Nrf2 Depletion Sensitizes Pancreatic Cancer Cells to Gemcitabine via Aldehyde Dehydrogenase 3a1 Repression

Running title:

Halofuginone increases gemcitabine sensitivity

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Number of text pages: 30
Number of tables: 1
Number of figures: 7
Number of references: 23
Number of words in the Abstract: 178
Number of words in the Introduction: 577
Number of words in the Discussion: 515

Abbreviations:
ABCG2, ATP-binding cassette subfamily G member 2; Aldh3a1, aldehyde dehydrogenase 3a1; CSC, cancer stem cell; DEM, diethyl maleate; DMEM, Dulbecco’s modified Eagle’s medium; eIF2α, eukaryotic translation initiation factor 2A; GEM, gemcitabine; H&E, hematoxylin and eosin; HF, halofuginone; HO-1, heme oxygenase 1; HPFs, high power fields; IHC, immunohistochemistry; Keap1, Kelch-like ECH-associated protein 1; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, NF-E2-related factor 2; NSCLCs, non-small-cell lung cancer cells; PanIN, pancreatic intraepithelial neoplasia; RIPA, radioimmunoprecipitation; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation.

Recommended section assignment:
Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

As the central regulator of the oxidative stress response, nuclear factor erythroid 2-related factor 2 (Nrf2) is attracting great interest as a therapeutic target for various cancers, and the possible clinical applications of novel Nrf2 inhibitors have been explored in Nrf2-activated cancers. In the present study, we specifically investigated halofuginone, which is derived from a natural plant alkaloid. We found that halofuginone administration decreased the number of pancreatic intraepithelial neoplasias in pancreas-specific Kras and p53 mutant (KPC) mice. In Nrf2-activated pancreatic cancer cell lines established from KPC mice, halofuginone rapidly depleted Nrf2 in Nrf2-activated cancer cells. Both in vitro and in vivo, it sensitized Nrf2-activated pancreatic cancer cells to gemcitabine, which is the first-line chemotherapy in clinical practice. In our mechanistic study, we found that halofuginone downregulated ALDH3A1 in mouse pancreatic cancer cells. The Nrf2 inducer diethyl maleate upregulated ALDH3A1, and knockdown of Aldh3a1 sensitized Nrf2-activated cancer cells to gemcitabine, strongly suggesting that ALDH3A1 is regulated by Nrf2 and that it contributes to gemcitabine resistance. The current study demonstrated the therapeutic benefits of halofuginone in Nrf2-activated pancreatic cancers.
Significance Statement

We identified Nrf2 and its downstream target ALDH3A1 as novel therapeutic targets in pancreatic cancer. They negatively impact the conventional chemotherapeutic agent, gemcitabine efficacy. We confirmed that Nrf2 plays a pivotal role in the induction of ALDH3A1.

Keywords: Nuclear factor erythroid 2-related factor 2, Halofuginone, Gemcitabine, Chemo resistance, Amino acid depletion
**Introduction**

Pancreatic cancer is an intractable gastrointestinal malignancy with poor prognosis. Therapeutic options for unresectable pancreatic cancers have improved in the last decade, but the median survival time remains shorter than 12 months (Okusaka and Furuse, 2020). Intrinsic and acquired resistance to chemotherapeutic agents promotes tumor re-growth. Numerous molecules, signaling pathways, and adaptive mechanisms are involved in this process, and all could be novel therapeutic targets in pancreatic cancer. Previous studies have clarified some of these mechanisms using *in vitro* approaches with cultured cells. For example, the Msh homeobox 2 transcription factor, which induces epithelial-to-mesenchymal transition, determines the chemosensitivity of pancreatic cancer cells by upregulating the ATP-binding cassette subfamily G member 2 (ABCG2) transporter (Hamada et al., 2012). Prolonged gemcitabine treatment led to resistant pancreatic cancer cell lines with increased cytidine deaminase activity, resulting in enhanced drug detoxification (Samulitis et al., 2015).

