



Combination Therapy of the Active KRAS-Targeting Antibody inRas37 and a PI3K Inhibitor in Pancreatic Cancer

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Abstract

KRAS activating mutations, which are present in more than 90% of pancreatic cancers, drive tumor dependency on the RAS/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways. Therefore, combined targeting of RAS/MAPK and PI3K/AKT signaling pathways may be required for optimal therapeutic effect in pancreatic cancer. However, the therapeutic efficacy of combined MAPK and PI3K/AKT signaling target inhibitors is unsatisfactory in pancreatic cancer treatment, because it is often accompanied by MAPK pathway reactivation by PI3K/AKT inhibitor. Therefore, we developed an inRas37 antibody, which directly targets the intra-cellularly activated GTP-bound form of oncogenic RAS mutation and investigated its synergistic effect in the presence of the PI3K inhibitor BEZ-235 in pancreatic cancer. In this study, inRas37 remarkably increased the drug response of BEZ-235 to pancreatic cancer cells by inhibiting MAPK reactivation. Moreover, the co-treatment synergistically inhibited cell proliferation, migration, and invasion and exhibited synergistic anticancer activity by inhibiting the MAPK and PI3K pathways. The combined administration of inRas37 and BEZ-235 significantly inhibited tumor growth in mouse models. Our results demonstrated that inRas37 synergistically increased the antitumor activity of BEZ-235 by inhibiting MAPK reactivation, suggesting that inRas37 and BEZ-235 co-treatment could be a potential treatment approach for pancreatic cancer patients with KRAS mutations.

Key Words: Pancreatic cancer, KRAS, Targeting antibody, PI3K, BEZ-235

INTRODUCTION

Pancreatic cancer accounts for 3% of all cancers and approximately 7% of all cancer-related deaths in the USA. The survival rates of pancreatic cancer patients are low relative to those of other cancers that are considered incurable (Cameron *et al.*, 1993; Perysinakis *et al.*, 2015). Additionally, only few pancreatic cancer patients can be treated with surgery at diagnosis. Furthermore, the lack of efficacy of current chemotherapy such as gemcitabine, FOLFIRINOX, and Nab paclitaxel, and the rapid drug resistance to these treatments, has accelerated the requirement for targeted therapeutic strategies. To date, treatment of the malignancy has been difficult because of the lack of known key actionable pathogenic drivers in pancreatic cancer; however, recent advances in the ge-

netic features of pancreatic cancer should lead to novel therapeutic strategies.

It is well documented that the KRAS gene is mutated in approximately 90% of pancreatic cancer patients and works in an early and initiating event, which is sufficient to drive pre-malignant lesion formation into pancreatic cancer (Biankin *et al.*, 2012). KRAS, a switch protein, works by alternating guanosine triphosphate (GTP)-bound form (active form) and guanosine diphosphate (GDP)-bound form (inactive form) at the inner membrane. This has been identified as an important driver of oncogenesis, which leads to alterations in the RAS pathway, resulting in cell growth, survival, and metastasis (Bryant *et al.*, 2014; Boutin *et al.*, 2017). Although many pharmacological approaches have been attempted to block RAS/MEK/ERK signaling, direct inhibition of KRAS has not been

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clinically successful and a lot of efforts have instead focused on targeting the downstream signaling proteins such as RAF, MEK, and AKT (John *et al.*, 1990; McCormick, 2015). Consequently, a few RAS downstream protein inhibitors, such as MEK, BRAF, and ERK, have been developed, but significant effects were not shown due to MEK/ERK negative feedback or other signal reactivation (Roberts and Der, 2007; Wilhelm *et al.*, 2008; Hatzivassiliou *et al.*, 2013).

Oncogenic KRAS drives the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways, which are important for proliferation, survival, and tumorigenesis in pancreatic cancer (Fresno Vara *et al.*, 2004). Because the PI3K/AKT pathway is often dysregulated in pancreatic cancer cells, targeting the PI3K/AKT signaling pathway has recently been considered as a potential therapeutic strategy (Ebrahimi *et al.*, 2017). Numerous PI3K inhibitors are undergoing clinical development; however, their efficacy may be limited by compensatory pro-oncogenic MEK signaling reactivation (Soares *et al.*, 2015). The induction of compensatory MEK signaling following PI3K/AKT inhibition provides a strong rationale for combining MEK inhibitors to enhance therapeutic efficacy. Specifically, the combined targeting of MEK and PI3K has attracted substantial interest for KRAS-driven tumor treatment. However, MEK/ERK inhibitors of RAS downstream target proteins exhibited unsatisfactory effects when combined with PI3K inhibitors because of MEK/ERK negative feedback or other signal reactivation.

Therefore, in the present study, we developed inRas37 KRAS-targeting antibody that demonstrated a strong binding affinity to the KRAS-GTP form and investigated whether inRas37 could enhance the antitumor effect of BEZ-235, a PI3K inhibitor, by overcoming MAPK pathway reactivation in pancreatic cancer.

MATERIALS AND METHODS

Cells and reagents

Human MIA PaCa-2 and PANC-1 pancreatic cancer cells were purchased from the American Type Culture Collection (ATCC, VA, USA). MIA PaCa-2 and PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan, Korea) with 1% antibiotic-antimycotic and 10% fetal bovine serum (FBS). Cultures were maintained in an incubator with 95% air and 5% CO₂ at 37°C.

