from N1 to N2 (*Figure 1B*).

Association between hCGβ expression in pancreatic cancer tissue and prognosis

We next performed a Kaplan-Meier analysis to assess the prognostic value of hCGβ expression in pancreatic cancer. Using the log-rank test, we found a significant association between hCGβ expression (*vs.* nonexpression) in pancreatic cancer and both OS and RFS (19 *vs.* 77 months for OS and 11 *vs.* 74 months for RFS for hCGβ expression and nonexpression, respectively, both P<0.001; *Figure 2*). Moreover, we performed univariate and multivariate Cox proportional hazard regression analyses, summarized in *Table 2*. In the univariate analysis, hCGβ expression was significantly associated with OS [hazard ratio (HR) 23.5; 95% CI: 2.9–190 months; P=0.003] and RFS (HR 6.0; 95% CI: 1.3–27 months; P=0.02). In the multivariate analysis, hCGβ expression was the only independent factor for OS (HR 14; 95% CI: 1.5–130 months; P=0.0019). Although not significant, hCGβ-positive patients tended to have poorer

Table 1 Correlation between hCGβ expression and clinicopathological features

Clinical characteristics	Total (N=30)	hCGβ-negative (N=9)	hCGβ-positive (N=21)	P value
Age				0.673
Median	63	63	63	
Sex				0.376
Male	17	4	13	
Female	13	5	8	
Pancreatic location of the tumor				0.193
Head	12	2	10	
Body and tail	18	7	11	
pT factor				0.108
pTis	4	2	2	
pT1	4	3	1	
pT2	11	2	9	
pT3	11	2	9	
pT4	0	0	0	
pN factor				0.418
Negative	13	5	8	
pN1	14	4	10	
pN2	3	0	3	
pStage				0.730
pPanIN1-3	4	2	2	
pStage IA	2	1	1	
pStage IB	4	1	3	
pStage IIA	3	1	2	
pStage IIB	14	4	10	
pStage III	3	0	3	
pStage IV	0	0	0	
Histopathology				0.388
Well	4	2	2	
Moderately	13	2	11	
Poor	4	1	3	
Others	9	4	5	
Adjuvant chemotherapy				0.936
Yes	17	5	12	
No	13	4	9	

Table 1 (*continued*)

Table 1 (*continued*)

Clinical characteristics	Total (N=30)	hCGβ-negative (N=9)	hCGβ-positive (N=21)	P value
Recurrence				0.011
Yes	20	3	17	
No	10	6	4	
Tumor marker				
CEA median	3	2	3.5	0.001
CA19-9 median	62	28	257.5	0.043

There were no statistically significant differences between the two groups. hCGβ, human chorionic gonadotropin free beta-subunit; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

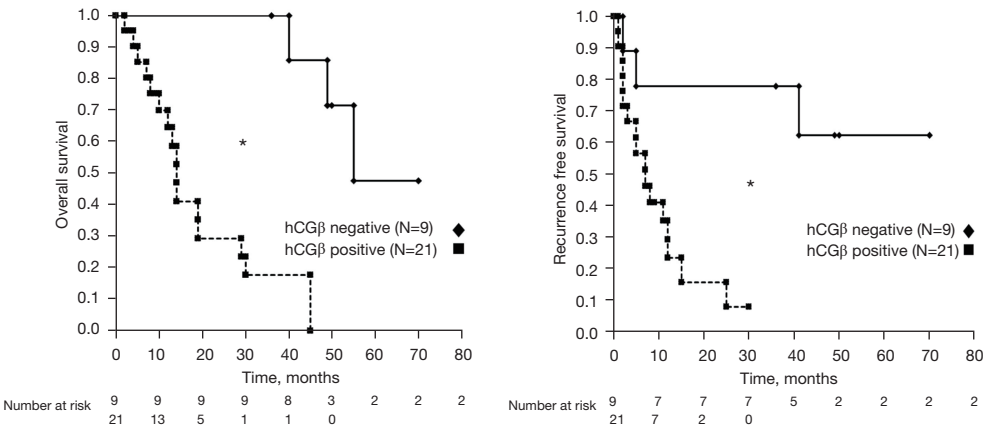

Figure 2 Histologically hCGβ-positive patients have significantly poorer overall survival and recurrence-free survival compared with hCGβ-negative patients. *, P<0.01 negative versus positive (log rank test). hCGβ, human chorionic gonadotropin free beta-subunit.