We have investigated the role of the Kelch-like ECH-associated protein 1 (KEAP1)-nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in pancreatic cancer cells (Hamada et al., 2017). The KEAP1-Nrf2 pathway functions as a major adaptive mechanism for oxidative and electrophilic stress by inducing antioxidant and detoxifying enzymes that result in cell survival and organ protection (Yamamoto et al., 2018; Biard et al., 2020). Nrf2 also protects cancer cells from reactive oxygen species and xenobiotics, including chemotherapeutic agents (Taguchi et al., 2020). To examine the contribution of Nrf2 to the development of pancreatic cancers, we introduced *Nrf2*-null background into
Kras<sup>G12D</sup>+/::p53<sup>R172H</sup>+/::Pdx-Cre (KPC) mice, which spontaneously develop invasive pancreatic cancer driven by mutant Kras and p53 (Hingorani et al., 2005). The loss of Nrf2 function in these mice resulted in fewer precancerous lesions, pancreatic intraepithelial neoplasias (PanIN), and invasive cancers (Hamada et al., 2017), indicating that Nrf2 is required to establish pancreatic cancer.

To gain further insights into how Nrf2 contributes to pancreatic cancer development, we established pancreatic cancer cell lines from Nrf2-null KPC mice, dubbing them KPCN cells. These cell lines have much higher sensitivity to the electrophilic stress inducer diethyl maleate (DEM), as well as to gemcitabine—a standard chemotherapeutic agent for pancreatic cancer (Hamada et al., 2017), suggesting that Nrf2 elicits the expression of cytoprotective and chemoresistant genes in KPC cells. However, the Nrf2-target genes responsible for gemcitabine resistance have not yet been identified.

Since Nrf2 plays a crucial role in cancer cell survival, its inhibition is likely an effective therapy (Yamamoto et al., 2018; Taguchi et al., 2020). Indeed, the small-molecule Nrf2 inhibitor ML385 selectively showed anti-tumor activity in combination with carboplatin in non-small cell lung cancer (NSCLC) cells harboring constitutive Nrf2 activation (Singh et al., 2016). A plant alkaloid-derived Nrf2 inhibitor, halofuginone, has been identified in high-throughput drug screening using a luciferase reporter cell line (Tsuchida et al., 2017). Halofuginone acts as a prolyl-tRNA synthetase inhibitor that suppresses protein synthesis and triggers amino acid depletion (Keller et al. 2012). Halofuginone sensitizes Nrf2-addicted cancer cell lines to chemotherapeutic agents.
In the present study, we addressed whether halofuginone could be used to treat pancreatic cancers. Using KPC mice and cell lines, we found that halofuginone pretreatment markedly sensitized pancreatic cancer cells to gemcitabine. Comprehensive analysis of differentially expressed genes after halofuginone pretreatment identified the downregulation of aldehyde dehydrogenase 3a1 (Aldh3a1) gene expression. A series of mechanistic analyses revealed that halofuginone pretreatment intensifies the efficacy of gemcitabine in Nrf2-activated cancers, and that ALDH3A1 is a key target molecule in the function of halofuginone. We believe that this regulatory mechanism is a novel therapeutic target for pancreatic cancer.
Materials and Methods

Materials

Gemcitabine and DEM were purchased from Wako (Osaka, Japan), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Dojindo (Kumamoto, Japan), halofuginone and anti-tubulin antibody (T5168) from Sigma-Aldrich (St. Louis, MO, USA), anti-ALDH3A1 antibody (ab76976), anti-heme oxygenase 1 (HO-1) antibody (ab137749) and peroxidase-conjugated anti-rat antibody (ab6734) from Abcam (Cambridge, UK), anti-caspase-3 antibody (9662), anti-eukaryotic translation initiation factor 2A (eIF2α) antibody (5324), anti-p-eIF2α antibody (3398), and peroxidase-conjugated anti-rabbit antibody (7074) from Cell Signaling Technology (Beverly, MA). The Anti-Nrf2 antibody has been described previously (Maruyama et al., 2008). Peroxidase-conjugated anti-mouse antibody (NA931) was purchased from GE Healthcare (Piscataway, NJ, USA).

Mice

Mice were handled according to the Guidelines for Proper Conduct of Animal Experiments of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. All animal experiments were performed according to the regulations for animal experiments and related activities at Tohoku University (Article No. 2019MdA-154 and 2018MdA-127-03, approved by Institution’s Animal Care and use Committee). Pdx-1-Cre transgenic mice, LSL-K-ras^{G12D} mice, and LSL-p53^{R172H} mice were obtained from...
the NCI Mouse Repository (Frederick, MD) (Hingorani et al., 2005, Jackson et al., 2001, Olive et al., 2004). Mice were back-crossed with the C57BL/6J strain for more than five generations. KPC mice were obtained by crossing the mice. Nrf2-knockout KPC (Kras\textsuperscript{G12D/+}; p53\textsuperscript{R172H/+}; Pdx-Cre::Nrf2\textsuperscript{−/−}; KPCN) mice were also obtained, as described previously (Hamada et al., 2017). At the age of 5 weeks, KPC mice received intraperitoneal injection (5 times/week) of halofuginone (0.25 mg/kg) or saline over 4 weeks and were then euthanized. The total number of PanINs within the pancreas was calculated at 9 weeks.