Preparation and purification of the inRas37 antibody

Based on our previous study, plasmids generating heavy chain (pciw3.4-inras37-HC) and light chain (pciw3.4-inRas37-LC) were produced. *Escherichia coli*-containing plasmids were cultured in Luria-Bertani medium on a small scale (3 mL) by incubating for 12 h at 37°C and 200 rpm, and then incubated on a large scale (1 L) for 12 h at 37°C and 200 rpm. After 12 h of incubation, *E. coli* were lysed and purified to obtain the plasmid DNA using a MN (Macherey-Nagel) kit (Düren, Germany), and the plasmid DNA products were transiently co-transfected in pairs at an equivalent molar ratio into 200 mL of HEK293F cells (2×10⁶ cells/mL) in Freestyle 293F medium (Invitrogen, CA, USA). The transfected cells were cultured for 7 days in an incubator at 37°C and 125 rpm, and the cell supernatants were centrifuged at 3,000 rpm and filtered (0.22 μm, Polyethersulfone; Corning, NY, USA). inRas37 was

purified from cell supernatant using a protein A-resin (Repligen, MA, USA) at a 1 mL/min flow rate and then dialyzed to achieve a final buffer composition of Histidine buffer (pH 7.4) using a sephadex G-25 desalting columns (GE Healthcare, Chicago, IL, USA). Then inRas37 in buffer was filtered using cellulose acetate membrane filters (0.22 μm, Corning), and its concentration was determined by the absorbance at 280 nm using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The 96-well Nunc Maxisorp™ ELISA plates (Nalgene Nunc, NY, USA) were coated for 1 h at 37°C with inRas37 and inCT37 (1, 10, and 100 nM), washed with washing buffer (Tris-buffered saline with 0.1% Tween 20 [TBST] and 10 mM MgCl₂, pH 7.4), and then blocked with blocking buffer (TBST, 10 mM MgCl₂, 4% BSA, pH 7.4) for 1 h at room temperature (RT). After washing, His-fused KRAS^{G12D}-GppNHp (1, 10, and 100 nM) and His-fused KRAS^{G12D}-GDP were incubated in each wells for 1 h at 37°C. After washing, bound proteins were detected by labeling with horseradish peroxidase (HRP)-conjugated goat anti-His antibody (Sigma Aldrich, MO, USA) and washed. Subsequent incubation with ultra TMB-ELISA solution (Thermo Fisher Scientific) was performed for 1 min, and then stopped with stop buffer (1 M H₂SO₄). The plate absorbance was read at 450 nm using a microplate reader (BioTek Instruments, VT, USA).

MTS assay

MIA PaCa-2 and PANC-1 cells were seeded at 8×10² cells/well in 96-well ultra-low attachment plates (Falcon, NY, USA) and were treated with inRas37 (0, 2, and 5 μM) and/or BEZ-235 (50 nM) every 2 days for 1 week. Subsequently, 13.5 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution (Promega, Madison, WI, USA) was added to each well and incubated for 3 h at 37°C. Absorbances were read at 490 nm using a microplate reader (BioTek Instruments). The MTS assay was performed in triplicate.

Anchorage-independent cell viability assay

Human pancreatic cancer cells were seeded at 1×10³ cells/well in ultra-low attachment round 96-well plates (Falcon) and were treated with inRAS37 and BEZ235 every 2 days for 1 week, followed by MTS solution at a 1:10 dilution in total volume for 4 h at 37°C. The absorbance was measured at 490 nm using a microplate reader (BioTek Instruments).

Western blotting

MIA PaCa-2 and PANC-1 cells were washed with Dulbecco's phosphate buffered saline (DPBS) and lysed with RIPA buffer (Biosesang, Korea) containing 1% Triton X-100, Xpert protease inhibitor, and phosphatase inhibitor Cocktail (Gen-DEPOT, TX, USA). Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, MA, USA). Protein transfer was verified using the Ponceau S staining solution (Amresco, OH, USA), and the blots were then incubated with the appropriate primary (1:500, except for β-actin [1:10,000]) and the secondary antibodies (1:1,000, except for β-actin [1:20,000]) conjugated to HRP. Antibody binding was detected using an

enhanced chemiluminescence reagent (Bio-Rad, CA, USA) using primary antibodies specific to the proteins of interest, and the proteins were detected using X-ray film and enhanced chemiluminescence reagent. Primary antibodies were used against the following: p-ERK, ERK, p-AKT, AKT, and β -actin (Cell Signaling Technologies, MA, USA) and p-BRAF (Santa Cruz Biotechnology, TX, USA), and the secondary antibodies were purchased from Cell Signaling Technologies.

Wound healing assay

MIA PaCa-2 and PANC-1 cells were seeded in 6-well plates at a density of 0.8×10^6 and 1.5×10^6 cells/well, respectively. After 24 h of incubation at 37°C, a straight scratch was made on the wells using a cell scratcher for the 6-well plate (SPL, Gyeonggi, Korea). The cells were then washed three times with PBS and further treated with inRas37 (1 μ M) and/or BEZ-235 (30 nM) in DMEM with 10% FBS and 1% antibiotic-antimycotic. After incubating for 72 h, the gap size on the wells was measured and recorded, and then compared with the initial gap size at 0 h. Using the Image J software (LOCI, WI, USA), the cells that moved over the scratched line were counted.

Invasion assay

The invasion assay was performed in a 24-well plate with 8.0- μ m pore size transwell (Corning). Inserts were coated with 10% Matrigel for 24 h at 37°C before cell seeding. Then, 5×10^4 MIA PaCa-2 and PANC-1 cells were seeded on the insert with serum-free medium treated with inRas37 (1 μ M) and/or BEZ-235 (30 μ M). The lower chambers were filled with DMEM complete medium (10% FBS and 1% antibiotics). After 72 h incubation at 37°C, the cells that passed through the 0.8- μ m pore were stained with 0.5% crystal violet. The invaded cells were photographed at 100 \times and counted (per microscopic field).

Tumor xenograft model

MIA PaCa-2 cells (7×10^6 cells/mouse) were implanted in the right flanks of 4-weeks-old male BALB/c nude mice (Orient Bio, Seoul, Korea) weighing 20 g. All animal experiments were approved by the Animal and Ethics Review Committee of Inha University (Incheon, Korea) and were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee. When tumor size reached approximately 50-100 mm³, the mice were divided into four groups. Mice were administered 30 mg/kg BEZ-235 and/or 30 mg/kg inRas37 intravenously three times a week for 23 days. Tumor volumes and weights were measured three times a week. Tumor volumes were calculated as (short axis)² \times (long axis)/2. At the end of the experiment, the mice were sacrificed, and the primary tumors were harvested.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining

TUNEL staining was performed in formalin-fixed, deparaffinized tumor tissue sections using an ApopTag peroxidase *in situ* apoptosis detection kit (Merck Millipore). Briefly, tumor tissue sections were incubated in 0.3% H₂O₂ in distilled water for 15 min at RT, and then TUNEL staining was performed using biotinylated secondary antibodies for 1 h at RT. Sections were then washed, developed with diaminobenzidine tetrahydrochloride substrate (Invitrogen), and viewed under a microscope (Olympus, Tokyo, Japan).

