Nrf2 promotes mutant K-ras/p53-driven pancreatic carcinogenesis

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Abstract

The Keap1-Nrf2 system contributes to the maintenance of homeostasis by regulating oxidative stress responses in normal tissues and organs, and is exploited in various cancers for proliferation, survival and acquisition of therapy resistance. Pancreatic cancer remains one of the intractable cancers, despite the improved clinical outcomes of other types of cancer, due to its invasive and refractory nature to therapeutic intervention. The current study aimed to clarify the contribution of Nrf2 to pancreatic carcinogenesis using a pancreas-specific mutant K-ras and p53 (KPC) mouse model. Deletion of Nrf2 in KPC mice (KPCN) decreased the formation of precancerous lesions as well as the development of invasive pancreatic cancer. The pancreatic tumor-derived cancer cell lines from KPCN mouse showed decreased expression of glutathione S-transferases (GST), UDP glucuronosyltransferases (UGT) and ABC transporters. Along with these biochemical changes, cell lines from KPCN mice revealed increased sensitivity to oxidative stress and chemotherapeutic agent. The current study revealed that Nrf2 contributes to pancreatic carcinogenesis in a way distinct from the chemoresistance of lung and esophagus, and that Nrf2 could be a novel therapeutic target of pancreatic cancer.

Introduction

The Keap1-Nrf2 system plays pivotal roles in oxidative stress responses. Nrf2 activation leads to cytoprotective gene induction, cellular proliferation and survival (1). These mechanisms have been reported to protect normal organ functions by upregulating detoxifying enzymes and metabolic enzymes (2,3). In normal conditions, Nrf2 binds to a Cullin 3-based E3 ubiquitin ligase adaptor, Keap1, which promotes the degradation of Nrf2 through the ubiquitin-proteasome system. However, Nrf2 activation has also been reported in various types of cancers, yielding growth advantage and resistance against therapeutic agents (4–6). Nrf2 is activated by loss of function mutations of Keap1 or degradation-resistant mutations of Nrf2 itself (5,7). Besides these genetic mutation-based mechanisms, several upstream regulators have also been shown to activate Nrf2. For example, an autophagy-related adaptor protein p62 competitively inhibits the interaction between Keap1 and Nrf2, resulting in the induction of Nrf2-target genes (8). This machinery promotes metabolic reprogramming of hepatocellular carcinoma cells that enhances the malignant features (9). Oncogenic mutations including the constitutively active K-ras mutation, which is frequently observed in pancreatic cancer, also induces Nrf2 target genes and lowers the levels of reactive oxygen species (ROS) (10). Since these oncogenic mutations and impaired autophagy are common phenomena during carcinogenesis, aberrant Nrf2 activation is inevitably evoked in cancer.

Pancreatic cancer is a lethal cancer due to resistance against chemotherapeutic agents and high frequency of recurrence after surgery. The molecular basis of the vicious nature of pancreatic cancer has been extensively studied, but no radical therapeutic strategy has yet been established. Even though constitutionally active K-ras mutation is one of the most frequent mutations
in pancreatic cancer, pharmacological inhibition of its downstream signals fails to achieve clinical efficacy (11). Another cancer-promoting factor, cell-to-cell interaction between pancreatic cancer cells and stromal cells (12) has also been targeted in pancreatic cancer. However, recent studies have found that simple removal of pancreatic stellate cells (PSCs), a major origin of pancreatic fibrosis, results in the paradoxical promotion of pancreatic cancer by inducing undifferentiated phenotypes and the spreading of metastasis (13,14). On the other hand, stromal cell programming by vitamin D receptor stimulation has a therapeutic effect, suggesting that the complex roles of the pancreatic cancer stroma may provide a target for intervention (15).

