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Knockdown or inhibition of Aldo-keto reductase 1B10 inhibits pancreatic carcinoma growth via modulating Kras - E-cadherin pathway

Wanying Zhang, Haonan Li, Yihe Yang, Jie Liao, and Guang-Yu Yang

Department of Pathology, Northwestern University, Feinberg School of Medicine, Chicago, IL60611, USA

Abstract

Aldo-keto reductase 1B10 (AKR1B10) has relatively specific lipid substrates including carbonyls, retinal and farnesal/geranylgeranial. Metabolizing these lipid substrates appears crucial to carcinogenesis, particularly for farnesal/geranylgeranial that involves in protein prenylation. Mutant Kras is a most common active oncogene in pancreatic cancer, and its activation requires protein prenylation. To directly determine the role of AKR1B10 in pancreatic carcinogenesis, we knocked down AKR1B10 in CD18 human pancreatic carcinoma cells using shRNA approach. Silencing AKR1B10 resulted in a significant inhibition of anchor-dependent growth (knockdown cells vs vector-control cells: 67 ± 9.5 colonies/HPF vs 170 ± 3.7 colonies/HPF, $P < 0.01$), invasion index (0.27 vs. 1.00, $p < 0.05$), and cell migration (at 16 hours $9.2 \pm 1.2\%$ vs $14.0 \pm 1.8\%$, at 24 hours $21.0 \pm 1.1\%$ vs $30.5 \pm 3.5\%$, and at 48 hours $51.9 \pm 5.7\%$ vs $88.9 \pm 3.0\%$, $P < 0.01$). Inhibition of AKR1B10 by oleanolic acid (OA) showed a dose-dependent inhibition of cell growth with IC₅₀ at 30 μ M. Kras pull-down and Western blot analysis revealed a significant down-regulation of active form Kras and phosphorylated C-Raf, and Erk, as well as an up-regulation of E-cadherin. A significant reduction of in vivo tumor growth was observed in nude mice implanted the CD18 pancreatic carcinoma cells with AKR1B10 knockdown (tumor weight: 0.25 ± 0.06 g vs. 0.52 ± 0.07 g, $P = 0.01$), and with OA treatment (tumor weight: 0.35 ± 0.05 g vs. 0.52 ± 0.07 g, $P = 0.05$). Our findings indicate AKR1B10 is a unique enzyme involved in pancreatic carcinogenesis via modulation of Kras-E-Cadherin pathway.

Keywords

AKR1B10; KRAS; E-cadherin; migration; invasion; pancreatic adenocarcinoma

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Address requests for reprints to: Guang-Yu Yang, M.D., Ph.D., Northwestern University, Feinberg School of Medicine, 303 E. Chicago Avenue, Ward 4-115, Chicago, IL 60611, Tel: 312-503-0645, Fax: 312-503-0647, g-yang@northwestern.edu, or, Jie Liao, M.D., Northwestern University, Feinberg School of Medicine, 303 E. Chicago Avenue, Ward 4-179, Chicago, IL 60611, Tel: 312-503-1260, Fax: 312-503-0647, jie-liao@northwestern.edu.

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Conflicts of Interest Statement

We do not have the Conflicts of Interest Statement for the all authors.

Introduction

Pancreatic ductal adenocarcinoma is one of the most lethal malignant neoplasms, and accounts for the fourth leading cause of cancer death, being responsible for 6% of all cancer-related deaths [1]. The prognosis of pancreatic cancer is grim, with a median survival time of 12 months, and a 5 year-survival rate of 4 % after diagnosis. Rates of pancreatic cancer have been slowly increasing over the past 10 years. Mutant Kras oncogene is the most common and earliest molecular event involved in human pancreatic carcinogenesis [2–5]. Targeting mutant Kras and its activated signaling would be significant for prevention and therapy of this lethal malignancy.

Aldo-keto reductase 1B10 (AKR1B10), also known as aldose reductase-like-1, is rarely expressed in normal human tissues, only seen in intestine and adrenal glands and minimally in liver [6–8]. AKR1B10 is an essential enzyme in the metabolism of carbonyls, retinal and farnesal/geranylgeranial for detoxifying the active carbonyls, maintaining cellular homeostasis of retinal-retinoid acid, and recycling farnesal/geranylgeranial, the key intermediate products of cholesterol synthesis [6, 9–13]. Metabolizing these lipid substrates is crucial for maintaining cellular homeostasis, proliferation and differentiation.