RFS in the multivariate analysis (HR 4.1; 95% CI: 0.87–19 months; P=0.074).

Association between hCGβ expression and EMT

To examine the potential molecular mechanisms of hCGβ in EMT, we performed western blotting to detect the expression of epithelial and mesenchymal protein markers. The results showed that the hCGβ-downregulated AsPC1 and PK45H cells demonstrated increased E-cadherin expression and reduced vimentin, slug, and α-SMA expression (*Figure 3*).

Cell proliferative capacity

We performed a WST-1 assay at 24, 48, and 72 hours after seeding the cell lines. The results of the WST-1 assay are shown in *Figure 4*. In both AsPC1 and PK45H, the cell proliferation of the hCGβ shRNA-transfected cell lines was significantly reduced (P<0.05).

Invasion capacity

In both AsPC1 and PK45H, the invasion of the hCGβ shRNA-transfected cells was significantly inhibited compared with the negative control (P<0.05) (*Figure 5A*).

Migratory ability

Intercellular spaces were measured at 24 and 48 hours after cell seeding. In both AsPC1 and PK45H, the closure rate of the hCGβ shRNA-transfected cell lines was significantly

Table 2 Univariate and multivariate analysis of overall survival and recurrence free survival in patients with pancreatic cancer

	Univariate analysis				Multivariate analysis			
	HR	Lower 95% CI	Upper 95% CI	P value	HR	Lower 95% CI	Upper 95% CI	P value
Overall survival								
Age (<65 or ≥65 years)	0.76	0.30	1.9	0.57				
Sex (male or female)	0.54	0.21	1.4	0.20				
hCGβ (negative or positive)	23.5	2.9	190	0.003	14	1.5	130	0.019
CA19-9 (<37 U/mL or ≥37 U/mL)	2.4	0.80	6.9	0.12	1.1	0.32	3.7	0.9
CEA (<5 ng/mL or ≥5 ng/mL)	3.8	1.34	10.8	0.010	1.9	0.60	6.0	0.28
Stage (I or ≥II)	5.9	0.79	44.6	0.083	3.6	0.41	32	0.25
T factor (1, 2 or 3)	3.1	1.25	7.8	0.015	1.6	0.58	4.5	0.36
N factor (0 or 1, 2)	1.9	0.72	5.1	0.19				
Recurrence free survival								
Age (<65 or ≥65 years)	0.55	0.21	1.4	0.21				
Sex (male or female)	0.58	0.23	1.5	0.25				
hCGβ (negative or positive)	6.0	1.3	27	0.02	4.1	0.87	19	0.074
CA19-9 (<37 U/mL or ≥37 U/mL)	1.5	0.56	4.1	0.41				
CEA (<5 ng/mL or ≥5 ng/mL)	2.5	0.95	6.8	0.064	1.1	0.36	3.1	0.91
Stage (I or ≥II)	6.9	0.92	52	0.060	3.4	0.41	28	0.25
T factor (1, 2 or 3)	3.9	1.5	1.8	0.004	2.4	0.87	6.6	0.090
N factor (0 or 1, 2)	2.3	0.87	6.2	0.092				

hCGβ positivity was an independent associated factor in overall survival. Although hCGβ positivity was not a statistically significant associated factor in recurrence free survival, it showed a very strong association. hCGβ, human chorionic gonadotropin free beta-subunit; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

reduced compared with the control (*Figure 5B*).

Discussion

To our knowledge, this study is the first to examine the relationship between hCGβ expression and tumor progression in pancreatic cancer. We investigated the clinicopathological significance of hCGβ expression in pancreatic cancer and the molecular role of hCGβ in pancreatic cancer progression. The results of immunostaining revealed that hCGβ-positive patients had a significantly higher recurrence rate, higher tumor markers, and a poorer prognosis. These results suggest that hCGβ is involved in tumor growth in pancreatic cancer. In addition, the IRS of hCGβ correlated with tumor T and N factors. These results also support the above-stated theory. Ectopic expression of hCGβ has been found in many epithelial carcinomas, including pancreatic cancer, and all of them had poor prognostic factors (13-15). Given the results of the present study were in agreement with the above finding, hCGβ could be a novel prognostic marker in pancreatic cancer.