Histopathological analysis

Xenograft tumor tissue samples were fixed in 10% buffered formaldehyde at 4°C overnight, embedded in paraffin, and sectioned to obtain 8- μ m-thick slices. Hematoxylin and eosin (H&E) staining were performed after deparaffinization. Finally, the pathological morphology of the tissue was examined in at least 3 randomly selected fields at 100 \times magnification.

Immunofluorescence assay

Xenograft tumor tissue Immunostaining was performed after deparaffinization. Antigen retrieval was performed by heating in citrate buffer (pH 6.0) for 20 min. Next, the sections were permeabilized with Triton X-100 for 15 min and endogenous peroxidase activity was quenched using 0.3% H₂O₂ for 10 min at RT. Sections were gently washed with distilled water, blocked with CAS block solution (Zymed Laboratories, CA, USA) for 1 h at RT, and incubated with 1:50 dilutions of primary antibodies (α -SMA (Sigma Aldrich) and p-AKT, fibronectin, and collagen I (Abcam, Cambridge, UK)) at 4°C overnight. After gently washing twice with PBS, tumor sections were incubated with 1:100 dilutions of secondary antibody conjugated to FITC or TEXAS RED for 1 h at RT and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) to visualize the nuclei. After gently washing twice with PBS, the slides were covered with mounting solution (Vectashield, CA, USA) before being viewed under a confocal laser scanning microscope (Olympus).

Statistical analysis

Statistical significance was determined using analysis of variance (ANOVA) or unpaired Student's t-test. Results are presented as the mean \pm standard deviation (SD), and *p*-values < 0.05 were considered statistically significant. Statistical calculations were conducted using SPSS software for Windows (version 10.0; SPSS, IL, USA).

RESULTS

KRAS-targeting antibody inRas37 selectively bound to the GTP-bound KRAS active form

We previously reported the KRAS-targeting antibody RT11-i which recognizes integrin α v β 3/5 on the cancer cell surface (Shin *et al.*, 2017). inRas37 is the second generation of KRAS-targeting antibody of RT11-i and has a stronger binding affinity to the KRAS-GTP form than RT11-i. inRas37 enhanced the endosomal escape ability by complementing the light chain variable domain (VL) of the RT11-i antibody and replacing the heavy chain variable domain (VH) for improved binding affinity to the KRAS active form (Shin *et al.*, 2020). Additionally, to improve the efficacy of tumor specificity in cancer, the integrin α v β 3/ α v β 5-targeting RGD10 cyclic peptide at the N-terminus of the light chain was replaced with an in4 cyclic peptide engineered to further increase affinity for integrin α v β 5. Finally, to minimize Fc γ receptor (Fc γ R)-mediated immune clearance, we mutated L234A, L235A, and P329G (so-called LALAPG mutations) in the Fc region (Fig. 1A).

To investigate the inRas37 targeting efficiency for GTP-bound KRAS active form, we used ELISA using non-hydrolyzable GTP analogs (KRAS^{G12D}-GppNHp) that bind to the active form of human KRAS^{G12D} mutants and the inactive form of GDP (KRAS^{G12D}-GDP). inRas37 strongly bound to the ac-

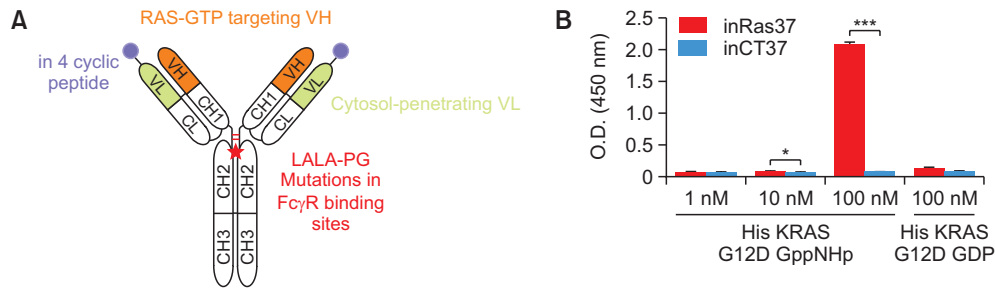


Fig. 1. Features of KRAS-targeting inRas37 antibody. (A) Schematics of the structures of inRas37 with integrin-targeting in4 cyclic peptide fused to N-terminal and cytosol-penetrating VL (light chain variable domain), active form of RAS•GTP-specific VH (heavy chain variable domain), and LALAPG mutations in the Fc region. (B) ELISA results showing the selective binding affinity of inRas37 compared to the control antibody inCT37 on His KRAS^{G12D}•GppNHp, an active form of KRAS, and KRAS^{G12D}•GDP, an inactive form of KRAS. Data are presented as the means \pm SD (* p <0.05 and *** p <0.001).