Nrf2 activation in pancreatic cancer has been described, especially in response to chemotherapeutic agents. An assessment of the cell lines resistant to gemcitabine (GEM), a standard chemotherapeutic agent for pancreatic cancer, revealed enhanced stress response including Nrf2 (16). The expression of nuclear NRF2 in human pancreatic cancer specimens correlates with poor survival in patients, indicating that the Keap1-Nrf2 contributes to the progression of pancreatic cancer (17). The contribution of Nrf2 to pancreatic cancer has been assessed utilizing pancreatic cancer model mice with active mutant K-ras. The expression of Nqo1, a typical Nrf2 target gene, is increased in mutant K-ras driven precancerous lesions (10). In this study, Nrf2 deletion caused decreased K-ras-induced pancreatic intraepithelial neoplasm (PanIN) formation. Recent study also identified that Nrf2 contributes to pancreatic cancer maintenance by modulating mRNA translation, using organoid models (18).

However, the net effect of the oxidative stress response on pancreatic carcinogenesis has not yet been described in detail at macroscopic cancer-occurring stage using an invasive cancer-causing model like KPC mice, which expresses mutant K-ras at macroscopic cancer-occurring stage using an invasive cancer-pancreatic carcinogenesis has not yet been described in detail. Recent study also identified that Nrf2 contributes to the progression of pancreatic cancer (17). The contribution of Nrf2 to pancreatic cancer has been assessed utilizing pancreatic cancer model mice with active mutant K-ras. The expression of Nqo1, a typical Nrf2 target gene, is increased in mutant K-ras driven precancerous lesions (10). In this study, Nrf2 deletion caused decreased K-ras-induced pancreatic intraepithelial neoplasm (PanIN) formation. Recent study also identified that Nrf2 contributes to pancreatic cancer maintenance by modulating mRNA translation, using organoid models (18).

In the establishment of metastasis (19), we introduced the Nrf2-null background to KC mice to assess the frequency of PanIN and invasive cancer. The establishment of mouse cell lines from KPC- or KPC::Nrf2-/--derived pancreatic tumors enabled a comparison of the gene expression profiles, which revealed that Nrf2 globally regulates cytoprotective genes in pancreatic cancer. Our study indicates the possibility of Nrf2-targeting therapy for pancreatic cancer.

Materials and Methods

Mice

The Nrf2-/- mouse was described previously (17). Pancreas-specific Pdx-1-Cre transgenic mice, LSL-K-rasG12D mice and LSL-p53R172H mice were obtained from NCI mouse repository (Frederick, MD) (21-23). Mice were crossed to generate K-rasG12D:p53R172H double mutant (KPC), K-rasG12D-single mutant (KC), p53R172H single mutant (PC) and Pdx-1-Cre expressing (C) mice.

Materials

Gemcitabine (GEM) and diethyl maleate (DEM) were purchased from Wako (Osaka, Japan). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Dojindo (Kumamoto, Japan). Buthionine-(S,R)-sulfoximine (BSO) and anti-Tubulin antibody (75168) was purchased from Sigma-Aldrich. Anti-Histone H3 antibody (4499), anti-Keratin 17/19 antibody (3984), anti-E-cadherin antibody (14472), anti-cleaved caspase-3 antibody (9664), anti-Ki-67 antibody (12202) and peroxidase-conjugated anti-rabbit antibody (7074) were from Cell Signaling Technology (Beverly, MA). Anti-Nqo1 antibody (ab2346) and anti-8-hydroxy-2'-deoxyguanosine (8-OhdG) antibody (ab48508) were from Abcam (Cambridge, United Kingdom). Anti-Nrf2 antibody was described previously (24). Peroxidase-conjugated anti-mouse antibody was from GE Healthcare (Piscataway, NJ). Peroxidase-conjugated anti-goat antibody (sc-2020) was from Santa Cruz Biotechnology (Dallas, TX).

Immunohistochemistry

Pancreatic tissues from mice were fixed in 4% paraformaldehyde (Wako) and paraffin-embedded for hematoxylin and eosin (H&E) staining or immunostaining. For 8-OhdG, cleaved caspase-3 and Ki-67 immunostaining, slides were autoclaved for 10 min in citrate buffer pH 6.0 and incubated with the primary antibody overnight. For Nqo1 immunostaining, slides were autoclaved for 10 min in target retrieval solution (Dako) and incubated with the primary antibody overnight. The visualization of immunostaining was performed using Histofine MOUSESTAIN KIT (Nichirei Biosciences INC, Tokyo, Japan) or biotin-labeled anti-goat immunoglobulin (Dako) and peroxidase-conjugated streptavidin (abcam) and diaminobenzidine.