Over expression of AKR1B10 has been identified in hepatocellular carcinoma, smoking-related non-small-cell lung cancer and oral squamous cell carcinoma, and pancreatic cancer [6–8, 14–16]. Metabolic events mediated by AKR1B10 could play a crucial role in pancreatic cancer; particularly the reduced products generated from farnesal and geranylgeranyl are intermediates of cholesterol synthesis involved in protein prenylation [6, 9]. Ras and Ras-related GTP-binding proteins (G proteins) are key proteins that need prenylation to exert its function [2]. Our previous study showed that AKR1B10 was over expressed in pancreatic carcinoma and in pancreatic intraepithelial neoplasia lesions (PanINs) [6]. Inhibition of AKR1B10 results in suppressed activity of Kras via reduction of membrane bound mutant Kras proteins, probably due to stopping the process of protein prenylation [6, 9]. But, direct experimental evidence of AKR1B10 in modulating mutant Kras activity and pancreatic carcinogenesis needs further investigation.

To directly determine the role of AKR1B10 in pancreatic cancer, silencing AKR1B10 was achieved in CD18 human pancreatic carcinoma cell line using shRNA approach. Nearly complete AKR1B10 knockdown clone/s of CD18 human pancreatic carcinoma cells were selected. Compared to vector-control CD18 cells, AKR1B10 knockdown CD18 cells displayed a significant decrease of cell growth, migration and invasion. Membrane-bound mutant kras and its activated signals were analyzed using Ras pull-down and western blot assays. CD18 human pancreatic carcinoma cells were further treated with Oleanolic acid (OA), the most potent competitive AKR1B10 inhibitor, and showed a dose-effect on inhibiting AKR1B10 activity and cell growth. *In vivo* tumorigenesis was analyzed in nude mice implanted with CD18 pancreatic carcinoma cells with AKR1B10 knockdown or OA treatment.

Materials and Methods

Cell culture

Human CD18/HPAF pancreatic carcinoma cell line (called CD18 cells) was cultured in DMEM media (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum, 0.1% gentamicin, and 0.1% insulin and maintained at 37°C with 5% CO₂.

shRNA AKR1B10 transfection

Human AKR1B10 shRNA in pGIPZ was purchased from Open Biosystems (RHS4430-101127443). Lentivirus express AKR1B10 shRNA were produced in HEK293T cells packaged by pMD2G and psPAX2. For stable infection, 8×10^4 cells were plated in each well of 6-well plates along with 2 mL of medium without antibiotics. After 18 hours incubation, remove media and replace with 1 ml medium containing lentiviral particles with 10 µg/mL polybrene. After incubation for another 24 hours, remove the media containing lentiviral particles and add 2ml fresh media to each well. Fresh medium containing 2 mg/mL puromycin was added to each well after another 48 hours. Fresh medium containing puromycin was replenished every 3 to 4 days. Single colonies were obtained after 2 weeks of puromycin selection. Western blot assay was performed to detect the silenced expression of AKR1B10 to select best single colonies.

Colony formation assay

Colony formation was detected using a plate colony formation assay. Logarithmically growing cells were seeded in duplicate at a density of 800 cells/well in 6-well flat-bottom plates with 3 ml DMEM containing 10% fetal bovine serum. Cells were incubated with or without treatment for 14 days at 37°C and 5% CO₂. Cell colonies were fixed in 100% ice cold ethanol and were visualized by crystal violet staining. Colonies (>50 cells) were counted and the average number of colonies from three separate experiments with duplicate wells per condition was represented.

Wound healing/cell migration assay

To evaluate cell migration, a wound-healing assay of CD18 cells was performed. Cells grown to subconfluence were scraped with a 1ml tip edge to make a cell-free area. Cells migrating into the scraped area were observed, and photographed at 0, 16, 24, and 48h after scraping. The width of the cell-free area was measured at each time point. The ratio of the reduction of width at each time point to the initial width of scraped area (0h) was expressed as percentage of migration at each time point.