**Immunohistochemistry**

Tissues from mice were fixed in 4% paraformaldehyde (Wako) and paraffin-embedded for hematoxylin-eosin (H&E) staining or immunohistochemistry (IHC). For mouse Aldh3a1 and HO-1, slides were boiled for 10 min in target retrieval solution (Dako, Glostrup, Denmark) and incubated with the primary antibody overnight. The visualization of IHC was performed using Histofine MOUSESTAIN KIT (Nichirei Biosciences Inc., Tokyo, Japan) and diaminobenzidine.

**Cell Viability Assay**

Cells were seeded in 96-well plates at 5000 cells/well and treated using 0-10 \( \mu \text{M} \) gemcitabine for 48 h, with or without 100 nM halofuginone pretreatment for 24 h. Cell viability was measured using the MTT assay, wherein the cells were treated using 5 \( \mu \text{g/mL} \) of MTT for 1 h and then solubilized in dimethyl sulfoxide. The optical density was measured using a
spectrophotometer at a wavelength of 570 nm, with a reference wavelength of 690 nm.

Cell Culture

Pancreatic cancer cell lines were established from either KPC or Nrf2-knockout KPC mice (KPC and KPCN cell lines, respectively), as described previously (Hamada et al., 2017). To establish stable Aldh3a1 knockdown cell lines, KPC cell line 1 was transfected with the predesigned shRNA expression vector pLKO.1-puro for mouse Aldh3a1 (TRCN0000438676; Sigma Aldrich), and the knockdown cells were selected. To establish the control line, KPC line 1 was transfected with predesigned non-targeting shRNA expression vector pLKO.1-puro (non-mammalian shRNA control plasmid; Sigma Aldrich), and the transfected cells were selected. Cells were maintained at 37 °C in a humidified incubator with 5 % CO₂ in Dulbecco’s modified Eagle’s medium containing 10 % fetal bovine serum and antibiotics.

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription PCR was performed using 1 μg of RNA and the SuperScript VILO™ Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The expression of each gene was quantified using the StepOnePlus™ real-time PCR system (Thermo Fisher Scientific) and Fast SYBR Green Master Mix (Thermo Fisher Scientific), with the following primers (Wang et al., 2012): β-actin, forward 5-GGCTGTATTCCCTCCATCG-3, reverse
5-CCAGTTGGTAAACAATGCCATGT-3; \textit{Aldh3a1}, forward

5-TGGCAAGACTCGTCAGACC-3, reverse 5-AGTTCCAAGCACCTATGACAAG-3′.

\textbf{Microarray}

Total RNA samples from cells were subjected to microarray analysis in duplicate using whole mouse genome Oligo DNA Microarray ver2.0 (Agilent Technologies, Santa Clara, CA) and the G2539A microarray scanner system (Agilent). Data were acquired using GeneSpring GX software (Agilent). To identify up- or down-regulated genes and compare the control and experimental samples, we calculated Z-scores and ratios (non-log-scaled fold change) from the normalized signal intensities of each probe (Quackenbush, 2002). We then established the following criteria for regulated genes: up-regulated genes, Z-score ≥ 2.0 (ratio ≥ 1.5-fold); down-regulated genes, Z-score ≤ -2.0 (ratio ≤ 0.66). The raw data are available at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) (accession No. GSE172046).

\textbf{Western Blot}

The cells were lysed in radioimmunoprecipitation (RIPA) buffer to obtain total proteins. Lysates were subjected to electrophoresis using NuPAGE 8 % Bis-Tris gel (Life Technologies) and transferred onto Immobilon-P membranes (Merck Millipore, Billerica, MA). The membranes were incubated overnight at 4 °C with primary antibodies. After incubation with peroxidase-conjugated secondary antibody for 1 h at room temperature, reactive bands were detected using ECL™ western blotting detection reagents (GE
Healthcare, Buckinghamshire, England). Densitometry analysis was performed using Image J (http://rsbweb.nih.gov/ij/index.html).