Establishment of mouse tumor-derived cell lines

Pancreatic cancer tissues from KPC or KPCN mice were cut into pieces and washed using Hank’s balanced salt solution. Tissues were incubated with 0.5 mg/ml collagenase P (Roche applied science, Mannheim, Germany) for 10 min, and the cell pellet was collected and seeded in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells showing epithelial morphology were selected (two lines from KPC or KPCN mice-derived tumors). Cells were kept at 37°C in a humidified incubator with 5% CO2.

Cell line authentication

KPC line1, KPC line2, KPCN line1 and KPCN line2 cell lines were established in between November and December 2015. For the confirmation of these cell lines from mouse origin, mouse-specific primer sets were used to perform quantitative reverse transcription PCR (qRT-PCR). This confirmation has been performed until September 2016.

Cell growth assay

The cellular proliferation of mouse tumor-derived cell lines was assessed by BrdU incorporation assay using cell proliferation
ELISA (Roche). Cells were seeded at 5000 cells/well in 96-well plates and incubated for 48 h before assay.

Western blot
Cells were lysed in RIPA buffer for total proteins. The nuclear fraction was prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA). Lysates were subjected to electrophoresis using NuPAGE 4–12% Bis-Tris Gel (Life Technologies) and transferred onto Immobilon-P Membrane (Merck Millipore, Billerica, MA). Membranes were incubated overnight at 4°C with the primary antibody. After incubation with peroxidase-conjugated antibody, reactive bands were detected using ECL™ Western blotting detection reagents (GE Healthcare, Buckinghamshire, England).

RNA extraction and qRT-PCR
Total RNA was extracted from cell lines using the RNeasy kit (Qiagen, Valencia, CA). One microgram of RNA was subjected to reverse transcription using SuperScript VIRO™ Master Mix (Thermo Fisher Scientific). The expression of each gene was quantified using StepOnePlus™ real-time PCR system (Thermo Fisher Scientific) and Fast SYBR Green Master Mix (Thermo Fisher Scientific) with primers as follows: β-actin (5′-GGCTGTATCCCTCCATCG-3′, 5′-CCAGTTGATCAATGCCATGT-3′ (25), Nrf2 (5′-CAAGACCTTG GCACTTTAAAGAC-3′, 5′-AGTAAGGCTTTCCATCCT-3′ (26), Nqo1 (5′-AGGCTCGGTATTAGCAGGC-3′, 5′-AGTACAACTACGGCTTCTCG-3′ (27), Gstm (5′-CTACCTGGCCGAAAGCAC-3′, 5′-ATGTCTGACGCGATCCTC-3′ (27).

Microarray
Total RNA samples from cell lines were subjected to microarray analysis in duplicate using whole mouse genome Oligo DNA Microarray ver2.0 (Agilent Technologies, Santa Clara, CA) and G2539A microarray scanner system (Agilent). Data acquisition was performed using GeneSpring GX software (Agilent).

Cell viability assay
Cells were seeded at 5000 cells/well in 96-well plates and incubated for 24 h, then treated at the indicated concentrations of chemical compounds for 48 h. Cell viability was measured by MTT assay. Cells were treated with 0.5 mg/ml of MTT solution for 2 h, and then solubilized in dimethyl sulfoxide. Optical density was measured by a spectrophotometer at a wavelength of 570 nm.

Quantification of ROS
For ROS quantification, cells were seeded at 10000 cells/well in 96-well plates and incubated for 24 h. Cells were washed and stained at 25 μM of 2',7'-dichlorofluorescin diacetate (DCFDA) for 1 h, followed by treatment with 50 μM of tert-butyl hydroperoxide (TBHP). Fluorescence measurement was performed 4 h later at 485 nm excitation and 535 nm emission.