Invasive study using Matrigel Invasion Chambers approach

BD BioCoat Matrigel Invasion Chambers (for a 24-well plate; BD Biosciences, San Jose, CA) were used to study the invasion activity of CD18 cells. CD18-Scr (CD18 scramble cells) and CD18-shAKR (CD18 cells with AKR1B10 knockdown using shRNA approach) cell suspension (5×10^4 cells/ well) in serum free DMEM was seeded in a Matrigel Invasion Chamber. After 22 hours of cultivation, invading cells on the underside of the chamber were fixed with 100% methanol, stained with Giemsa, and counted under HPF microscope. BD

BioCoat Control Insert (for a 24-well plate; BD Biosciences, San Jose, CA) was used as negative control. The number of cells that migrated through the membrane was determined by averaging 5 random fields of view. The ratio of cells number migrated through matrigel-coated membrane to cells number migrated through non-coated membrane was expressed as the percentage of cells that migrated through matrigel-coated membrane. Invasion index was also calculated by comparison to CD18 scramble shRNA treated cells.

GTP-RAS pull-down assay

The activation of RAS was detected using an Active Ras Pull-Down and Detection Kit (Pierce Biotechnology, Rockford, IL, USA). Briefly, cell lysate (500 µg) was incubated with immobilized Raf1 Ras binding domain fused to glutathione S-transferase (GST- Raf1-RBD). Precipitates were washed 3 times, and bound proteins were eluted by boiling for 5 minutes. Proteins were separated on a 12% polyacrylamide gel, transferred to a PVDF membrane, and subjected to immunoblot analysis using anti-Ras antibodies.

Protein extraction and western blot analysis

Cultured cells were washed with cold 1× PBS buffer, scraped with sterile cell lifters and pelleted using a table-top centrifuge at 1,350 rpm for 5 min. Cells were then lysed on ice by vortexing every 5 min for 30 min using RIPA lysis buffer system (Santa Cruz biotechnology, Santa Cruz, CA) containing 1% protease inhibitor cocktail, 1% PMSF and 1% sodium orthovanadate and 1% phosphatase inhibitor cocktail 1 and 2 (Sigma, St. Louis, MO). The extracts were collected by centrifugation at 13,000 rpm for 10 min at 4°C, followed by transfer of the supernatant to a new tube. All protein concentrations were determined using Bradford reagent (Thermo). 30µg of protein was loaded on a 10% SDS-polyacrylamide gel and transferred to a Immune-Blot PVDF Membrane (Bio-Rad), the wash buffer using Tris buffered saline containing 0.1% Tween-20 (TBST) and blocking buffer using TBST with 5% non-fat milk or 5% bovine serum albumin (BSA) depending on the primary antibodies, followed by membrane incubation with the primary antibody solution overnight at 4°C. The antibodies used were: polyclonal rabbit anti-human AKR1B10 (1:300; LifeSpan BioSciences, Inc., Seattle, WA), phospho-C-Raf and total Raf (1:1000, Cell Signaling Technology), phospho-ERK1/2 and total ERK1/2 (1:1000; Cell Signaling Technology), phospho-NF-κB (1:1000, Cell Signaling Technology) and NF-κB (1:1000, Cell Signaling Technology), IKK-α (1:1000, Cell Signaling Technology) and Akt (1:1000, Cell Signaling Technology), and E-cadherin (1:2000, BD Biosciences). Mouse anti-human β-actin (1:5000, Sigma-Aldrich) was served as an internal control. The membrane was then washed with TBST and incubated with HRP-linked anti-rabbit (1:2000) or anti-mouse (1:2000) IgG and HRP-linked anti-biotin antibodies (1:1000, Cell Signaling Technology, Inc., Danvers, MA) corresponding to the primary antibodies for 1 hour at room temperature. The protein-antibody complexes were detected using the 1× LumiGLO® chemiluminescent substrates (Cell Signaling Technology) according to the manufacturer's instructions and the emitted light captured on X-ray film.

