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Suppression of Wnt/β-catenin and RAS/ERK pathways provides a therapeutic strategy for gemcitabine-resistant pancreatic cancer

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ABSTRACT

Pancreatic cancer is a major malignant tumor without an effective treatment. *KRAS* mutations occur in 90% of the pancreatic cancer patients and are a major obstacle for treatment of pancreatic cancer. Pancreatic cancer patients have been treated with limited chemotherapeutic agents such as gemcitabine. However, patients often develop resistance to gemcitabine that is attributed to *KRAS* mutations. Gemcitabine treatment activates both the Wnt/β-catenin and RAS/ERK pathways. These signaling pathways are also activated in the gemcitabine-resistant pancreatic cancer cell lines, suggesting that they play an important role in gemcitabine resistance in pancreatic cancer. The gemcitabine-resistant cell lines show enhanced migratory and invasive capabilities than their parental lines. Therefore, we investigated the effects of a small molecule, KYA1797K that degrades both β-catenin and RAS, on pancreatic cancer. KYA1797K decreased the expression level of both β-catenin and KRAS in pancreatic cancer cell lines expressing either wild-type or mutant *KRAS*. It also suppressed migration and invasion of gemcitabine-resistant and parental pancreatic cancer cells. Overall, we demonstrated that inhibiting the Wnt/β-catenin and RAS/ERK pathways by destabilizing β-catenin and RAS could be a therapeutic approach to overcome gemcitabine resistance in pancreatic cancer.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common and lethal malignant tumor of the pancreas and the fourth leading cause of cancer mortality [1]. PDAC patients are usually diagnosed at the advanced stage of the disease and are rarely treated with surgery, thus, chemotherapy is a first-line option [2].

Gemcitabine (2′, 2′-difuluorodeoxycytidine) is a standard chemotherapy agent used to treat advanced PDAC that prolong patient survival by several months [3]. However, because PDAC patients rapidly develop resistance to gemcitabine, there is an urgent need to identify a target-specific drug that can prevent gemcitabine resistance to prolong the effectiveness of the chemotherapy.

The Wnt/β-catenin pathway is a major pathway associated with gemcitabine-resistance in pancreatic cancer [4,5]. Gemcitabine-resistant pancreatic cells show increased levels of β-catenin [5]. Especially, β-catenin is found predominantly in the nucleus, leading to upregulated expression of its transcriptional target genes [5]. Thus, a therapeutic agent targeting the Wnt/β-catenin pathway in combination with gemcitabine could be a novel strategy for treating pancreatic cancer.

Approximately 90% of pancreatic cancer patients have *KRAS* mutations, a major feature of PDAC [2]. *KRAS* mutations play a crucial role in the development and tumorigenesis of PDAC [6]. Currently, an effective therapy directly targeting KRAS is not available despite significant efforts [7–10].

In clinical trials of first-line gemcitabine-based chemotherapy, PDAC patients with *KRAS* mutations had a worse response and poorer survival rates than those with wild-type KRAS [11]. Combining gemcitabine treatment with a KRAS targeting antibody produces a synergistic anti-cancer effect in pancreatic cancer [12]. Therefore, controlling KRAS levels could be an additional treatment option for PDAC patients with *KRAS* mutations and gemcitabine resistance.

We recently identified compounds that degrade both β-catenin

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and RAS proteins [13,14]. One of the derivatives from the initial hit compounds, KYA1797K, interacts with the RGS domain of Axin and activates GSK3β by enhancing the β-catenin destruction complex [14]. KYA1797K successfully suppressed tumorigenesis in several types of cancers driven by activated the Wnt/β-catenin and RAS-ERK pathways, such as colorectal cancer, triple-negative breast cancer, intestinal-type gastric cancer, and non-small cell lung cancer [15–18].

In this study, we investigated the effect of KYA1797K on *KRAS*-mutant pancreatic cancer cells by focusing on their β-catenin and pan-RAS levels including KRAS and their transforming abilities. The suppression of growth and the transforming abilities of PDAC cells by KYA1797K treatment was confirmed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays. To better understand the mechanism of gemcitabine resistance, we treated pancreatic cancer cell lines with gemcitabine and observed increased levels of β-catenin and pan-RAS. Combination treatment with KYA1797K and gemcitabine suppressed gemcitabine-induced cellular transformation and metastasis characteristics that contribute to resistance. To further characterize the effects of KYA1797K on PDAC cells, we established gemcitabine-resistant cell lines activated both Wnt/β-catenin and RAS/ERK pathways. Using these cell lines, we confirmed that KYA1797K inhibited transformation and metastatic characteristics of gemcitabine-resistant cells.