Statistical analysis
Statistical analysis was performed using JMP software (12.2.0 SAS Institute Inc. Cary, NC). The differences between two groups were analyzed by Student's t-test. The differences between more than two groups were analyzed using the Tukey-Kramer method. P < 0.05 was regarded as statistically significant. The error bars indicate standard deviations.

Results

Introduction of Nrf2⁻/⁻ background to KPC mice attenuates precancerous lesion formation
A mouse model with K-ras and p53 mutations using Pdx-1-Cre (C) has been established as a useful tool for developing pancreatic cancer (19). We crossed mice harboring K-rasG12D::p53L85R/+ with mice harboring Pdx-1-Cre to generate Pdx-1-Cre (C), K-rasG12D:: p53L85R/+::Pdx-1-Cre (KC), and K-rasG12D::p53L85R/+::Pdx-1-Cre (KC) mice (Figure 1A). To examine the contribution of Nrf2 to pancreatic cancers with K-ras and/or p53 mutations, we then generated K-rasG12D::p53L85R/+::Pdx-1-Cre::Nrf2⁻/⁻ (KPN) mutant mice. KPN mice were crossed with Pdx-1-Cre::Nrf2⁻/⁻ (CN) mice to generate p53L85R/+::Pdx-1-Cre::Nrf2⁻/⁻ (PCN), K-rasG12D::Pdx-1-Cre::Nrf2⁻/⁻ (KC), and K-rasG12D::p53L85R/+::Pdx-1-Cre::Nrf2⁻/⁻ (KPCN) mice (Figure 1B).

We then followed up these mutant mice and, since most KPC mice were reported to die of pancreatic cancer within 12 months of age (19), we attempted to perform histological analyses of these mice at the age of 90 days. While C and PC mice showed no histological changes (data not shown), KC, KPN, KPC mice developed acinar ductal metaplasia and pancreatic intraepithelial neoplasia (PanIN) regardless of gender (Figure 2A). However, the total number of PanIN in KCN mice was significantly lower than that in KC (arrow heads in Figure 2A), and the total number of PanIN in KPCN mice was also much lower than that of KPC mice (Figure 2B). The infiltration of neutrophils around PanINs in KCN and KPCN mice were also significantly lower than those in KC and KPC mice, respectively (Supplementary Figure 1). Number of PanINs significantly increased in KPC mice than in KC mice, suggesting tumor-promoting role of mutant p53 as described previously (Figure 2B) (19). These results thus indicate that Nrf2 is necessary for K-ras mutant-driven PanIN formation.

Nrf2 activation suppresses oxidative stress in precancerous lesions of KPC mice
We then assessed the expression of Nqo1, a typical Nrf2-target gene product, by immunohistochemistry. Of note, PanIN lesions showed no histological changes (data not shown), KC, KCN, KPC mice developed acinar ductal metaplasia and pancreatic intraepithelial neoplasia (PanIN) regardless of gender (Figure 2A). However, the total number of PanIN in KCN mice was significantly lower than that in KC (arrow heads in Figure 2A), and the total number of PanIN in KPCN mice was also much lower than that of KPC mice (Figure 2B). The infiltration of neutrophils around PanINs in KCN and KPCN mice were also significantly lower than those in KC and KPC mice, respectively (Supplementary Figure 1). Number of PanINs significantly increased in KPC mice than in KC mice, suggesting tumor-promoting role of mutant p53 as described previously (Figure 2B) (19). These results thus indicate that Nrf2 is necessary for K-ras mutant-driven PanIN formation.

Loss of Nrf2 represses invasive growth of pancreatic cancer in KPC mice
By 90 days after birth, 4 of 26 KPC mice (15%) developed macroscopic pancreatic cancers accompanied by distant metastasis and invasion into surrounding organs, such as the liver, spleen and lymph nodes (Figure 3A). An additional 6 of the 26 KPC mice (23%) were found to harbor microscopically invasive cancers. Collectively, 10 KPC mice (38%) developed invasive pancreatic cancers. On the other hand, only 2 of 16 KPCN mice (12%)
developed invasive pancreatic cancers, and no organ metastasis was observed in the KPCN mice.