Animal experiments and tissue processing for histopathology

CD18-vector control or CD18-shAKR cells (5×10⁶ cells per 100µl per mouse) were injected subcutaneously to two hind legs of 4- to 5-week-old balb/c nude mice. CD18-V1+OA group

animals were given AKR1B10 inhibitor oleanolic acid 20mg/kg/day by i.p. injection after CD18-V1 cells implantation (n=5). CD18-V1 group and CD18-shAKR group received no OA treatment after cells implantation (n=5/ group). AIN93G diet was administered to the mice until the end of experiment. Food and water consumption, and body weight were monitored weekly. Mice were housed under pathogen-free conditions in the facilities of Laboratory Animal Services, Northwestern University. All studies were conducted in compliance with the Northwestern University IACUC guidelines. Tumor development was monitored daily. Mice were sacrificed at the end of 21 days when tumor size reached to 1 cm in diameter. Blood and plasma were collected via heart acupuncture after euthanized by CO₂ and stored in a -80°C freezer until analysis. Tumors were dissected and measured for size (length and width) and weight. Tumor volume was determined using the equation $V \text{ (mm}^3\text{)} = L \times W^2/2$. Tumors were then fixed in 10% buffered formalin and processed for paraffin sections for histopathological and immunohistochemical analyses.

Immunohistochemistry

Immunohistochemical staining was performed using avidin-biotin-peroxidase complex method on paraffin-embedded sections as previously described. The primary antibodies are phosphor-ERK antibody (1:100) and Ki67 antibody (Rabbit mAb, 1:100 diluted, Vector Laboratories Inc, Burlingame, CA). Biotinylated anti-rabbit IgG secondary antibody (1:200) and ABC complex (Avidin-Biotin-Complex) were purchased from Vector Laboratories Inc, Burlingame, CA. Diaminobenzidine (Sigma-Aldrich, St.Louis, MO) was used as the chromogen. Slides were washed with 1XTBST buffer between incubations and counterstained with Mayer's hematoxylin for 1 minute. The number of phosphor-ERK positive-stained cells per high power (40× objective lens) was counted for each specimen. Cell proliferation was analyzed by Ki67-labeled cells and proliferation index was the percentage of Ki67 positive cells in the total number of cells counted.

Results

Silencing of AKR1B10 inhibits cell growth, migration and invasion of human pancreatic carcinoma CD18 cells

To directly determine the role of AKR1B10 in malignant behavior of pancreatic cancer, knockdown of AKR1B10 expression was achieved in human pancreatic carcinoma cells CD18 using shRNA approach. As seen in Fig.1A, Western blotting assay showed nearly complete loss of AKR1B10 expression in four selected clones (A1–A4) in shRNA-AKR1B10 transfected CD18 cells compared to vector control clones (V1–V4). Malignant behavior of these cells with knockdown of AKR1B10 expression were further determined for colony formation, cell migration and invasion. Two AKR1B10 knockdown clones (A1–A2) of CD18 cells showed the smaller size of clones as compared to Vector control clone (V1), as seen in Fig. 1B; as well as the average clone numbers ($67 \pm 9.5/\text{HPF}$ and $66 \pm 12.4/\text{HPF}$ versus $170 \pm 13.7/\text{HPF}$ colony formation, $P < 0.01$, Fig. 1C),

Cell migration was analyzed using wound-healing assay in CD18 cells with silenced AKR1B10 at different time points. The results showed that CD18-shAKR cells (Clone A1) displayed a significant reduction of cell migration, as seen in Fig. 2A, CD18-Vector cells

displayed significantly more rapid migration to heal the scratched wound compared to CD18-shAKR cells. Further quantitation of wound healing process (reduced width of cell-free scratched area/initial width of the scraped cell-free area) showed that at 16, 24 and 48 hours time points, CD18-Vector cells had $14 \pm 1.8\%$, $30.5 \pm 3.5\%$ and $88.9 \pm 3.0\%$ of migration, while CD18-shAKR (A1) only had $9.2 \pm 1.2\%$, $21 \pm 1.1\%$ and $51.9 \pm 5.7\%$ of migration ($p < 0.05$, Fig. 2B).