2. Materials and methods

2.1. Cell culture and drug treatment

Human PDAC stable cell lines (Panc1, Cfpac1, and BxPC1 cells) were provided by S. Y. Song (Yonsei University College of Medicine, Korea). AsPC1 cells were provided by J. S. Noh (Yonsei University, Korea). Cells were cultured in DMEM, IMDM, or RPMI (Gibco) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco), and were maintained in 5% CO_2 at 37 °C.

To established gemcitabine-resistant PDAC cells, Panc1 and AsPC1 cells were adapted by growing in DMEM and RPMI medium, respectively, with increasing concentrations of gemcitabine (Sigma-Aldrich; G6423), starting from 10 nM to 1000 nM. Cells

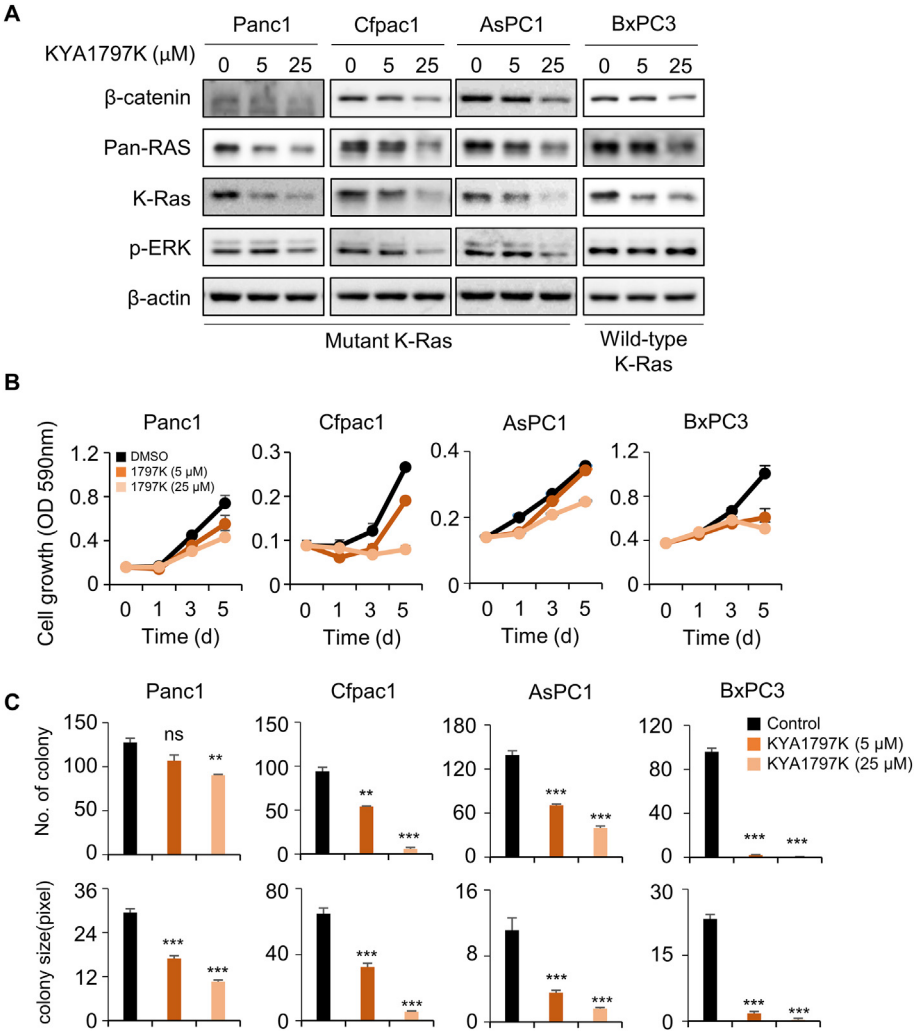

Fig. 1. Effects of KYA1797K on PDAC cell lines. (A) PDAC cell lines were cultured and treated with KYA1797K at indicated concentrations for 24 h. Immunoblot (IB) analyses of whole cell lysates (WCLs) were conducted with the indicated antibodies. (B) PDAC cells were treated with 5 or 25 μM KYA1797K for a 5-day period. Cell growth was measured and quantified via MTT assay ($n = 3$). (C) PDAC cells were treated with 5 or 25 μM KYA1797K for 14 days. Colonies were photographed after 14 days at the end of experiment. (C) The colonies were quantified from independent experiments (mean ± SEM; n = 3) by using the Image J software based on two-sided Student's *t* tests between control and experimental samples. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