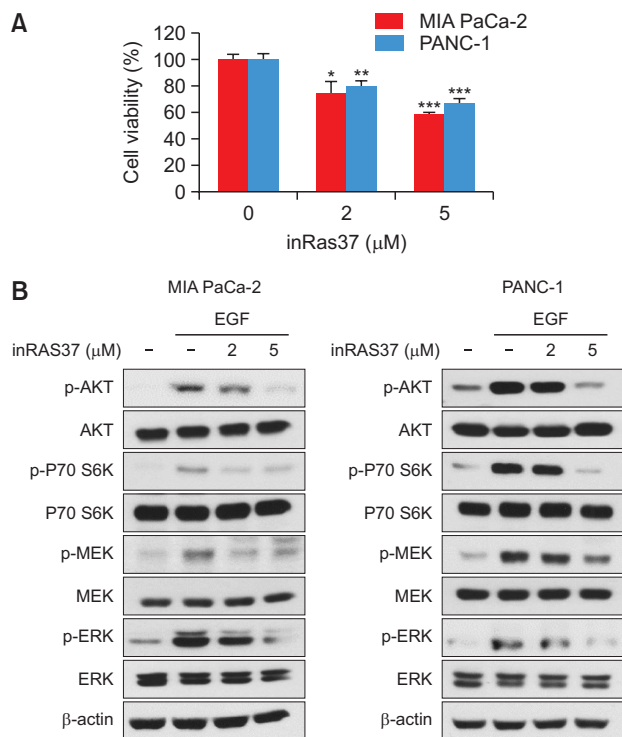


Fig. 2. Therapeutic efficacy of inRas37 on cell viability and KRAS downstream signaling. (A) 3D-cultured anchorage-independent cell viability was measured using an MTS assay in MIA PaCa-2 and PANC-1 cells treated with inRas37 (2 or 5 μ M) every 2 days for 1 week. (B) Both pancreatic cancer cells (MIA PaCa-2 and PANC-1) were treated with inRas37 (2 or 5 μ M) for 6 h after serum starvation for 2 h, and then stimulated with EGF (10 ng/mL) for 10 min. The expressions of p-AKT, p-P70 S6K, p-MEK, and p-ERK levels were determined using western blotting. Data are presented as the means \pm SD (* p <0.05, ** p <0.01, and *** p <0.001).

tive form of KRAS (KRAS^{G12D}•GppNHp); however, it did not bind to the inactive form of KRAS (KRAS^{G12D}•GDP; Fig. 1B). Moreover, as a control antibody, inCT37 containing the same components as inRas37 without KRAS-binding activity did not bind to active form of KRAS. These results indicated that inRas37 selectively bound to the active form of KRAS•GTP.

inRas37 inhibited anchorage-independent growth and KRAS signaling in pancreatic cancer cells

Next, we investigated whether inRas37 has anticancer effects on pancreatic cancer cells with KRAS mutations. Previously, we found α v β 3 and α v β 5 to be highly expressed in various pancreatic cancer cells (Kang *et al.*, 2018). Among them, the antiproliferative activity of inRas37 was evaluated in MIA PaCa-2 and PANC-1 cells, with the highest expression of integrins α v β 3 and α v β 5 at the indicated concentrations (2 and 5 μ M). inRas37 exhibited significant antiproliferative activity in pancreatic cancer cells in a dose-dependent manner (Fig. 2A). We investigated whether inRas37 could inhibit KRAS signaling, including MAPK and PI3K/AKT pathways, by targeting GTP-bound KRAS active form. When the cells were treated with inRas37, we observed that inRas37 dose-dependently inhibited the expression of p-AKT, p-P70 S6K, p-MEK, and p-ERK. These results indicated that inRas37 showed antiproliferative effects by inhibiting KRAS signaling, including the MAPK and PI3K/AKT pathways, by targeting the GTP-bound KRAS active form (Fig. 2B).

BEZ-235, a PI3K inhibitor, inhibited pancreatic cancer cell proliferation, but induced MAPK pathway reactivation

In previous study, a dual PI3K/mTOR inhibitor, BEZ-235, was reported to inhibit cell growth and proliferation in various cancer cells (Serra *et al.*, 2008; Herrera *et al.*, 2011). Therefore, to verify whether BEZ-235 could inhibit cell growth and proliferation in pancreatic cancer cells, we evaluated cell viability under 3D cultured conditions using the MTS assay (Fig. 3A). As a result, BEZ-235 dose-dependently decreased 3D cultured cell viability (0.01–5 μ M) and inhibited PI3K/AKT signaling. Despite these effects, a major problem with PI3K inhibitors is that they induce rapid MAPK pathway signaling reactivation, which subsequently leads to drug resistance, as previously reported (Soares *et al.*, 2015). Indeed, our study showed p-ERK reactivation after BEZ-235 treatment in MIA PaCa-2 and PANC-1 pancreatic cancer cells (Fig. 3B). These results indicated that BEZ-235 could have limited therapeutic efficacy in MIA PaCa-2 and PANC-1 cells because of ERK reactivation.

inRas37 blocked MAPK pathways reactivation by BEZ-235 and synergistically inhibited cell growth in combination with BEZ235

KRAS drives tumor progression by regulating the MAPK

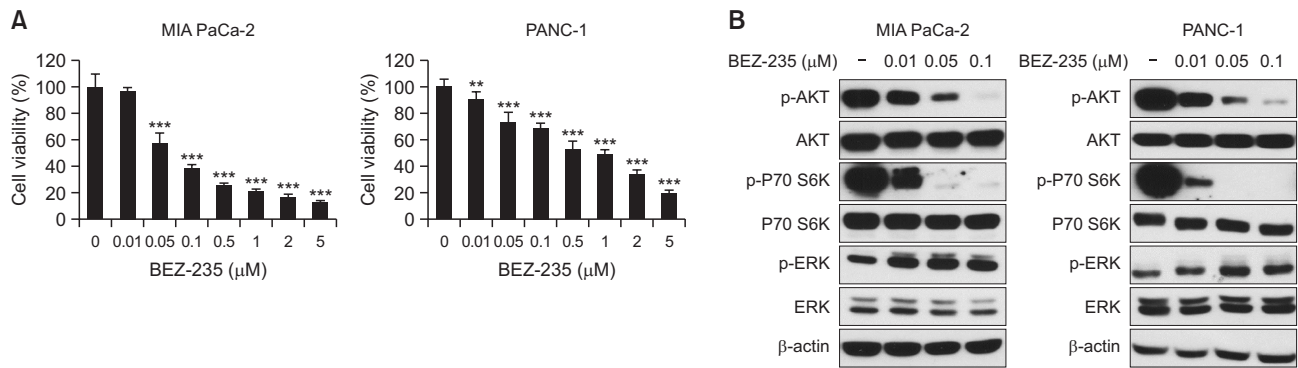


Fig. 3. Therapeutic efficacy of BEZ-235 on cell viability and PI3K/AKT signaling. (A) MIA PaCa-2 and PANC-1 cells were treated with BEZ-235 (0.01-5 μM) for 72 h, and cell viability was measured using the MTS assay. (B) Pancreatic cancer cells (MIA PaCa-2 and PANC-1) were treated with BEZ-235 (0.01, 0.05, and 0.1 μM) for 2 h. The expressions of p-AKT, p-P70 S6K, and p-ERK levels were determined using western blotting. Data are presented as the means ± SD (** $p < 0.01$ and *** $p < 0.001$).