Tumors in the KPCN mice tended to be smaller compared to those in the KPC mice. The histology of the tumors was similar between the KPC and KPCN mice, showing moderately to poorly differentiated cancers (Figure 3B). However, the KPCN tumor cells showed increased 8-OHdG staining, suggesting an increase in the oxidative stress in or surrounding the KPCN tumors (Figure 3C). To identify whether tumor-size difference relays on decreased cell proliferation or increased cell death, we performed Ki-67 and cleaved caspase-3 staining. The KPC mouse tumor revealed increased staining for Ki-67, while cleaved caspase-3 staining were almost undetectable in both KPC and KPCN mice tumors (Figure 3D-I). Based on these findings, Nrf2 appeared to be involved in the progression to advanced pancreatic cancer from precursor lesions.

Establishment of pancreatic cancer-derived cell lines

To further assess the molecular mechanisms involved in the differential progression of pancreatic cancer between KPC and KPCN mice, we established mouse pancreatic cancer-derived cell lines. Two cell lines from KPC mouse tumors (KPC cells) and two cell lines from KPCN mouse tumors (KPCN cells) were established. These cell lines revealed an epithelial phenotype with cell-to-cell contact (Figure 4A). The cellular proliferation of KPCN cells was not reduced under the normal culture condition compared with the KPC cells (Figure 4B). These cells

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**Figure 1.** (A) Mating strategy for mouse model with K-ras and p53 mutations using Pdx-1-Cre. (B) mating strategy for mouse model with K-ras and p53 mutations using Pdx-1-Cre with Nrf2−/− background.
expressed epithelial markers such as E-cadherin and cytokeratin 17/19 (CK17/19) to a similar extent. The loss of expression of nuclear Nrf2 and its target Nqo1 were also confirmed in KPCN cells (Figure 4C). Cell lines from KPC or KPCN tumors were established that maintained their features in the presence or absence of Nrf2.

Figure 2. (A) H&E staining of PanIN lesions in KC, KPC, KCN and KPCN mice at the age of 90 days. Arrow head indicates PanIN foci. White bar shows 500 μm. Black bar shows 100 μm. (B) Quantification of total number of PanIN in pancreas of male and female mice. **P < 0.01 (N = 6–15). (C) Immunohistochemistry for Nqo1 in KC, KPC, KCN and KPCN mice at the age of 90 days. Arrow head indicates positive staining for Nqo1. Black bar shows 100 μm. (D) Immunohistochemistry for 8-OHdG in PanIN lesions in KC, KPC, KCN and KPCN mice at the age of 90 days. Arrow head indicates positive staining for 8-OHdG. Black bar shows 100 μm.

Nrf2-target gene expression in pancreatic cancer cells from KPC or KPCN mice using microarray analysis

The gene expression profiles of cell lines from KPC or KPCN tumors were examined by microarray analysis. Genes showing 0.5-fold or less expression were regarded as down-regulated.
Commonly (KPCN line 1 versus KPC line 1, KPCN line 1 versus KPC line 2, KPCN line 2 versus KPC line 1 and KPCN line 2 versus KPC line 2) down-regulated genes in KPCN-derived cell lines were elucidated as shown in Figure 4D. Among 476 down-regulated probes, multiple Nrf2-target genes were found (Table 1), including Nqo1, Gpx2, ABC transporters (Abcc2, Abcb6), GST family (Gsta1, Gsta2, GSTa3, Gsta4, Gstm1, Gstm3), UGT family (Ugt1a9, Ugt1a6b, Ugt2b5, Ugt2b34, Ugt2b36, Ugt2b37, Ugt2b38). In addition to these Nrf2-target genes, several solute carrier family genes such as Slc23a2 and Slc27a6 were down-regulated in KPCN lines. Among these genes, Nrf2, Nqo1 and Gstm were validated by qRT-PCR (Figure 4E). Cell lines from the KPCN tumors exhibited down-regulated gene expressions responsible for detoxification or antioxidation compared to cell lines from the KPC tumors.