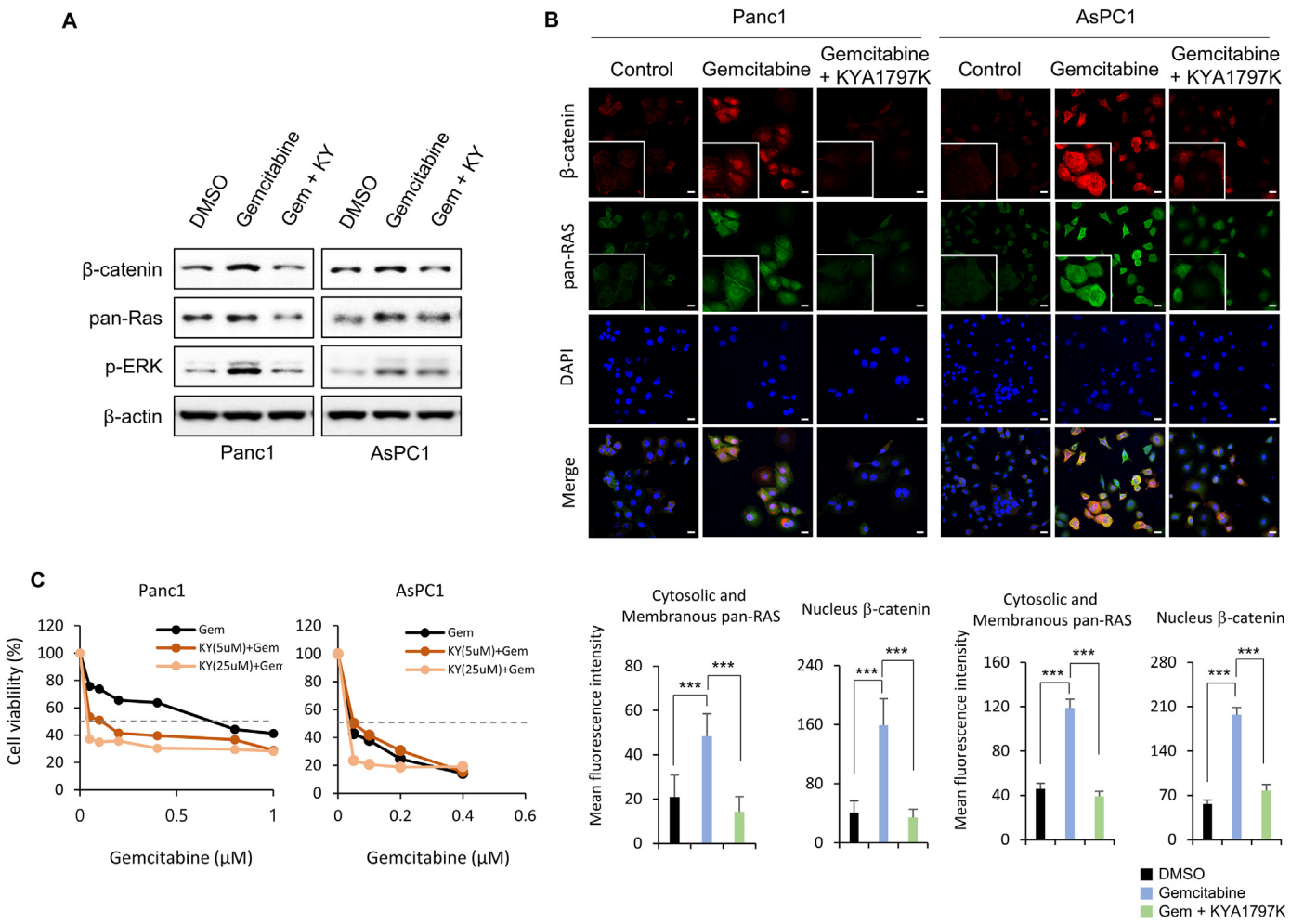

Fig. 2. Effects of gemcitabine and KYA1797K on PDAC cells. (A) PDAC cells were cultured and treated with 0.1 μM gemcitabine and/or 25 μM KYA1797K for 24 h. Immunoblot analyses were performed with the indicated antibodies. (B) Immunocytochemical analyses were performed using β-catenin or pan-RAS antibodies. Cells were counterstained with DAPI. Original photos were captured using a Zeiss confocal microscope. Scale bar = 20 μm. Mean fluorescence intensity of each marker was quantified for three different samples using ZEN microscope software (Carl Zeiss Microscopy). (C) PDAC cells were treated with gemcitabine and/or KYA1797K. Cell proliferation was measured and quantified using MTT assay ($n = 3$). Data represent the mean ± SEM. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

were adapted to each concentration of gemcitabine for at least 2 weeks. The derived gemcitabine-resistant cells were named Panc1-GR and AsPC1-GR. KYA1797K was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) for *in vitro* studies. Unless otherwise indicated, KYA1797K was used at a concentration of 25 μM for 24 h.