Invasive growth is a key feature of malignant behavior. Cell invasion was analyzed using matrigel-coated membranes approach in cell culture in vitro. In general, 2.5×10^5 CD18-shAKR (A1) or CD18-Vector cells were seeded on matrigel-coated and non-coated membranes and incubated for 22 hours. 10% fetal bovine serum in the lower chamber was used as chemo-attractant. Non-coated membrane was used as positive base-line control, as majority of cells can migrate through non-coated membrane. The cells migrated through the membrane were stained and quantified the number of invasion cells under the microscope. The number of cells that migrated through the membrane was determined by averaging 5 random fields of view. The ratio of cells number migrated through matrigel-coated membrane to cells number migrated through non-coated membrane was expressed as the percentage of cells that migrated through matrigel-coated membrane. As shown in Fig. 2C and D, CD18-shAKR cells showed a significant decreased percentage of migration ($17 \pm 1\%$) compared to that of CD18-vector cells ($64 \pm 4\%$) ($P < 0.01$), or 3.7 folds decrease of invasion ability of CD18-shAKR cells.

Silencing of AKR1B10 results in up-regulation of E-cadherin via targeting Kras-pathway

To determine if AKR1B10 knockdown that inhibits malignant behavior - cell migration/invasion is via suppressing Kras function by targeting protein prenylation, we analyzed protein prenylation such as membranous bound Kras protein. Using Kras-pull down and Western blot assays, a significant decrease of cell membrane-bound Kras protein, but not total Kras protein was observed in CD18-shAKR cells (A1) compared to CD18-vector cells (β -Actin as loading protein control), as seen in Fig. 3A. Kras downstream signals were further evaluated using Western blot assay and showed that the phosphorylation of C-Raf and phosphorylation ERK were down-regulated, as well as the phospho-NF- κ B, I κ B- α , and total Akt in CD18-shAKR cells, as seen in Fig. 3B. Consequently, the expression of E-cadherin in CD18-shAKR cells was significantly increased compared to CD18-vector cells (Fig. 3B).

To determine if Kras pathway is crucial on up-regulating E-cadherin, silencing Kras expression using siRNA was performed on CD18 cell line. As shown in Fig. 3C, silencing Kras protein expression by siRNA resulted in the reduction of phospho-C-Raf and phospho-ERK and the up-regulation of E-cadherin.

Oleanolic acid, an AKR1B10 inhibitor, inhibits pancreatic cancer cell growth via suppressing Kras-E-Cadherin pathway

Oleanic acid (OA) has been reported to be a potent AKR1B10 inhibitor [17]. AKR1B10 enzymatic activity was analyzed using oxidized NADPH as a monitor at 340nm in CD18 cells lysates treated with or without OA [6]. Dose-dependent inhibition of AKR1B10 by OA

was observed with dose ranges of 0, 0.4, 0.8, and 1.6 μ M (Fig. 4A). Using colony formation assay, a significant dose-dependent inhibition of CD18 cell growth by OA was observed with IC₅₀ at 30 μ M, as seen in Fig. 4B. Due to differences in absorption, distribution and metabolism in the biologic system, the discrepancy for the biologic activity of these inhibitor/s in cell-free enzyme assay (Cell lysates) versus *in vitro* cells have been recognized well. Thus, 10 $\times$ difference between enzyme activity assay in cell lysates and *in vitro* growth inhibition by OA is reasonable.

Parallel results from Kras-pull down and western blot assays demonstrated that a significantly reduction of membrane-band Kras expression was observed in CD18 cells with 60 – 100 μ M OA treatment compared with the CD18 control cells (Fig.4C). Consequently downstream signals such as phosphorylation of C-Raf and phosphorylation ERK were further decreased (Fig. 4C). The expression of E-cadherin was significantly increased in 30–100 μ M OA treated CD18 cells compared to CD18 control cells (Fig. 4C).