and AKT signaling pathways, which are highly activated in human pancreatic cancer cells with KRAS mutation, which involve cell proliferation, survival, and metastasis (Hancock, 2003; Keleg *et al.*, 2003). Therefore, we investigated whether inRas37 and BEZ-235 co-treatment could synergistically inhibit PI3K/AKT and MAPK signaling and suppress cell growth in pancreatic cancer cells. inRas37 and BEZ-235 co-treatment synergistically inhibited the expression of p-AKT, p-BRAF, and p-ERK which are effector proteins of the MAPK and PI3K/AKT pathways, compared with single treatment in MIA PaCa-2 and PANC-1 cells (Fig. 4A). Since 3D culture is more similar to the *in vivo* environment than 2D culture, and integrin as mediators of cell ECM interactions could be generated, we evaluated the combined effect of inRas37 and BEZ-235 in 3D culture systems using 3D-clutred MTS and tumor spheroid formation assays. Our results show that the combination synergistically inhibits the anchorage-independent cell growth and tumor spheroid formation of MIA PaCa-2 and PANC-1 cells compared with only inRas37 or BEZ-235 treatment (Fig. 4B, 4C). Collectively, these results demonstrated that inRas37 and BEZ-235 combination treatment inhibited growth by blocking the MAPK and PI3K/AKT pathways.

The combination of inRas37 and BEZ-235 synergistically inhibited cancer cell migration and invasion

Pancreatic cancer is characterized by metastasis via migration and local invasion into surrounding tissues, and KRAS, in particular, drives invasion and maintains metastasis (Keleg *et al.*, 2003); therefore, we conducted migration and invasion assays to determine whether the combination of inRas37 and BEZ-235 inhibited the migration and invasion of pancreatic cancer cells with KRAS mutation. Cell migration was reduced by 20-50% upon treatment with inRas37 or BEZ-235 and by 60-80% after co-treatment versus controls in MIA PaCa-2 and PANC-1 cells (Fig. 5A). Similarly, co-treatment inhibited the invasion more than that in the single treatment groups in both cell lines (Fig. 5B). Collectively, these results indicated that combination treatment of inRas37 and BEZ-235 remarkably inhibited pancreatic cancer cell metastasis.

Combination of inRas37 and BEZ-235 synergistically inhibited tumor growth in MIA PaCa-2 xenograft model

To evaluate the therapeutic potential of inRas37 and BEZ-

235 co-treatment *in vivo*, we used the xenograft mouse model using human MIA PaCa-2 pancreatic cancer cells. During the 23 days treatment, exposure to inRas37 and BEZ-235 alone delayed tumor growth compared with that in the control group, and the combination group showed a more significant antitumor effect (Fig. 6A). This observation was consistent with the tumor weight after harvesting (Fig. 6B). No adverse effect on body weight change was observed in any group (Fig. 6C). Furthermore, co-treatment increased necrotic tumor regions as determined by H&E staining, increased TUNEL- positive cells, and decreased the expressions of p-AKT, p-MEK, and p-ERK levels as determined by immunofluorescence assay (Fig. 6D).

DISCUSSION

Pancreatic cancer patients have few viable therapeutic options. It is clear that KRAS mutations frequently occur and are early drivers of pancreatic cancer progression. KRAS accelerates the activity of the RAF/MEK/ERK and PI3K/AKT pathways, which are the drivers of tumor growth in the majority of pancreatic cancer (Jones *et al.*, 2008; Biankin *et al.*, 2012; Waddell *et al.*, 2015; Witkiewicz *et al.*, 2015). Therefore, inhibitors of the major KRAS-mediated signaling pathways, such as MEK/ERK and PI3K/AKT, have been developed; however, adaptive resistance has been a significant hurdle in achieving durable remission. Particularly, when MEK/ERK and PI3K/AKT inhibitors were used alone in pancreatic cancer management, the drug activity was reduced by reactivating the upstream pathways because of a positive feedback loop. Therefore, it is critical to improve the adaptive resistance to these inhibitors and develop combinatorial therapeutic strategies to mitigate resistance (Roberts and Der, 2007; Wilhelm *et al.*, 2008). In this study, we developed an inRAS37 antibody, a selectively targeted GTP-bound KRAS active form to effectively inhibit RAS signaling, including the MEK/ERK and PI3K/AKT pathways, and investigated whether it could enhance the anti-tumor effect of BEZ-235, a PI3K inhibitor in pancreatic cancer. We discovered that inRAS37 and BEZ-235 co-treatment synergistically inhibited the RAS signaling pathway by inhibiting the RAS/RAF/MEK and PI3K/AKT pathway and cell growth, migration, and invasion in human pancreatic cancer.