2.2. Trans-well and invasion assays

PDAC cells were seeded at a density of 1×10^5 or 3×10^5 cells per 300 μl onto uncoated or Matrigel-coated chambers for 24 well plates (SPL, Korea) with 25 μM KYA1797K, 1 μM gemcitabine, or DMSO. Cells were allowed to invade for 24 h. After clearing the cells on the inner surface of the chamber, cells on the outer surface were fixed in 4% paraformaldehyde (PFA) for 15 min, and stained with crystal violet for 20 min. Stained chambers were dipped in distilled water to remove excess staining and allowed to dry. Representative images were captured on a brightfield microscope (TE-2000U, Nikon). Data are presented as mean ± S.D. based on three biological replicates.

2.3. Immunoblotting analyses

PDAC cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in radio-immunoprecipitation assay (RIPA) buffer containing 20 mM NaF, 1 mM sodium vanadate, 10 mM Tris-HCl at pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor cocktail (Millipore; Billerica, MA, USA). Proteins were separated on 10–12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Immunoblotting was performed with the following primary antibodies: anti-pan-RAS monoclonal (clone Ras10; Millipore; MABS195; 1:3000), anti-β-catenin (Santa Cruz, Dallas, TX, USA; sc-7199; 1:3000), anti-p-ERK (Cell Signaling Technology, Beverly, MA, USA; #9101S; 1:1000), anti-α-SMA (abcam; ab5694; 1:1000), anti-vimentin (Abcam; ab92547; 1:2000) or anti-β-actin (Santa Cruz; sc-47778; 1:5000). Horseradish peroxidase-conjugated anti-mouse (Cell Signaling Technology; #7076; 1:3000) or anti-rabbit (Bio-Rad, Hercules, USA; 1:3000) secondary antibodies were used. Proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Issaquah, WA, USA) using a luminescent image analyzer (LAS-3000; Fuji Film,

Tokyo, Japan).

2.4. Immunocytochemistry

Cells grown on gelatin-coated cover glasses were fixed in 4% PFA for 10 min and permeabilized with 0.1% Triton X-100 for 15 min. Cells were blocked in 5% bovine serum albumin for 1 h and incubated with primary antibodies overnight at 4 °C. Cells were washed with PBS and incubated for 1 h at room temperature with either Alexa Fluor 488- or Alexa Fluor 555-conjugated IgG secondary antibody (Invitrogen). Cell nuclei were counterstained by incubating cells with 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescent images were captured using a confocal microscope (LSM510; Carl Zeiss).

2.5. Cell proliferation and colony formation assays

To analyze cell proliferation, PDAC cell lines (Panc1, Cfpac1, AsPC1, and BxPC1) were plated at a density of $1-5 \times 10^3$ cells/well in 96-well plates. Cells were treated with 5 or 25 μM KYA1797K or DMSO for 120 h. Subsequently, 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT; AMRESCO, Solon, OH) reagent was added to each well at a concentration of 0.5 mg/ml. After incubation for 1 h at 37 °C, insoluble purple formazan was obtained by dissolving in 200 μl (96-well plate) DMSO for 10 min. The absorbance of formazan was determined at 590 nm every 24 h. For colony formation assays, cells were seeded in 12-well plates (100−500 cells/well for PDAC cells). DMSO control or KYA1797K was added to the cells and media was changed every 3 days until visible colonies formed. At the end of the experiment, cells were fixed in 4% PFA for 30 min and stained with 0.5% crystal violet in 20% ethanol for 30 min.

2.6. Statistical analyses

Statistical analyses were performed using Microsoft Excel. All data are presented as the mean ± S.D. Statistically significant differences were determined using Student's *t*-tests. Statistically significant *P*-values are denoted as *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$.

3. Results

3.1. KYA1797K inhibits proliferation and transformation of pancreatic cancer cells through degradation of both KRAS and β-catenin

To analyze the effect of KYA1797K on pancreatic cancer cells, we treated pancreatic cancer cell lines expressing wild-type or mutant *KRAS* with KYA1797K. KYA1797K treatment reduced the levels of both β-catenin and pan-RAS in all examined cell lines (Fig. 1A). In addition, it also decreased KRAS levels in wild-type *KRAS* expressing BxPC3 cells and in mutant-*KRAS* expressing Panc1, Cfpac1, and AsPC1 cells (Fig. 1A).