AKR1B10 knockdown or inhibition reduces *in vivo* growth of human CD18 pancreatic carcinoma cells implanted in nude mice

To determine the effect of AKR1B10 knockdown or inhibition on tumorigenesis *in vivo*, we injected 5×10^6 cells of CD18-shAKR A1 (n=5) or CD18-vector (n=10) into both sides of hind limbs in Balb/c nude mice. Five mice injected with CD18-vector cells were further treated with OA for three weeks (20mg/kg/day, 0.3mg OA solved in 2% Tween 80 saline, 0.1ml/mouse by *i.p.* injection once per day). The animals were monitored twice per week for tumor development. Visible tumor was observed after 14 days of inoculation in animals. When tumor size reached to 10mm in diameter at the end of 3 weeks, the experiment was terminated. The xenograft tumors were removed from the sites, and were measured and weighted. As seen in Fig.5A–C, Morphology showed that compared to vector control cells (Fig.5A), lumen formation and mucin production were frequently observed in the tumors with AKR1B10 knockout or OA treatment. The xenograft tumors formed by CD18-shAKR cells were significantly smaller in size ($275.9 \pm 68.9\text{mm}^3$ vs. $582.7 \pm 10.5.6\text{mm}^3$, $p<0.03$), and in weight ($0.25 \pm 0.06\text{g}$ vs. $0.52 \pm 0.07\text{g}$, $p<0.01$) than the CD18-vector control. The xenograft CD18-vector tumor follows by OA treatment also showed significantly smaller in size ($315.6 \pm 50.8\text{mm}^3$ vs. $582.7 \pm 10.5.6\text{mm}^3$, $p<0.03$) as well as in weight ($0.35 \pm 0.05\text{g}$ vs. $0.52 \pm 0.07\text{g}$, $p<0.05$) compared to the CD18-vector tumor with no OA treatment, (Fig. 5D–E).

To further determine the effect of AKR1B10 knockdown or inhibition on modulating cell proliferation, phosphorylated Erk1/2, and E-cadherin, immunohistochemical analysis was performed for the xenograft tumors. Figure 6 showed that numerous Ki-67 nuclear staining of proliferative cells were identified in the xenograft tumors of CD18-vector control cells, and markedly decreased Ki-67-labeled proliferative cells were observed in the xenograft tumors of CD18-shAKR cells or CD18-vector control cells treated with OA. The semi-quantitative analysis showed that the proliferation index (percentage of Ki-67-positive cells in the total cells counted) was $63 \pm 8.8\%$ in the xenograft tumors of CD18-vector control cells and a statistically significant reduction in Ki-67-labeled proliferation index was observed in the xenograft tumors of CD18-shAKR cells ($31 \pm 12.2\%$, $p<0.05$) and CD18-

vector control cells treated with OA ($33 \pm 11.8\%$, $p < 0.05$). A decreased positive staining intensity of phosphorylated ERK1/2 and an increased staining intensity of E-cadherin were found in the xenograft tumors of CD18-shAKR cells or CD18-vector control cells treated with OA compared to xenograft tumors of CD18-vector control cells (Fig.6).

Immunohistochemical staining intensity of phosphorylated ERK1/2 and E-cadherin were quantitatively analyzed for at least 10 snapped images (under $\times 20$ objective lens) of each tumor ($n = 3$ mice/group) in Photoshop. Compared to xenograft tumors of CD18-vector control cells, the significant differences in staining intensity of phosphorylated ERK1/2 and E-cadherin were observed the xenograft tumors of CD18-shAKR cells (113 ± 18.9 vs. 62 ± 22.9 for phosphorylated ERK1/2, and 52 ± 21.3 vs 107 ± 18.8 for E-cadherin, $p < 0.05$) or CD18-vector control cells treated with OA (113 ± 18.9 vs. 59 ± 19.4 for phosphorylated ERK1/2, and 52 ± 21.3 vs 98 ± 21.2 for E-cadherin, $p < 0.05$).

Discussion

Our previous studies have demonstrated that AKR1B10 is over-expressed in pancreatic adenocarcinoma and in pancreatic intraepithelial neoplasia lesions (PanINs), and inhibition of AKR1B10 by Sulindac increases animal survival from development of pancreatic cancer in mice. To further determine the profound role of AKR1B10 in pancreatic cancer, we constantly knocked down AKR1B10 in human CD18 pancreatic carcinoma cells using shRNA approach, and first demonstrated that knockdown of AKR1B10 resulted in a significant reduction of malignant behavior of pancreatic cancer cells as evidenced by the analyses of *in vitro* colony formation, cell migration and invasion as well as *in vivo* xenograft growth in nude mice. Further mechanistic studies revealed that Knock down of AKR1B10 resulted in a significant inhibition of Kras and its downstream CRAF-MEK-ERK pathway and a significant up-regulation of E-cadherin.