Next, 2 hCGβ-downregulated pancreatic cancer cell lines were used to confirm the molecular effects of hCGβ in prompting cell proliferation, migration, and invasion. The hCGβ-downregulated cell lines had significantly reduced proliferative ability, invasion capacity, and migratory ability compared with the control cell lines. Moreover, the western blot analysis showed that the expression of cadherin, an epithelial marker, was upregulated, and the expressions of the mesenchymal markers vimentin, slug, and α-SMA were downregulated in the hCGβ shRNA-transfected pancreatic cancer cell lines compared with the control cell lines. Downregulation of epithelial markers and upregulation of

mesenchymal markers occur during EMT, which is one of the key processes by which cancers acquire invasive and metastatic properties (16,17). Therefore, the results of the present study suggest that hCGβ induces EMT in pancreatic cancer cells, resulting in the cells acquiring proliferative, invasive, and migratory capacities.

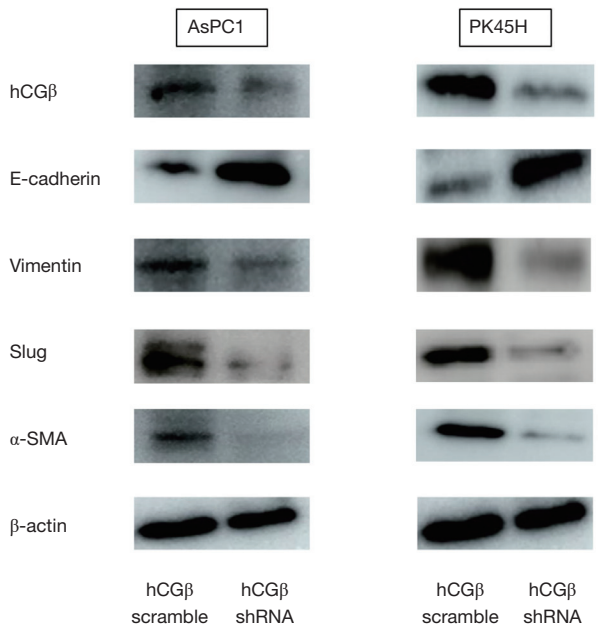

Figure 3 hCGβ induces EMT in pancreatic cancer cell lines. Expression levels of hCGβ and EMT markers in small hairpin RNA (shRNA)-transfected AsPC1 and PK45H cell lines by western blotting. hCGβ, human chorionic gonadotropin free beta-subunit; EMT, epithelial mesenchymal transition.

The biological role of tumor-produced hCGβ in cancer is not completely understood. Most choriocarcinomas and germ cell tumors produce an intact heterodimeric hormone composed of alpha and beta subunits; however, many nonchorionic tumors produce only hCGβ (18). hCGβ is structurally homologous to the cysteine knot family of growth factors consisting of TGF, PDGF, and NGF, and also has a 4-peptide cysteine knot structure in the β-subunit in common with TGFβ (4,8). In particular, it is important that hCGβ has structural homology with TGFβ, which is a significant inducer of EMT. In early-stage pancreatic cancer, TGFβ plays a role as a tumor suppressor by promoting apoptosis and inhibiting epithelial cell cycle progression; in its advanced stage, however, TGFβ plays a role as a tumor progression factor based on genomic instability, neoangiogenesis, immune evasion, cell motility, and metastasis (19). In addition, TGFβ might induce apoptosis and tumor suppression in cancer cells, induce EMT, or promote cancer stem cell heterogeneity and drug resistance (20,21). The structural homology of hCGβ to TGFβ suggests that tumor-produced hCGβ might bind to TGF β receptor complex components, blocking their ligand binding sites, thus inhibiting their interaction with other receptor components necessary to initiate the cytoplasmic signaling and leading to apoptosis (7). Furthermore, hyperglycosylated hCGβ shows a potent angiogenic effect through the activation of TGFβ (22). Ectopically expressed hCGβ not only has an anti-apoptotic effect, but also promotes invasion capacity by downregulating E-cadherin, which plays an important role in epithelial cell-to-cell adhesion and is an invasion suppressor (6,23). Further clarification of the hCGβ-related pathways underlying