Furthermore, KYA1797K inhibited active ERK in Panc1, Cfpac1, and AsPC1 cells, but not in BxPC3 cells that have a *B-Raf* mutation (Fig. 1A). KYA1797K suppressed proliferation of pancreatic cancer cell lines in a dose-dependent manner (Fig. 1B). The growth of BxPC3 cells were also inhibited by KYA1797K treatment although phosphorylation of ERK (p-ERK) was not regulated (Fig. 1A *vs* B). Similar to its effect on cell growth, KYA1797K inhibited the transforming capacity of pancreatic cancer cell lines (Fig. 1C). KYA1797K reduced both the number and size of colonies in a dose-dependent manner, although it showed only a weak effect in Panc1 cells at low concentrations (Fig. 1C). Overall, KYA1797K inhibited the proliferation and transformation of pancreatic cancer cells independently of their *KRAS* status.

3.2. KYA1797K suppresses gemcitabine-induced activation of the Wnt/β-catenin and Ras/ERK pathways in pancreatic cancer cells

Given that the Wnt pathway is known to be involved in gemcitabine resistance [4,5], we investigated the effect of short-term gemcitabine treatment on Wnt/β-catenin signaling. Gemcitabine treatment increased the levels of β-catenin and pan-RAS in Panc1 and AsPC1 cells (Fig. 2A and B). It also significantly increased ERK activity, although cell growth was decreased (Fig. 2A and C).

KYA1797K treatment abolished the gemcitabine-induced increase of β-catenin and pan-RAS (Fig. 2A). The effect of KYA1797K on suppression of Wnt/β-catenin signaling was evident by significant reduction of nucleus β-catenin which was increased by gemcitabine treatment (Fig. 2B). KYA1797K treatment also suppressed the gemcitabine-induced accumulation of cytosolic and membranous pan-RAS in pancreatic cancer cells (Fig. 2B). We also examined the combined effect of gemcitabine and KYA1797K on cell proliferation. Combination treatment with gemcitabine and KYA1797K reduced PDAC cell proliferation more than gemcitabine alone (Fig. 2C). These results show that short-term treatment with gemcitabine activated both Wnt/β-catenin and Ras/ERK signaling pathways, possibly acting as a resistance mechanism in PDAC. However, the activation of these pathways can be blocked by KYA1797K.

3.3. Gemcitabine resistance in pancreatic cancer cells involves the activation of Wnt/β-catenin and Ras/ERK pathways that induces metastatic capacity

To understand the significance of the Wnt/β-catenin and Ras/ERK pathways in the development of gemcitabine resistance, we established gemcitabine-resistant PDAC cell lines from Panc1 (Panc1-GR) and AsPC1 (AsPC1-GR) cells. Although gemcitabine effectively inhibited the growth of Panc1 and AsPC1 cells (IC_{50} = 0.15 and 0.04 μM, respectively), it did not affect their gemcitabine-resistant derivatives (Fig. 3A). The IC_{50} values of the cells resistant to gemcitabine were at least 20 times greater than those of the parental cell lines (Supplementary Fig. S1).

Panc1-GR and AsPC1-GR cells showed increased levels of β-catenin as a previous report [5]. They also expressed up-regulated levels of pan-RAS and activated ERK (Fig. 3B). Moreover, their increased levels of the nucleus β-catenin and cytosolic and membranous pan-RAS further showed the activation of the Wnt/β-catenin and RAS-ERK pathways (Fig. 3C and D).

Among chemoresistance mechanisms, the epithelial-to-mesenchymal transition (EMT) is considered an important process for inducing resistance in pancreatic cancer [19,20]. Therefore, we compared the metastatic capacity of gemcitabine-resistant PDAC cells and their parental cells. Panc1-GR and AsPC1-GR cells showed increased migration and invasion capabilities along with activation of metastatic markers (Fig. 3B and E). Panc1 and AsPC1 cells that were short-term treated with gemcitabine also showed increased migration and invasion capacities compared to their gemcitabine-resistant derivatives (Supplementary Fig. S2).