AKR1B10 is a member of aldo-keto family that exhibits more restricted substrate specificity and lipid substrates including farnesal, geranylgeranyl, retinal and carbonyls. It has been hypothesized that metabolizing farnesal and geranylgeranyl substrates is crucial and possible new mechanism for cells to recycle these metabolites that is functionally important for protein post-translational modification called prenylation [6, 9, 11, 12]. Ras is one of the proteins that required prenylation for its activity. Our previous study revealed that transient AKR1B10 knockdown using small interfering RNA technology resulted in decreased level of farnesylated HDJ-2 and membrane-bound Kras protein [6]. In the present study, we further demonstrated that shRNA constant knockdown of AKR1B10 in pancreatic carcinoma cells led to a significant down-regulation of active form K-Ras and its downstream phosphorylated C-Raf, and ErK, as well as a significant up-regulation of E-cadherin. These results imply that intracellular recycling of farnesal and geranylgeranyl metabolites is crucial for protein prenylation.

E-cadherin is essential for homophilic adhesion of epithelial cells, and plays an important role in the formation of epithelial architecture. Loss or reduction of E-cadherin expression in carcinoma promotes invasion and metastasis, due to loosened intercellular adherin [18]. Constant activated oncogenic Kras in neoplastic cells appears key mechanism to down-regulation of E-cadherin [19]. There are two potential signal transduction pathways involved

in the modulation of E-cadherin via activated Kras. One is to activate focal adhesion kinase (FAK) leads to the activation of Akt, following by up-regulation of its downstream signal NF- κ B via IKK-mediated phosphorylation-induced proteasomal degradation of the I κ B inhibitor, which allows the active NF- κ B transcription factor subunits to translocate to the nucleus and induce the expression of another transcription factor SNAIL, and finally SNAIL in turn, repress the expression of E-cadherin. Another one is that mutant Kras-activated ERK2 directly phosphorylates SNAIL [20]. In our study, we demonstrated that up-regulation of E-cadherin by AKR1B10 knocked down or inhibition in CD18 pancreatic carcinoma cells appeared via down-regulation of mutant Kras-activated ERK2, Akt and I κ B- α /NF- κ B signaling, which was parallel with the effect of siRNA-knockdown K-Ras in CD18 pancreatic carcinoma cells.

Oleanolic acid or oleanic acid (OA) is a naturally occurring triterpenoid, widely distributed in food and medicinal plants [21, 22]. OA is relatively non-toxic, and has been used in cosmetics and health products, and has been recognized to have antiinflammatory and antihyperlipidemic properties and to protect against chemically induced liver injury in laboratory animals [21, 22]. But the mechanism for these beneficial effects is not known. Recent study indicates that OA is the most potent AKR1B10 competitive inhibitor with inhibition constant, 72 nM and the highest AKR1B10/AKR1B1 selectivity ratio of 1370 [17]. Molecular docking and site-directed mutagenesis studies reveal that the non-conserved residues Val301 and Gln303 in AKR1B10 are important for inhibitory potency and selectivity by OA [17]. Our study showed a dose-dependent inhibition of AKR1B10 enzyme activity and pancreatic cancer cell growth *in vitro* and *in vivo*. Mechanistic study revealed a comparable effect on inhibiting active form K-Ras and its down-stream signals as well as up-regulation of E-Cadherin as shRNA knockdown AKR1B10 in pancreatic carcinoma. These results imply that OA would be a highly potential compound for targeting AKR1B10 and pancreatic cancer.

In conclusion, silencing of AKR1B10 by shRNA approach or AKR1B10 inhibitor resulted in a significant reduction of pancreatic carcinoma cell proliferation, migration and invasion. Mechanistic studies revealed that inhibition of active membrane bound Kras and its downstream effectors, and up-regulation of E-cadherin were crucial for suppression of pancreatic cancer malignant behavior. Taken together, these results strongly suggest that inhibition of AKR1B10 could serve as a highly potential target for the prevention and treatment of highly lethal neoplasm-pancreatic adenocarcinoma.