Antibody drugs have the advantages of long half-life in hu-

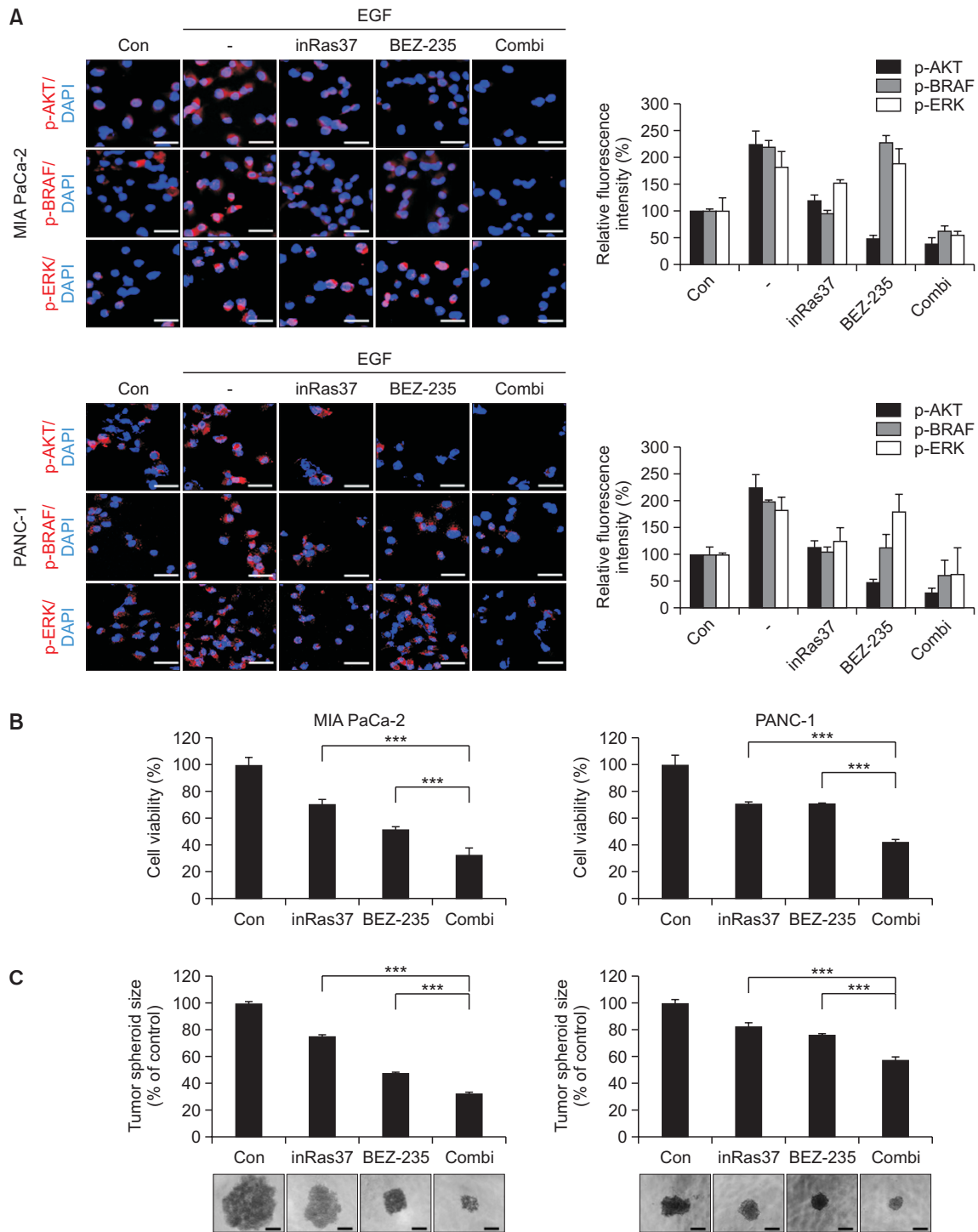


Fig. 4. Inhibition of KRAS downstream signaling and 3D-cultured cell growth by inRas37 and BEZ-235 co-treatment. (A) MIA PaCa-2 and PANC-1 cells were treated with inRas37 (5 μ M) and/or BEZ-235 (0.05 μ M) for 6 h and then stimulated with EGF (200 ng/mL for MIA PaCa-2, and 50 ng/mL for PANC-1) for 10 min after serum starvation for 2 h. The expression levels of p-AKT, p-BRAF, and p-ERK were assessed using immunofluorescence staining. Scale bar, 30 μ m. (B) Cell viabilities of MIA PaCa-2 and PANC-1 cells treated with inRas37 (5 μ M) and/or BEZ-235 (0.05 μ M) on every 2 days for 1 week were measured using the MTS assay. (C) Tumor spheroid assays were performed on MIA PaCa-2 and PANC-1 cells treated with inRas37 (5 μ M), and/or BEZ-235 (0.05 μ M) on every 2 days for 1 week, and sphere sizes were measured using Image J software. Scale bar, 200 μ m. Data are presented as the means \pm SD (***) p <0.001.