3.4. KYA1797K significantly inhibits transformation and the metastatic capacity of gemcitabine-resistant pancreatic cancer cells

KYA1797K treatment reduced growth of the gemcitabine-resistant Panc1-GR and AsPC1-GR cells (Fig. 4A). It decreased the expression levels of β-catenin and pan-RAS, as well as the activity of ERK, in PDAC-GR cells (Fig. 4B and C). Colony formation assays

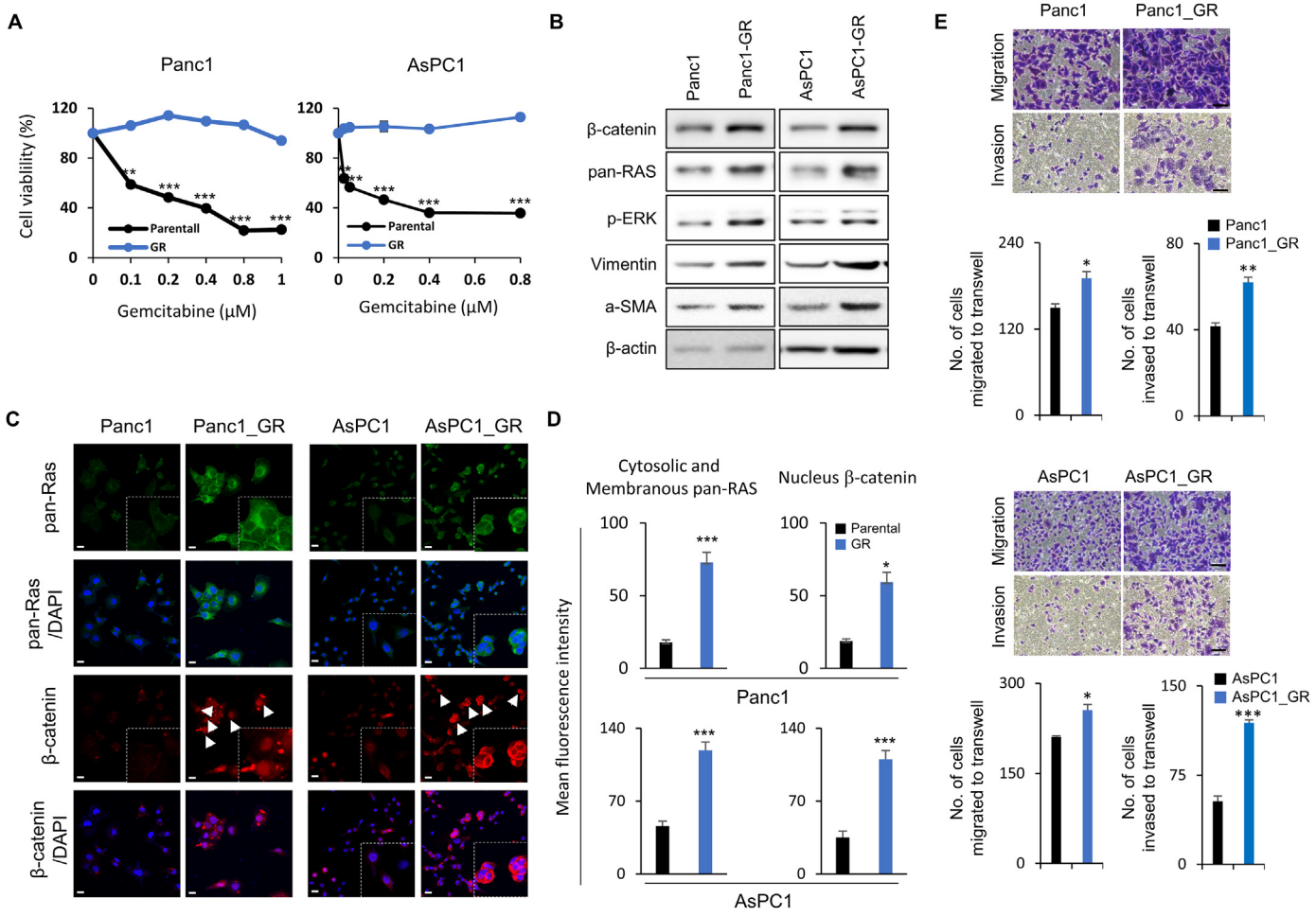

Fig. 3. Characteristics of the established gemcitabine-resistant PDAC cells. (A) Parental Panc1 and AsPC1 cells and their gemcitabine-resistant derivatives were incubated with gemcitabine for 72 h. Cell proliferation was measured and quantified using MTT assay ($n = 3$). (B) Immunoblot analyses of WCLs were performed with indicated antibodies. (C) Immunocytochemical analyses were performed using β-catenin or pan-RAS antibodies. Cells were counterstained with DAPI and images were captured using a confocal microscope. Scale bar = 20 μm. (D) Each group was quantified for at least three different samples using ZEN microscope software. (E) Migration and invasion assays were performed on parental or gemcitabine-resistant cells using noncoated or Matrigel-coated chambers. Cells were stained with 0.25% crystal violet. Representative images were captured with a Nicon TE-2000U microscope. Scale bar = 100 μm Data represent the mean ± SEM. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