Nrf2-null cancer cells were vulnerable to GEM treatment and oxidative stress
Since commonly down-regulated genes in KPCN-derived cell lines were indispensable for oxidative stress responses or...
detoxification such as glutathione S-transferase family, UDP-glucuronosyltransferase family and ABC transporters, we hypothesized that Nrf2-null cancer cells, KPCN cells, are vulnerable to oxidative stress and xenobiotics. Treatment of KPCN cells with diethyl maleate (DEM), a typical Nrf2 inducer, and GEM, an anti-cancer drug, resulted in significantly decreased cell viability compared to KPC cells (Figure 5A and B). Intracellular reactive oxygen species (ROS) level after tert-butyl hydrogen peroxide (TBHP) treatment were measured by DCFDA staining. The ROS level was increased in KPCN cells compared with KPC cells, suggesting a decreased ROS scavenging capacity of Nrf2-null cancer cells (Figure 5C). These data suggest that GEM exerts anti-cancer effects more strongly in Nrf2-null tumor cells and Nrf2 deletion leads to vulnerability to oxidative stress.

Vulnerability to GEM involves alternative pathway

To further address the mechanism that caused vulnerability to GEM in Nrf2-null cancer cells, we assessed ROS increase after GEM treatment in these cell lines. However, GEM treatment did not increase intracellular ROS in both KPC and KPCN-derived pancreatic cancer cell lines compared to KPC-derived pancreatic cancer cell lines (Figure 4).

Figure 4. (A) Cell morphology of KPC or KPCN mouse-derived cell lines. Black bar shows 100 μm. (B) Cellular proliferation by BrdU assay (N = 6). (C) Protein expressions of nuclear Nrf2, Nqo1 as an Nrf2 target gene product, and E-cadherin and CK17/19 as epithelial markers in KPC or KPCN mouse-derived cell lines by Western blotting. Histone H3 and tubulin are displayed as loading controls of nuclear fraction or whole cell lysate. (D) Venn diagram of commonly down-regulated genes in KPCN-derived pancreatic cancer cell lines compared to KPC-derived pancreatic cancer cell lines. (E) Quantification of down-regulated gene mRNA expression in KPC and KPCN-derived pancreatic cancer cell lines. ND, not detected. **P < 0.01 (N = 6).
mice-derived cell lines (data not shown). Co-administration of DEM, an oxidative stress inducer and Nrf2 inducer, with GEM did not show synergistic anti-cancer effect, even increased cell viability in KPC line 1 (Supplementary Figure 2A). We also tested BSO in combination with GEM to deplete glutathione, and this treatment also failed to show synergistic anti-cancer

Table 1. Genes commonly down-regulated in KPCN lines

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Fold change is average KPCN line 1, 2 versus average KPC line 1, 2.

Figure 5. Cell viability after treatment of DEM (A) or GEM (B) at indicated concentration for 48 hours by MTT assay. **P < 0.01 (N = 6). (C) Quantification of Intracellular ROS after TBHP treatment for 4 h by DCFDA staining. **P < 0.01 (N = 4).
effect (Supplementary Figure 2B). Together with the lack of ROS increase after GEM treatment in these cell lines, we surmise vulnerability to GEM in Nrf2-null cancer cells involves glutathione-independent pathway at least in part.

Discussion

The results of the current study suggest that Nrf2 plays pivotal roles during mutant K-ras and p53-driven pancreatic carcinogenesis. This is in agreement with previous studies that described K-ras induced Nrf2 activation plays promoting roles in the formation of precancerous lesion PanINs (10) and Nrf2-deleted pancreatic organoids from KPC mice show less proliferative properties (18). The Keap1-Nrf2 system has multifaceted roles depending on the cellular context. For example, the oxidative stress response via the Keap1-Nrf2 system in cancer cells contributes to the resistance against chemotherapeutic agents or increased proliferation (7,16). In contrast, myeloid lineage-specific deletion of Nrf2 leads to increased ROS production from myeloid-derived suppressor cells (MDSCs) that create an immunosuppressive microenvironment allowing metastatic colonization (28). Similar observations have also been reported in a lung cancer model, in which the Nrf2 level elevated by Keap1 knockdown prevented urethane-induced lung carcinogenesis (29). This study also revealed increased tumorigenicity in Keap1 knockdown lung cancer cells, suggesting a tumor-promoting role of Nrf2.