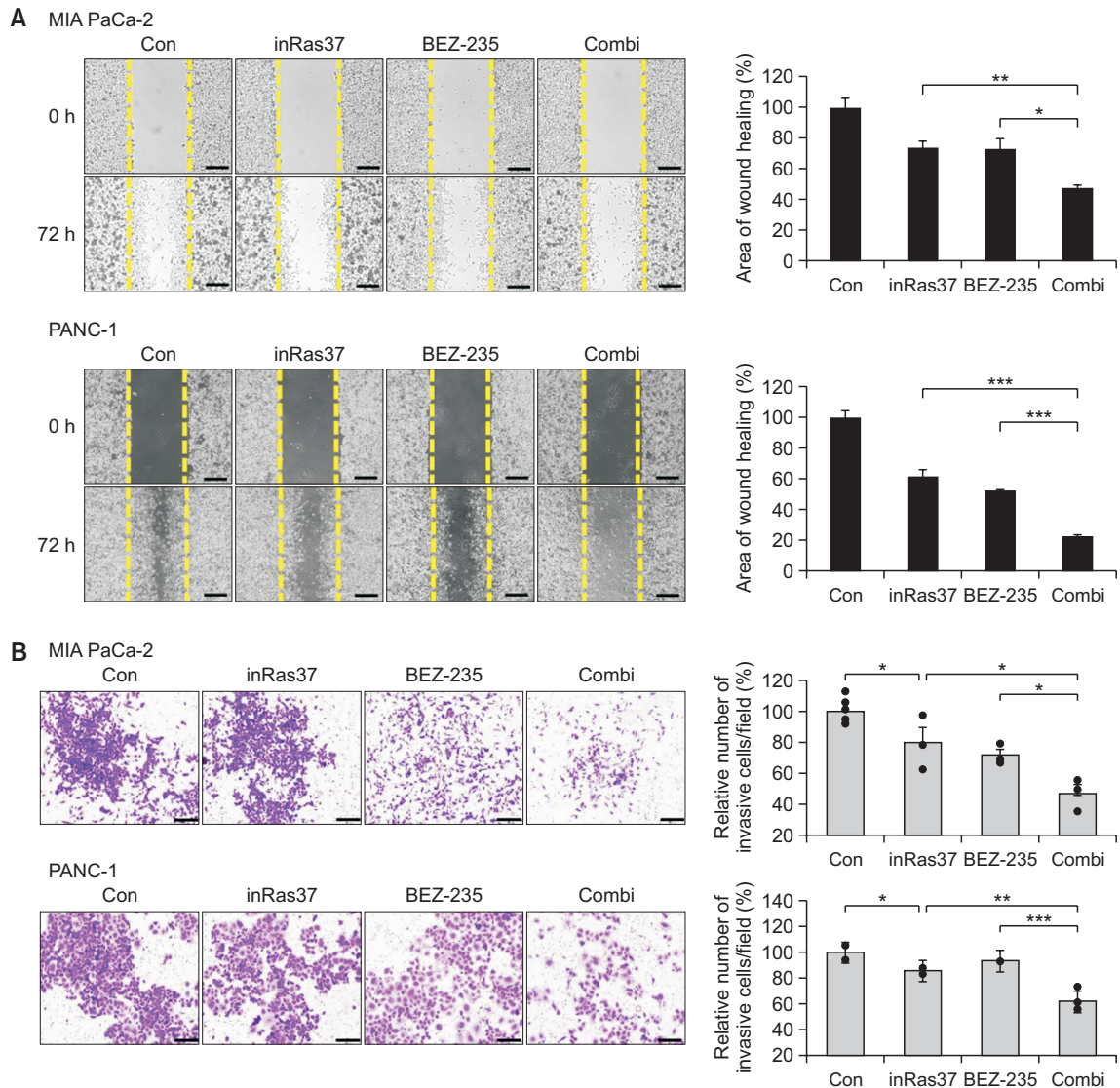


Fig. 5. Effect of inRas37 and BEZ-235 co-treatment on cancer cell migration and invasion. (A) MIA PaCa-2 and PANC-1 cells were scratched and treated with inRas37 (1 μ M) and/or BEZ-235 (0.03 μ M) for 72 h. The number of migrated cells was measured using the ImageJ software. (B) MIA PaCa-2 and PANC-1 cells were seeded into upper Transwell chambers and treated with inRas37 (1 μ M) and/or BEZ-235 (0.03 μ M) for 72 h in serum-free media; lower chambers contained completed media. Invading cells were counted using the ImageJ software. Data are presented the means \pm SD (* p < 0.05, ** p < 0.01, and *** p < 0.001).

man blood and less toxicity to normal cells, which offer targeted therapy in pancreatic cancer compared with cytotoxic drugs or small molecule inhibitors (Scott *et al.*, 2012; Herschel *et al.*, 2016). Recently, we generated an antibody, a full-length human IgG protein (RT11-i), which could penetrate the cell cytosol and selectively target the GTP-bound KRAS active form (Shin *et al.*, 2017). The antibody binds to integrin α β 3 and α β 5, which are expressed on the tumor cell surface, and then enters the cytosol through integrin receptor-mediated endocytosis, thereby inhibiting cell growth by targeting GTP-bound KRAS active form. inRas37 is the second generation of the KRAS-targeting antibody of RT11-i and has a stronger binding affinity to the KRAS-GTP form than RT11-i, particularly by binding to the in4 cyclic peptide, which increases the affinity of integrin α β 5 (Shin *et al.*, 2020). Indeed, in this study,

competitive screening with KRAS^{G12D}-GppNHP in the presence of excess KRAS^{G12D}-GDP, which amounts as a competitor, resulted in the successful isolation of VH-dependent active KRAS form-specific inRas37, which selectively bound to active forms of KRAS mutations, although not to GDP-bound inactive forms. Therefore, inRas37 treatment effectively inhibited downstream signaling mediated by KRAS, such as the RAF/MEK/ERK and PI3K/AKT signaling pathways.

BEZ235 inhibits multiple class I PI3K isoforms and mTORC1/2 kinase activity, exerts potent anticancer activity, and attenuates PI3K reactivation and mTORC2-mediated AKT reactivation (Chiarini *et al.*, 2015). It has exhibited antitumor effects in various cancers, including pancreatic cancer, and its synergistic effects have been revealed in combination with numerous drugs, including cytotoxic drugs and target inhibitors