showed that KYA1797K treatment significantly reduced the size and number of colonies in PDAC-GR cells compared to DMSO-treated cells (Supplementary Fig. S3). KYA1797K treatment also abolished the increased migration and invasion capabilities of PDAC-GR cells (Fig. 4D and Supplementary Fig. S4). It had a similar effect on PDAC cells that were short-term treated with gemcitabine (Supplementary Fig. S2). Therefore, KYA1797K effectively inhibited the tumorigenic and metastatic potential of gemcitabine-resistant pancreatic cancer cells.

4. Discussion

Gemcitabine is used as a first-line therapy in PDAC. Approximately 90% of PDAC patients show mutations in *KRAS*, which is deemed a cause of recurrence at the early stage of treatment and an obstacle for successful prolonged treatment [21]. Many attempts to overcome early recurrence have failed. The repeated failures in clinical trials has been attributed largely to *KRAS* mutations [11]. In this study, we showed that activation of β-catenin and RAS is important for the development of gemcitabine resistance in PDAC. We verified that the levels of β-catenin and RAS proteins were increased in PDAC cells that were short-term treated with gemcitabine or that acquired resistance to gemcitabine.

Co-stabilization of β-catenin and RAS plays an important role in the development of many other types of cancer. We identified compounds destabilizing these proteins and uncovered their anti-transforming effects on several cancer types [13–18,22]. These findings suggest that destabilizing oncogenic proteins could be an effective approach to treating cancer types that are driven by activated Wnt/β-catenin and EGFR-RAS pathways.

In this study, we hypothesized that inhibiting both signaling pathways with small molecules that degrade β-catenin and RAS is a potential therapeutic approach to treat pancreatic cancer, particularly the gemcitabine-resistant cases. As proof of principle, KYA1797K successfully suppressed the transforming capabilities of PDAC cells, thus, repressing transformation, migration, and invasion. KYA1797K treatment also provides a potential approach for overcoming problems associated with *KRAS* mutations, a major hurdle in the treatment of pancreatic cancer. Although mutant *KRAS* has been known to be an important oncogene in PDAC for decades, most attempts to control mutant *KRAS* have failed [11]. With the current lack of effective PDAC treatments, our finding that