**Subcutaneous Implantation Model**

Male ICR-nu mice at 5 weeks of age were used in the subcutaneous implantation model. The mice received bilateral dorsal injection of $1 \times 10^6$ cells/injection of KPC cell line 2. After 1 week, they received intraperitoneal injections of saline for 3 weeks, twice weekly gemcitabine (50 mg/kg), 5 times weekly halofuginone (0.25 mg/kg), or a combination of gemcitabine and halofuginone. Tumor size was calculated 3 weeks after treatment using the following formula: $V = 0.5 \times \text{width}^2 \times \text{length} (\text{mm}^3)$. The mice were then euthanized and the subcutaneous tumors were subjected to histological analysis.

**Statistical Analysis**

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using JMP Pro version 15.0.0 (SAS Institute, Cary, NC). Differences between the two factors were assessed using the Student’s $t$-test, while differences between more than two groups were assessed using the Tukey–Kramer method. Statistical significance was set at $P$-values $< 0.05$. 
Results

*Halofuginone Treatment Decreased the Number of Precancerous Lesions in KPC Mice*

To confirm the anti-cancer effects of halofuginone on pancreatic cancer *in vivo*, we administered halofuginone to 5-week-old KPC mice, which were expected to develop Nrf2-activated PanINs at 13 weeks of age (Hamada et al. 2017). Administration of halofuginone for 4 weeks did not cause adverse effects, such as loss of body weight or weakness. At 9 weeks of age, vehicle-treated KPC mice developed precancerous lesion PanINs, but halofuginone treatment significantly decreased the number of PanINs in KPC mice. Typical histological images of vehicle-treated and halofuginone-treated mouse pancreas are shown in Fig. 1A, with lower and higher magnifications (top and bottom panels, respectively). The number of PanINs was significantly lower in halofuginone-treated mice than in vehicle-treated mice (Fig. 1B). These results indicate that halofuginone attenuates the formation of PanINs in KPC mice without eliciting unwanted adverse effects. We also assessed HO-1 expression in the PanINs of vehicle-treated and halofuginone-treated mice by immunohistochemistry. Halofuginone decreased HO-1 expression in the PanINs (Fig. 1C).

*Halofuginone Depletes Nrf2 and Sensitizes Pancreatic Cancer Cells to Gemcitabine*

We next explored the mechanisms by which halofuginone inhibits Nrf2 in Nrf2-activated pancreatic cancer cell lines established from KPC mice (Hamada et al. 2017). To this end, we used two KPC cell lines that expressed Nrf2 abundantly (KPC line 1) and moderately (KPC line 2). Treating these cell lines with halofuginone (100 nM) for 1–3 h markedly depleted
Nrf2 expression (Fig. 2A). This rapid decrease in Nrf2 expression was accompanied by an increase in phosphorylated eIF2α. This is consistent with the known functions of halofuginone, which inhibits prolyl-tRNA, leading to amino acid starvation and thus repressing protein synthesis. Nrf2 in particular is severely affected by halofuginone because it is a very short-lived protein (Tsuchida et al., 2017).

Next, we examined KPC cell viability after treatment with halofuginone (100 nM) and an incremental amount of gemcitabine (0.01 to 10 µM). We treated the KPC cell lines first with halofuginone for 24 h and then with gemcitabine for 48 h. Halofuginone pre-treatment significantly enhanced the cytotoxic effect of gemcitabine, compared to cells treated with gemcitabine alone (Fig. 2B). This synergistic effect of halofuginone and gemcitabine was reproducible in the two KPC cells, although KPC line 1, which had higher Nrf2 expression, showed weaker sensitivity than the moderate Nrf2-expressing KPC line 2. We also examined the expression of cleaved caspase-3 to determine whether apoptosis increased. The expression of cleaved caspase-3 was induced by 10 µM gemcitabine and promoted by halofuginone pretreatment in both KPC cell lines (Fig. 2C), suggesting that combined gemcitabine and halofuginone increased apoptosis. KPC line 1 treated with halofuginone and gemcitabine expressed more cleaved caspase-3 than KPC line 2 (Fig. 2C). These differences between KPC line 1 and 2 might be due to additional factors induced by Nrf2, sensitizing cancer cells to gemcitabine-induced apoptosis, which were depleted by halofuginone. These results indicate that halofuginone depletes Nrf2 in pancreatic cancer cells and enhances their sensitivity to gemcitabine.
Halofuginone Potentiates the Anti-Tumor Effect of Gemcitabine In Vivo

To validate the in vitro results with KPC cells, we examined whether concomitant halofuginone improves the anti-cancer activity of gemcitabine in vivo. To this end, we subcutaneously implanted KPC cell line 2 into male immunodeficient ICR-nu mice at 5 weeks of age to generate tumor-bearing mice, which were then treated using halofuginone (0.25 mg/kg; i.p.; 5 times/week for 3 weeks) and/or gemcitabine (50 mg/kg; i.p.; twice per week for 3 weeks).