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Fig. 1. shRNA-mediated sequence-specific silencing of AKR1B10 and Colony formation assay (A) Western blot assay shows the silencing expression of AKR1B10 in CD-18 shRNA-AKR1B10 knockdown cells compared to CD-18 vector control cells. Western blot showed levels of AKR1B10 expression compared to β -actin, a protein loading control. (B) Colony formation assay: Morphology of colony growth of CD18 shRNA-AKR1B10 knockdown cells and CD18 vector control cells. (C) Histogram of quantitative analysis of colony formation in the CD18 shRNA-AKR1B10 knockdown cells compared to CD18 vector control cells.

Fig. 2. Assays for cell migration and invasion *in vitro*

(A) Wound-healing/cell migration assay. Representative images for healing/cell migration process in scramble wound was examined for CD18 shRNA-AKR1B10 knockdown cells and CD18 vector control cells at 0, 16, 24 and 48h after wound formation. (B) The histogram showed a ratio of cell migration/healing to the 0 hour scramble wound at 16, 24 and 48h after wound and revealed a significant decrease of cell migration in CD18 shRNA-AKR1B10 knockdown cells compared to CD18 vector control cells. (C – D) Matrigel Invasion Chamber Assay: the cell invasion was analyzed as % invasion (the % of Mean # of cells invading through Matrigel insert membrane to Mean # of cell migrating through control insert membrane) as shown in histogram (C); and as Invasion Index (%Invasion of CD18 shRNA-AKR1B10 knockdown cells to % Invasion of CD-18 vector control cell) as shown in (D).

Fig. 3. Active Ras Pull-down assay and Western blot for CD18 shRNA-AKR1B10 knockdown cells and CD-18 vector control cell

(A) Active Ras Pull-down assay and Western blot showed the levels of active form Kras protein compared to total Kras protein (β-actin as a protein loading control). (B) Western blot assay showed the levels of Kras downstream signals including c-RAF, ERK1/2, Akt, IKK-α, NF-κB and E-Cadherin (β-actin as a protein loading control). (C) Active Ras Pull-down assay and Western blot for CD18 cells with siRNA Kras knockdown: showing the levels of active form Kras protein and Kras downstream signals including ERK1/2 and E-Cadherin (β-actin as a protein loading control).

Fig. 4. Analysis of AKR1B10 enzyme activity, the active form and its downstream signals in CD18 pancreatic carcinoma cell line treated with oleanolic acid (OA)

(A) Oxidized reduced nicotinamide adenine dinucleotide phosphate was used as a monitor at 340 nm for measuring AKR1B10 enzymatic activity and showed a dose-dependent inhibition of enzyme activity by OA. (B) Dose-dependent inhibition of CD-18 cell growth by OA (0 – 100 μM). (C) Active Ras Pull-down assay and Western blot showed the levels of active form Kras and its downstream signals including cRaf, ERK, and E-Cadherin (β -actin as a protein loading control).

Fig.5. Reduction of *in vivo* tumor growth by AKR1B10 knockdown or inhibition in nude mice Subcutaneous inoculation of 5×10^6 CD18-vector control or CD18-shAKR cells into the hints of nude mice (n=5) or further treated with oleanolic acid (20mg/kg/day, *i.p.* injection) for mice inoculated with CD18-vector control (n=5). (A–C) Morphology of the xenograft tumors of CD18-vector control cells, CD18-vector control cells plus OA treatment, and CD18-shAKR knockdown cells, respectively; (D) Histogram of average tumor weights; (E) Histogram of average tumor sizes. Results were reported as mean $\pm$ SD. The statistically significant difference was labeled in the figure.

Fig. 6. Representative photos of immunohistochemical analysis of Ki-67-labeled cell proliferation, phosphorylated ERK1/2, and E-cadherin in the xenograft tumors of CD18-vector control or CD18-vector control treated with OA or CD18-shAKR knockdown cells.