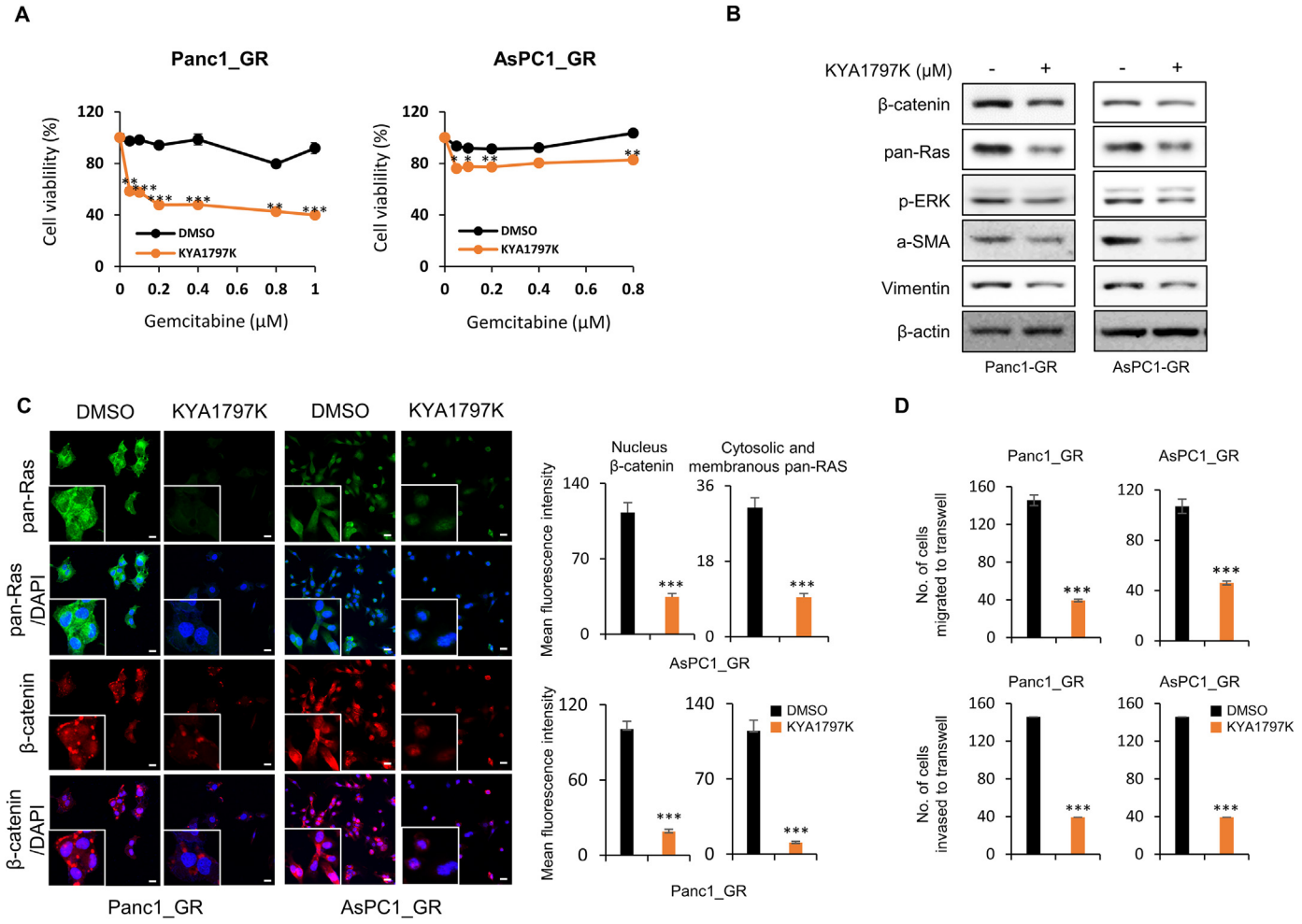

Fig. 4. Effect of KYA1797K on gemcitabine-resistant PDAC cells. (A) Panc1-GR and AsPC1-GR cells were co-treated with gemcitabine (at the indicated concentration) and 25 μM KYA1797K for 72 h. Cell proliferation was measured and quantified by MTT assay ($n = 3$). (B) Panc1-GR and AsPC1-GR cells were treated with DMSO or KYA797K for 24 h. Immunoblot analyses of WCLs were performed with the indicated antibodies. (C) After treatment with DMSO or KYA1797K for 24 h, immunocytochemical analyses were performed using the indicated antibodies. Mean fluorescence intensities were quantified ($n = 3$) Scale bar = 20 μm. (D) Migration and invasion assays were performed on Panc1-GR and AsPC1-GR cells using noncoated or Matrigel-coated chambers. The number of migrated or invaded cells was quantified ($n = 3$). Data represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

KYA1797K inhibits transformation of parental and gemcitabine-resistant PDAC cells resistant to gemcitabine, suggests that using small molecules to degrade β-catenin and RAS is a potential therapeutic strategy to treat PDAC.

The approach of destabilizing β-catenin and RAS is further supported when considering the acquired invasive characteristics of PDAC cells following gemcitabine treatment [23] and the involvement of the Wnt/β-catenin pathway in PDAC metastasis [24]. Long-term gemcitabine treatment induces EMT-like changes that sustain invasion and chemoresistance in PDAC cells [19]. By establishing the gemcitabine-resistant PDAC cell lines, Panc1-GR and AsPC1-GR, and by evaluating their migration, invasion, and transformation ability in response to KYA1797K, we confirmed that destabilizing β-catenin and RAS effectively suppresses transformation of gemcitabine-resistant PDAC. These results together with the support of *in vitro* studies demonstrate that the combined use of gemcitabine and KYA1797K could be a potential therapy, as KYA1797K prevents resistance caused by gemcitabine treatment alone. The inhibitory effect of KYA1797K on metastatic markers in gemcitabine-resistant cells suggests that KYA1797K or its functional analogs could be developed as potential therapeutic agents for PDAC.

Overall, co-suppressing the Wnt/β-catenin and Ras/ERK pathways by destabilizing β-catenin and RAS protein is a potential therapeutic approach to treat pancreatic cancer.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.02.076.

Author contributions

Experiments were designed by W-J. R. Data interpretation was performed by W-J. R., S–H. L., G. H., and K–Y., C. Experiments were performed by W-J. R. The manuscript was written and edited by W-J. R., S–H. L., and K–Y., C.

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