Treatment with gemcitabine alone tended to reduce the size of subcutaneous tumors in immunodeficient mice injected with KPC cell line 2, but there was no significant difference between the vehicle control and gemcitabine (Fig. 3A and B). Halofuginone alone had no substantial effect on tumor size. In contrast, the combination of gemcitabine and halofuginone significantly reduced the tumor size. Halofuginone alone and the combination of gemcitabine and halofuginone reduced Aldh3a1 expression in these tumors (Fig. 3C). None of the groups showed any adverse effects, such as body weight loss or weakness. These results demonstrate that halofuginone sensitizes pancreatic cancer cells to gemcitabine, both in vitro and in vivo. Administration of halofuginone alone had little effect on the size of subcutaneous tumor, suggesting additional mechanisms supporting tumor growth besides Aldh3a1 in in vivo setting.

Halofuginone Reduces ALDH3A1 Expression both in Vitro and in Vivo
To gain mechanistic insights, we next attempted to identify the downstream target genes of Nrf2 that are responsible for the sensitization to gemcitabine. For this purpose, we conducted microarray analyses using KPC cell lines 1 and 2 treated with or without halofuginone (100 nM, 24 h). We identified a number of downregulated genes in individual cell lines and selected nine genes that were commonly downregulated by halofuginone treatment in both KPC line 1 and KPC line 2 cells (Table 1). In addition to the canonical Nrf2 target genes, such as the glutathione S-transferase family and ATP-binding cassette (ABC) transporter family, we identified Aldh3a1. A previous study showed that Nrf2 silencing in pancreatic cancer cells led to reduced ALDH1A1 and ALDH3A1 and resulted in enhanced sensitization to 5-fluorouracil (Duong et al., 2017). We hypothesized that Aldh3a1 might be an important Nrf2-target gene in pancreatic KPC cells, so we conducted a series of experiments to clarify whether Aldh3a1 is responsible for gemcitabine sensitivity in an Nrf2-dependent manner.

We first assessed ALDH3A1 expression after halofuginone treatment (100 nM; 24 h) in KPC cell lines 1 and 2. Halofuginone treatment significantly reduced ALDH3A1 mRNA expression (Fig. 4A). Western blotting experiments revealed that ALDH3A1 protein levels were also markedly decreased by halofuginone treatment (Fig. 4B).

We then asked whether halofuginone treatment actually reduced ALDH3A1 protein levels in xenografted KPC tumor cells. To investigate this, we treated KPC mice with a series of intraperitoneal injections of halofuginone (0.25 mg/kg/injection; 5 times/week for 4 weeks) and analyzed ALDH3A1 protein expression using immunohistochemical staining. The results revealed that halofuginone led to significantly lower ALDH3A1 expression in PanINs than in
control KPC mice (Fig. 4C). Taken together, these results demonstrate that halofuginone can repress ALDH3A1 expression both in vitro and in vivo.

**Nrf2 Deletion Suppresses ALDH3A1 Induction via the Electrophilic Stress Inducer DEM**

Thus far, the results strongly suggest that ALDH3A1 is regulated by Nrf2. To confirm this, we next examined whether ALDH3A1 can be induced by the electrophilic Nrf2 inducer DEM, and whether Nrf2 depletion can reverse this induction. We found that treating KPC cell line 1 with DEM (100 μM, 24 h) resulted in increased ALDH3A1 expression at both the mRNA and protein levels (Fig. 5A and B). In contrast, the KPC cell line established from Nrf2-knockout mice, which we refer to as KPCN cell line 1, showed no increase in ALDH3A1 after DEM treatment (Fig. 5A and B). We also assessed ALDH3A1 expression in the PanIN lesions of KPC and KPCN mice using immunohistochemistry. In KPCN mice, ALDH3A1 expression was markedly lower than that in KPC mice (Fig. 5C).