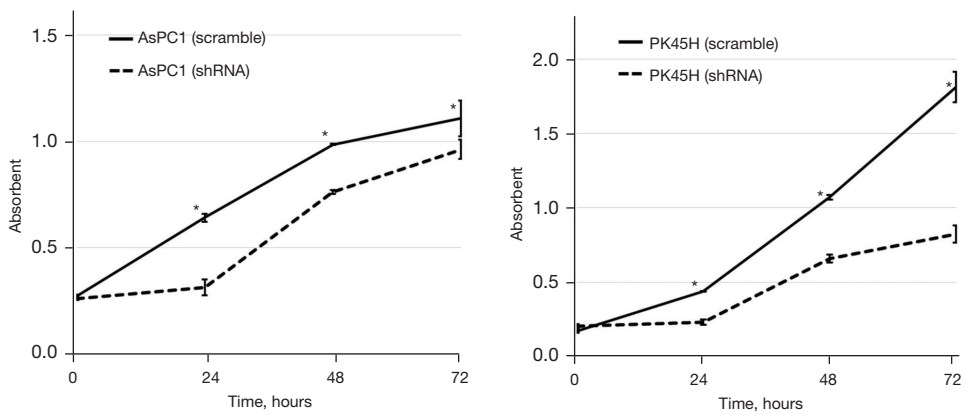

Figure 4 Proliferation ability of AsPC1 and PK45H cell lines transfected with hCGβ small hairpin RNA (shRNA) or scramble shRNA measured using a WST-1 assay at 24, 48, and 72 hours after seeding. *, P<0.05.