From this point of view, the introduction of the systemic Nrf2-null background into KPC mouse resulted in a decreased frequency of precancerous lesions and invasive cancer development, indicating potential immunosuppressive changes did not significantly assist pancreatic carcinogenesis. In addition, cancer cells isolated from KPCN mice revealed a growth ratio similar to those of the KPC mice-derived cell lines in vitro, suggesting that the growth advantage of Nrf2 is limited under an unstressed condition. Similar intracellular ROS amount in KPC and KPCN mice-derived cell lines at normal culture condition is an additional evidence indicating normal 2D culture has less oxidative stress. Since Ki-67 staining was less and 8-OHdG staining increased in KPCN mouse tumor, we surmise cancer cells are under more oxidative stress in vivo, which might result in the current discrepancy. Considering the increased sensitivity to increased ROS levels after the treatment with an oxidative stress inducer, the attenuation of pancreatic carcinogenesis by Nrf2 deletion could be attributed to the vulnerability of cancer cells to specific conditions. Furthermore, sensitization to GEM treatment in Nrf2-null cancer cells raises additional roles of Nrf2. Lack of ROS increase by GEM and no synergistic effect with BSO suggest that resistance to GEM by Nrf2 might involve alternative mechanism. Since several soluble factor family genes’ expression were decreased in KPCN lines, their contribution to gemcitabine resistance need further validation. Interestingly, recent organoid study showed that pancreatic organoid revealed similar sensitivity to GEM regardless of the Nrf2 status (18). Compared with this result, current KPC-derived cell lines might gain intrinsic resistance to GEM, depending on Nrf2. These cell lines would be useful to identify acquired malignant phenotype during pancreatic carcinogenesis.

The reduced stromal reaction also suggests the involvement of the Keap1-Nrf2 system in the cancer cell-stromal cell interaction. Administration of L-cysteine as an antioxidant attenuated pancreatic fibrosis in a chronic pancreatitis model rat induced by trinitrobenzene sulfonic acid (30). A recent report described that soluble factors activate Nrf2 in pancreatic cancer cells, leading to ROS detoxification (31). Since stromal Nrf2 was also deleted in the current KPC mouse model, Nrf2 may activate pancreatic stellate cells (PSCs) directly or indirectly through unknown activating factors. To address this issue, a comparison between pancreas-specific Nrf2 deletion or PSC-specific Nrf2 deletion will be the next approach.

In conclusion, Nrf2 contributes substantially to pancreatic carcinogenesis. Deletion of Nrf2 sensitizes cancer cells to oxidative stress and a chemotherapeutic agent, GEM. An Nrf2 inhibitor could be applied to improve the response to current standard therapies. In addition, systemic Nrf2 deletion does not paradoxically increase metastatic spread and frequency of undifferentiated tumors like simple deletion of PSCs (13,14), indicating the potential safety of Nrf2-targeting therapy. To achieve novel Nrf2-targeting therapy, a specific Nrf2 inhibitor suitable for administration is required. Brusatol has been reported to inhibit Nrf2 activity, but a recent report described that brusatol globally functions as a protein synthesis inhibitor (32). The results of the present study suggest that the cytotoxicity of brusatol is independent of Nrf2, indicating limitation for clinical application. Novel candidates for specific Nrf2 inhibition need to be explored by extensive study in order to establish a Nrf2-targeting therapy for cancer.

Supplementary Material

Supplementary data are available at Carcinogenesis online.

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Conflict of Interest Statement: None declared.

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