**Aldh3a1 Knockdown Sensitized Pancreatic Cancer Cells to Gemcitabine**

We addressed whether loss of ALDH3A1 function in KPC cells contributes to the increase in gemcitabine sensitivity. To answer this, we performed Aldh3a1 knockdown in KPC line 1 and examined the relationship between knockdown and gemcitabine sensitivity. We established two cell lines stably expressing shRNA for ALDH3A1, with each cell line showing a distinct reduction in ALDH3A1 expression (Fig. 6A). Both showed reduced viability after gemcitabine treatment; the reduction correlated with the decreased ALDH3A1 expression in
both cell lines and was significant compared with the control cell line (Fig. 6B). In conclusion, these results strongly suggest that ALDH3A1 reduction is responsible for the gemcitabine sensitization induced by Nrf2 depletion.

**Nrf2 and Aldh3a1 are responsible for sensitization to gemcitabine**

Finally, we assessed whether loss of ALDH3A1 or NRF2 in KPC cells attenuates the sensitization to gemcitabine caused by halofuginone treatment. Pretreatment with halofuginone (100 nM) for 24 hours did not sensitized sh Aldh3a1 line 1 and KPCN line 1 to gemcitabine at 1 or 10 μM (Fig. 7A and B). These results indicate that halofuginone sensitizes pancreatic cancer cells to gemcitabine via depletion of NRF2/ALDH3A1 to certain extent.
Discussion

In this study, we found that the Nrf2 inhibitor halofuginone sensitized pancreatic cancer cells to gemcitabine. Through a study exploring halofuginone activity, we discovered that halofuginone reduces the expression of ALDH3A1. Furthermore, by establishing stable Aldh3a1 knockdown cell lines, we confirmed that ALDH3A1 potentiates the sensitivity of pancreatic cancer cells to gemcitabine treatment. We also found that halofuginone has suppressive effects on ALDH3A1 expression in vivo in mice with Nrf2-activated pancreatic cancers. The PanINs of KPCN mice showed low ALDH3A1 expression, and DEM treatment failed to induce ALDH3A1 in KPCN cell lines, indicating that Nrf2 contributes to ALDH3A1 expression. Notably, the PanINs of KPC mice that had received halofuginone administration showed markedly decreased ALDH3A1 expression, suggesting that halofuginone combined with gemcitabine could be used in pancreatic cancer therapy.

ALDH3A1 plays pivotal roles in other types of cancers. For instance, Aldh3a1 knockdown potentiates the sensitivity of human pancreatic cancer cells to 5-fluorouracil (Duong et al., 2017). Higher expression of ALDH3A1 in the cancer stem cell fraction has also been reported in prostate cancer, along with elevated expression of the cancer stem cell marker ABCG2 (Wang et al., 2020b). Pharmaceutical inhibition of ALDH3A1 by shikonin, a natural naphthoquinone, sensitizes cancer cells to the chemotherapeutic agent cabazitaxel (Wang et al., 2020b). Irradiated lung cancer cells release ALDH3A1-containing exosomes, which increase the invasive capacity of cancer cells receiving them (Wang et al., 2020a). In cancer cells that receive ALDH3A1-containing exosomes, glycolysis is accelerated,
suggesting that metabolic reprogramming occurs. The KEAP1-Nrf2 system also impacts the metabolic status of cancer cells by inducing metabolic reprogramming (Mitsuishi et al., 2012). In one study, comprehensive metabolome analysis in NSCLC cells enabled the activation status of Nrf2 to be classified based on characteristic metabolites of Nrf2, which could be used as diagnostic markers (Saigusa et al., 2020).

The expression levels of ALDH3A1 in surgically resected cancer tissues correlate with the prognosis of patients with gastric cancer. Combined with other differentially expressed genes, ALDH3A1 shows diagnostic efficacy in patients with gastric cancer (Zhao et al., 2017). The impact of ALDH3A1 expression status on the prognosis of pancreatic cancers needs further validation in relationship with therapeutic intervention such as adjuvant chemotherapy. Several studies have targeted multi-isoform ALDH, including ALDH3A1. Newly synthesized ALDH inhibitors, such as 2-[4-(2,3-dioxo-5-trifluoromethyl-2,3-dihydroindol-1-ylmethyl)benzyl] isothiourea hydrobromide, have shown anticancer effects in melanoma cells, colon cancer cells, and multiple myeloma cells (Dinavahi et al., 2020). This cytotoxic effect caused by ALDH loss was accompanied by increased reactive oxygen species and lipid peroxidation, indicating greater oxidative stress. Based on the present study and these reported studies, we propose that ALDH3A1 plays a pivotal role as a target gene of Nrf2 in the response to oxidative stress in pancreatic cancers.