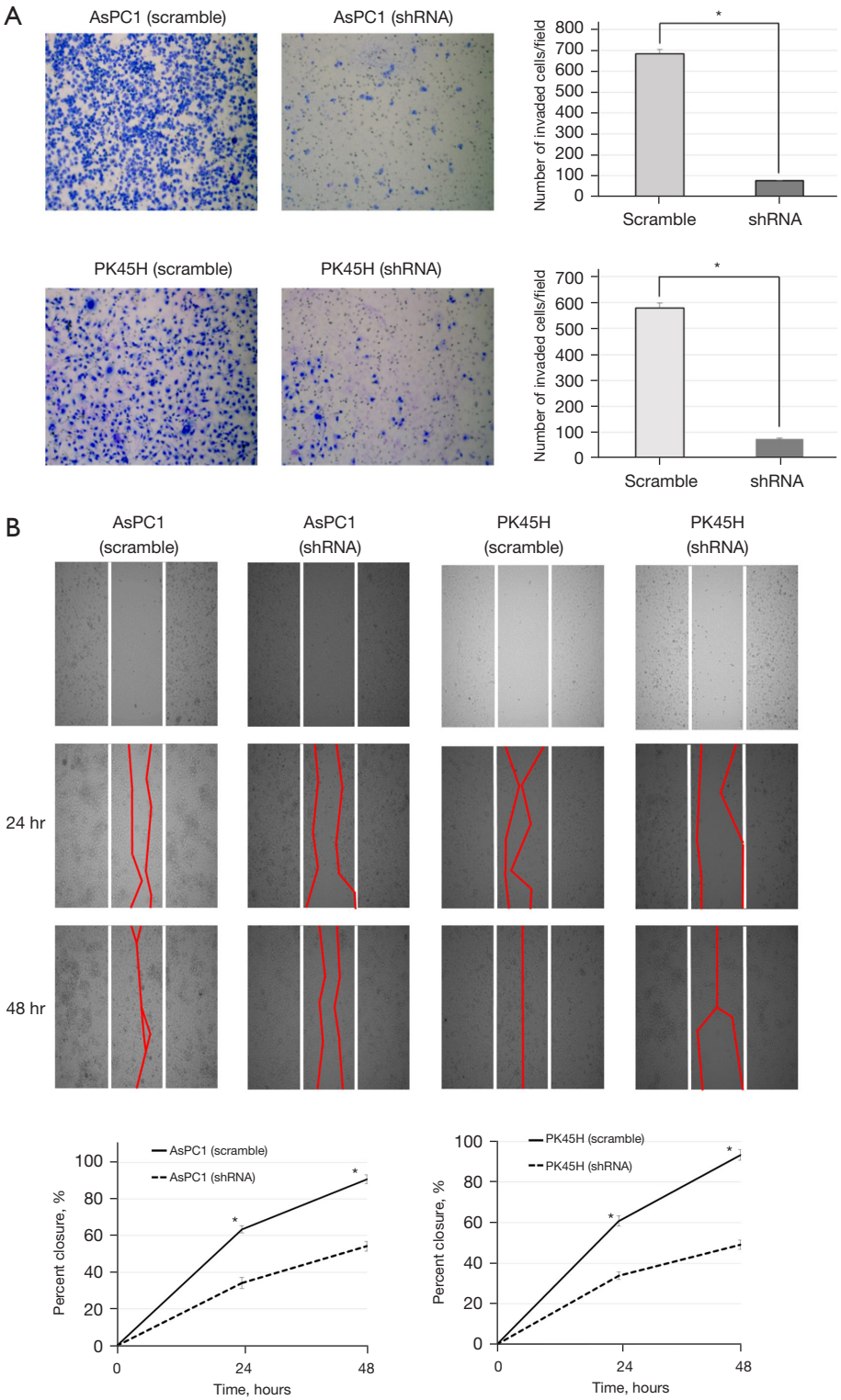

Figure 5 (A) Invasion capacity of AsPC1 and PK45H cell lines transfected with hCGβ small hairpin RNA (shRNA) or scramble shRNA was measured using Matrigel invasion assay. (B) Migratory ability of AsPC1 and PK45H cell lines transfected with hCGβ small hairpin RNA (shRNA) or scramble shRNA was measured using wound healing assay. Diff Quik stain, magnification: ×4. *, P<0.05.

the interaction between hCGβ and TGFβ receptors will lead to the development of targeted therapeutics such as hCGβ antagonists, which are expected to be used in pancreatic cancer treatment. To elucidate the hCGβ-related pathway underlying the interaction between hCGβ and TGFβ receptors, it is necessary to confirm not only hCGβ expression but also TGFβ and TGFβ receptor expression in pancreatic cancer cells; therefore, further research is warranted. Although speculative, other hCGβ-activated receptors, such as the hCG/LH receptor, may be important in pancreatic cancer progression, and further studies are also needed.

In the future, hCGβ measurements could change the progression diagnosis and treatment of pancreatic cancer. The addition of hCGβ immunostaining to pathology could allow for a more detailed determination of pancreatic cancer progression. Stronger adjuvant chemotherapy could be required for patients with positive hCGβ immunostaining, even if the histopathology shows early-stage cancer. Although not investigated in the present study, measurement of serum hCGβ levels could also be useful as a prognostic factor. It has been reported that patients with high serum hCGβ levels had poor prognoses in non-germ cell carcinoma, including colorectal and gastric cancer (13,24-27), and similar results might be obtained in pancreatic cancer. It could also be useful to determine the effects of treatment by performing serial measurements during chemotherapy.

Several limitations of this study should be acknowledged. First, the number of cases for hCGβ immunostaining was small and there was a slight bias in the number of cases between the hCGβ-positive and hCGβ-negative groups. With a larger number of cases, a multivariate analysis might have revealed the statistical relevance of hCGβ expression in RFS. Future studies with large sample sizes are required to accurately evaluate hCGβ. Second, we did not conduct comparative experiments using hCGβ-upregulated pancreatic cancer cell lines. We used only hCGβ-downregulated cell lines and were able to show a reduction in cell proliferation, invasion, and migration; however, it might have been desirable to show the opposite results using hCGβ-upregulated cell lines.

In summary, this present study suggests that hCGβ promotes the EMT signaling pathway, which in turn stimulates pancreatic cancer invasion and metastasis. In the future, novel agents targeting hCGβ are expected to improve the prognosis of patients with pancreatic cancer.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-907/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-907/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-907/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Internal Review Board on Ethical Issues of the University of the Ryukyus (Approval No. 1600) and informed consent was obtained from all patients.

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