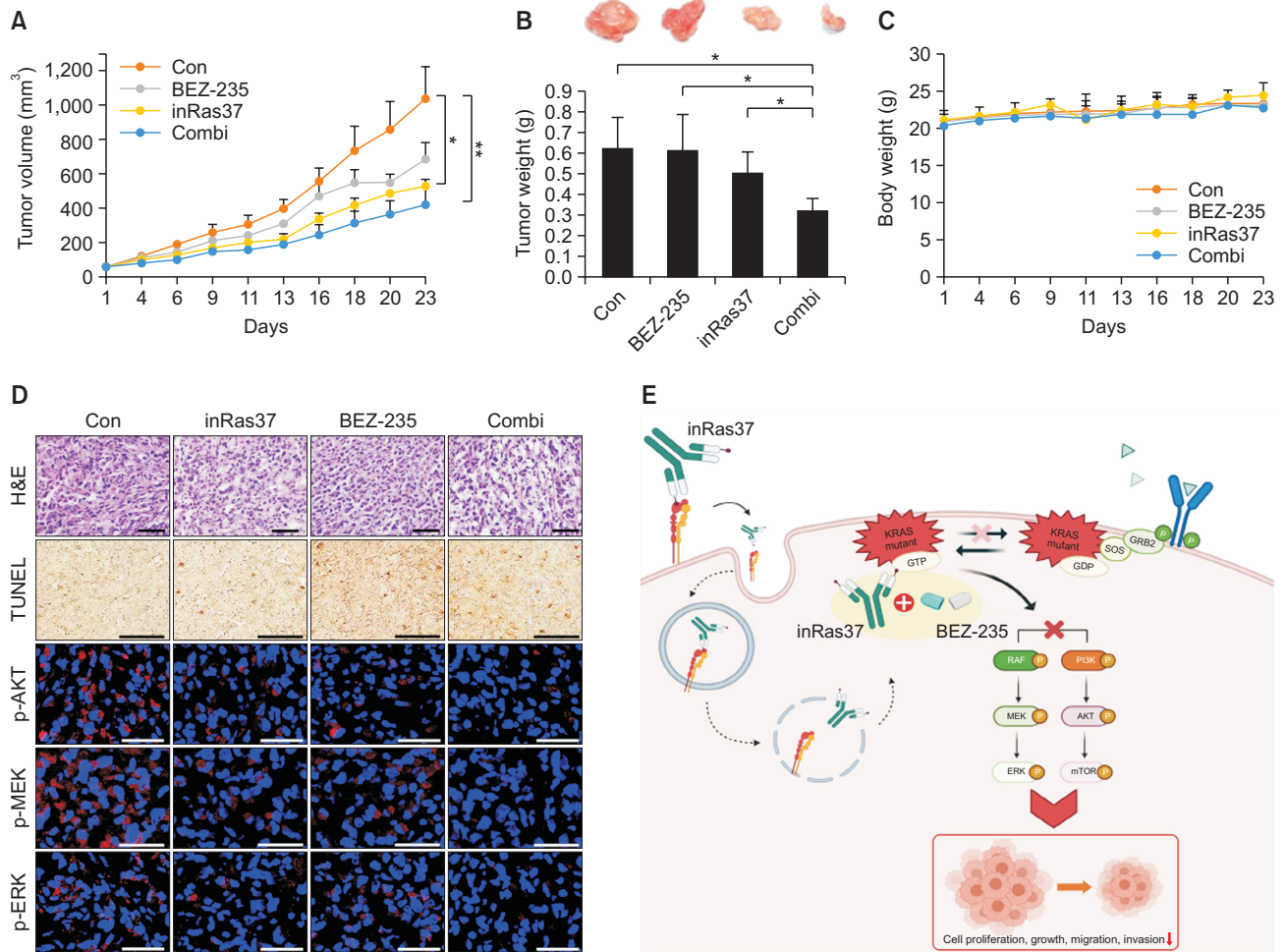


Fig. 6. Anti-tumor efficacy of inRas37 and BEZ-235 co-treatment on MIA PaCa-2 xenograft tumor model. (A-C) The antitumor efficacy of inRas37 and BEZ-235 co-treatment were analyzed by measuring the tumor sizes. All xenograft tumors were established by subcutaneous injection of MIA PaCa-2 (5×10^6 cells/200 μ L PBS) cells in the flank. As the tumor volume reached between 50 and 100 mm³, mice were intravenously administered 30 mg/kg inRas37 three times a week and intraperitoneally administered 30 mg/kg BEZ-235 three times a week for 23 days ($n=5$, per group). After 23 days of treatment, the tumor was excised, and the tumor weight was measured. (D) H&E and TUNEL staining of tumor tissues, and expressions of the KRAS downstream signaling (p-AKT, p-MEK, and p-ERK) in tumor tissues were assessed using immunofluorescence staining after co-treatment with inRas37 and/or BEZ-235. Scale bar, 50 μ m. (E) A schema depicting the mechanism of inRas37 and BEZ-235 co-treatment. Data are presented as the means \pm SD * $p < 0.05$, and ** $p < 0.01$.

(Murakami *et al.*, 1987; Zhu *et al.*, 2015; Chen *et al.*, 2018; Luszcak *et al.*, 2020; Ruan *et al.*, 2020). However, PI3K inhibitors, including BEZ-235, have been reported to enhance MEK/ERK pathway activation through a negative feedback loop mediated by mTORC2 in pancreatic cancer cells, leading to drug resistance (Soares *et al.*, 2015). Therefore, a combination of MEK and PI3K inhibitors may be necessary for optimal therapeutic activity in pancreatic cancer. Several clinical studies are currently evaluating the combination of PI3K and MEK inhibitors in various cancers with specific types of molecular alterations, with unsatisfactory results. Accordingly, given that there is no effective combination of PI3K and MEK inhibitors and no studies on the combination of PI3K and RAS-targeting inhibitors that can inhibit KRAS downstream signaling, including the MAPK and PI3K pathways in cancer, we expected that a combination of inRAS37, RAS-target antibody, and BEZ235 would be effective in pancreatic cancer with MAPK signaling

activation by KRAS mutation. Here, we found that the combination of KRAS-targeting antibody inRas37 and BEZ-235 effectively inhibited RAF/MEK/ERK and PI3K/AKT signaling and inhibited pancreatic cancer cell viability and tumor sphere size, as well as cell migration and invasion, which are associated with metastasis. Additionally, the combined treatment effects were observed in the mouse models. Our results concur with the findings of Kim *et al.* (2020), who demonstrated that the combination of KRAS gene silencing and a PI3K inhibitor suppressed cell proliferation and migration in ovarian cancer (Kim *et al.*, 2020). Although PI3K/AKT signaling inhibition by BEZ-235 results in a compensatory increase in ERK phosphorylation, combinational treatment with inRAS37 appears to result in synergistic cytotoxicity and cell death by MAPK signaling inhibition overactivated by BEZ-235.

In conclusion, MEK reactivation by BEZ-235 was inhibited by a combination treatment with inRAS37, a KRAS-targeting

antibody. In addition, this combination synergistically suppressed pancreatic cancer cell growth *in vitro* and *in vivo* by blocking the RAF/MEK/ERK and PI3K/AKT pathways and inhibits cell migration and invasion, which are important steps of the metastasis process. Our results suggest that combination therapy of inRAS37 and BEZ-235 offers an innovative therapeutic approach for managing pancreatic cancer patients with KRAS mutations.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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