In conclusion, the present study demonstrated that the Nrf2 inhibitor halofuginone shows a synergistic anti-cancer effect with gemcitabine. The suppression of Nrf2 activity led
to ALDH3A1 reduction. Nrf2 inhibition and ALDH3A1 reduction could be used to potentiate the conventional chemotherapeutic agent gemcitabine in pancreatic cancers. Halofuginone and/or ALDH inhibitors may be the next important innovation in the treatment of pancreatic cancers, which are an intractable gastrointestinal malignancy.

**Authorship Contributions**

Participated in research design: Atsushi Masamune and Shin Hamada.

Conducted experiments: Shin Hamada, Yu Tanaka and Ryotaro Matsumoto.

Contributed new reagents or analytic tools: Keiko Taguchi.

Performed data analysis: Shin Hamada.

Wrote or contributed to the writing of the manuscript: Shin Hamada, Masayuki Yamamoto and Atsushi Masamune.
References


Footnotes

This work was supported in part by JSPS KAKENHI [19H03631, 20K08300], and Smoking Research Foundation (to Shin Hamada). The authors acknowledge the technical support of the Biomedical Research Core of Tohoku University Graduate School of Medicine.

Financial disclosure

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

**Fig. 1.** Decreased precancerous lesion pancreatic intraepithelial neoplasias (PanINs) in *Kras* and *p53* mutant (KPC) mice treated with halofuginone. (A) Hematoxylin and eosin (H&E) staining of the pancreas in KPC mice injected intraperitoneally with control (saline) or halofuginone (HF; 0.25 mg/kg) 5 times/week for 4 weeks. White bar shows 500 μm and black bar shows 100 μm. (B) Average number of PanINs in four random high power fields (HPFs) in the pancreases of four KPC mice that underwent control treatment and four that received HF (N=16; **P < 0.01). Error bars show standard deviation. (C) Immunohistochemistry for HO-1 expression in KPC mice injected intraperitoneally with control (saline) or halofuginone (HF; 0.25 mg/kg) 5 times/week for 4 weeks. Black bar shows 100 μm.

**Fig. 2.** Nuclear factor erythroid 2-related factor 2 (Nrf2)-inhibitory effects of halofuginone in *Kras* and *p53* mutant (KPC) cell lines. (A) Western blot for Nrf2 depletion and eukaryotic translation initiation factor 2A (eIF2α) phosphorylation indicating amino acid depletion after halofuginone (HF; 100 nM) treatment. Total eIF2α and αTubulin are displayed as loading controls. (B) 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability 48 hours after gemcitabine (GEM; 0.01–10 μM) treatment, with or without HF pretreatment for 24 hours (5000 cells/well; N = 6, **P < 0.01 compared with control at the same concentration of GEM). The error bars show standard deviation. (C) Western blot for expression of cleaved caspase-3 after gemcitabine treatment with or without HF pretreatment.
αTubulin is displayed as a loading control. Lower panel shows the result of densitometry analysis (N=3, **P < 0.01).

**Fig. 3.** Effects of combination treatment with gemcitabine and halofuginone (HF) in the subcutaneous implantation model. Macroscopic view (A) and the quantified size (B) of subcutaneous tumors in immunodeficient mice injected with the Kras and p53 mutant (KPC) cell line 2 (1 x 10^6 cells) 3 weeks after treatment with saline, gemcitabine (GEM; 50 mg/kg, i.p.; twice weekly), HF (0.25 mg/kg; i.p.; 5 times weekly), or the combination of GEM and HF (N = 6–8; *P < 0.05, **P < 0.01; ns, not significant). Error bars show standard deviation. (C) Immunohistochemistry for ALDH3A1 in subcutaneous tumors with control treatment, GEM, HF and GEM+HF. Black bar shows 100 μm.

**Fig. 4.** Decreased expression of ALDH3A1 after halofuginone (HF) treatment. (A) Quantitative RT-PCR for ALDH3A1 mRNA expression 24 h after treatment with HF (100 nM; N = 6; *P < 0.05; **P < 0.01). Error bars show standard deviation. (B) Western blot for ALDH3A1 expression 24 h after treatment with HF (100 nM). αTubulin is displayed as a loading control. (C) Immunohistochemistry for ALDH3A1 expression in Kras and p53 mutant (KPC) mouse pancreas 4 weeks after HF treatment (0.25 mg/kg/injection; i.p.; 5 times/week). Black bar shows 100 μm.

**Fig. 5.** Effects of nuclear factor erythroid 2-related factor 2 (Nrf2) deletion on the expression
of ALDH3A1. (A) Quantitative RT-PCR for ALDH3A1 mRNA expression 24 h after diethyl malate (DEM; 100 μM) treatment in KPC line 1 and KPCN line 1 (N = 6, *P < 0.05). Error bars show standard deviation. (B) Western blot for Aldh3a1 expression after DEM (100 μM, 24 hours) treatment in KPC line 1 and KPCN line 1. αTubulin is displayed as a loading control. (C) Immunohistochemistry for ALDH3A1 expression in KPC and KPCN mouse pancreas at 90 days after birth. Black bar shows 100 μm.

**Fig. 6.** Establishment of Aldh3a1 knockdown cell lines and gemcitabine (GEM) sensitivity. (A) Western blot for ALDH3A1 expression in stable Aldh3a1 knockdown cell lines. αTubulin is displayed as a loading control. (B) 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability of stable Aldh3a1 knockdown cell lines 48 h after GEM treatment (0–10 μM; 5000 cells/well, N = 6, **; P < 0.01). Error bars show standard deviation.

**Fig. 7.** Effects of halofuginone in Nrf2-deleted and Aldh3a1 knockdown cell lines. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability of (A) Nrf2-deleted KPCN line 1 and (B) stable Aldh3a1 knockdown cell line sh Aldh3a1 line 1 at 48 hours after gemcitabine (GEM; 0.01–10 μM) treatment, with or without HF pretreatment for 24 hours (5000 cells/well; N = 6, **P < 0.01 compared with control at the same concentration of GEM). The error bars show standard deviation.
Table 1. Commonly down-regulated genes in halofuginone treated KPC line 1 and 2

<table>
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<tr>
<th>Gene symbol</th>
<th>Fold change in KPC line 1</th>
<th>Description</th>
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<tr>
<td>Aldh3a1</td>
<td>0.14</td>
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<td>ATP-binding cassette, sub-family C (CFTR/MRP), member 5</td>
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</table>
A

Control

HF

B

![Bar graph showing the number of PanINs per HPF.](image)

Control

HF

C

HO-1 IHC

Control

HF

Matsumoto Fig.1
Figure A: Immunoblot analysis of Nrf2, Phospho-eIF2α, Total-eIF2α, and Tubulin in KPC line 1 and KPC line 2 treated with HF 100 nM for 0, 1, 2, and 3 hours.

Figure B: Graph showing relative cell viability (%) of KPC line 1 and KPC line 2 treated with GEM 10 μM and HF 100 nM for 0, 0.01, 0.1, 1, and 10 GEM (μM).

Figure C: Immunoblot analysis of Caspase-3, Cleaved caspase-3, and Tubulin in KPC line 1 and KPC line 2 treated with GEM 10 μM and HF 100 nM.
**Matsumoto Fig. 4**

**A**

![Bar graph showing the relative expression of Aldh3a1 in KPC line 1 and 2 with and without HF (100 nM).](image)

**B**

![Western blot analysis of Aldh3a1 and Tubulin in KPC line 1 and 2 with and without HF (100 nM).](image)

**C**

![Immunohistochemistry (IHC) images of Aldh3a1 staining in Control and HF 0.25 mg/kg 4 weeks groups.](image)
A

**Aldh3a1**

Relative expression (β-actin)

KPC line 1

KPCN line 1

0 0.03 0.06

DEM (μM)

0 100

B

KPC line 1  KPCN line 1

DEM (100 μM) - + - +

Aldh3a1

Tubulin

C

Aldh3a1 IHC

KPC mouse

KPCN mouse

Matsumoto Fig. 5
Matsumoto Fig. 7

A

KPCN line 1

B

sh Aldh3a1 line 1

Control

HF 100 nM

GEM (μM)

0

0.01

0.1

1

10

0

50

100

0

50

100

***

***

GEM (μM)