Pancreas

The roles of interferon regulatory factor (IRF) 1 and IRF2 in the progression of human pancreatic cancer.

--Manuscript Draft--

Manuscript Number:	PANCREAS 13362R1
Full Title:	The roles of interferon regulatory factor (IRF) 1 and IRF2 in the progression of human pancreatic cancer.
Short Title:	IRF1 and IRF2 in human pancreatic cancer
Article Type:	Full Manuscript
Keywords:	interferon regulatory factor (IRF); IRF1; IRF2; pancreatic cancer; tumor progression
Corresponding Author:	Hirosato Mashima, MD, PhD Akita University Graduate School of Medicine Akita, JAPAN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Akita University Graduate School of Medicine
Corresponding Author's Secondary Institution:	
First Author:	Toshitaka Sakai, MD
First Author Secondary Information:	
Order of Authors:	Toshitaka Sakai, MD
	Hirosato Mashima, MD, PhD
	Yumi Yamada, MD
	Takashi Goto, M.D., PhD
	Wataru Sato, M.D., PhD
	Takahiro Dohmen, M.D., PhD
	Kentaro Kamada, M.D., PhD
	Masato Yoshioka, M.D., PhD
	Hiroshi Uchinami, M.D., PhD
	Yuzo Yamamoto, M.D., PhD
	Hirohide Ohnishi, M.D., PhD
Order of Authors Secondary Information:	
Manuscript Region of Origin:	JAPAN
Abstract:	Objectives: Pancreatic cancer is one of the most malignant diseases worldwide. Interferon regulatory factor (IRF) 1 and IRF2 function as a tumor suppressor and oncoprotein, respectively, in several types of cancers. We investigated whether IRF1 and IRF2 are involved in the progression of pancreatic cancer. Methods: We examined the expressions of IRF1 and IRF2 in pancreatic cancer specimens and analyzed the association with clinicopathological features. We evaluated the biological effects of IRF1 and IRF2 using a pancreatic cancer cell line. Results: The expression levels of IRF1 and IRF2 were decreased and increased, respectively, in the pancreatic cancer cells compared to those observed in the paired normal areas. A higher expression of IRF1 was associated with better features of tumor differentiation, infiltration depth, tumor size and survival, while that of IRF2 was associated with a worse feature of tumor infiltration depth. IRF2-overexpressing PANC-1 cells exhibited an increase in cell growth, less apoptotic features and chemoresistance to gemcitabine

	treatment. In contrast, IRF1-overexpressing cells exhibited the opposite characteristics. Conclusions: IRF1 and IRF2 may regulate the progression of pancreatic cancer by functioning as an anti-oncoprotein and oncoprotein, respectively. These molecules may serve as potential targets of therapy.

Dear the Editor,

We would like to submit our revised manuscript (Pancreas 13362) entitled "The roles of interferon regulatory factor (IRF) 1 and IRF2 in the progression of human pancreatic cancer." to be considered for publication in ***Pancreas***.

According to the valuable comments of editors and reviewers, we revised our manuscript intensively. The point-by-point responses to the comments are provided as follows and in the section of answers to the comments of reviewer. We highlighted all significant changes to the manuscript in red.

Your kind consideration of this paper would be greatly appreciated. This study was reviewed and approved by the ethics committee of Akita University Faculty of Medicine. These data have not been reported elsewhere and all of the authors took part in the work and agreed with the contents of the manuscripts. We have no financial interests. We hope that you will find this revised manuscript suitable for publication. Thank you in advance for your considerations.

Sincerely yours,
Hirosato Mashima, MD., PhD.

Answers to the Editorial Comments

1. We added a Conflict of Interest Disclosure section on the title page.
2. We repeated the immunostaining experiments and changed some of the images in Figure 1A and Supplementary Figure 1. We recreated the figures in Figure 1B & 1C in 300 dpi resolution. We also have them in 1200 dpi resolution but the file size became too large (more than 500MB). Then, we uploaded Figure 1 in 300 dpi resolution. If we need to upload the file in 1200 dpi, please let us know. We have noticed that the figures converted to pdf file in the manuscript system became somehow blurry. So, please check the figures as high resolution images.
3. We have recreated Table 1 using Word software.
4. Each of the authors signed the copyright transfer agreement form and we uploaded all of them.

Answers to the comments of the reviewer

Thank you very much for the valuable comments to our manuscript. According to your kind suggestions, we intensively revised our manuscript and cited the papers you listed. We highlighted all significant changes to the manuscript in red. We also had it reviewed by a native English speaker.

1. We described the antitumor activity of type I IFNs in pancreatic cancer briefly in “Introduction” and cite the papers. (Page4, L10 – Page4, L13)
2. We added the potential clinical application and future perspectives arising from our results in the “Discussion” section. We underscored that our strategy (IRF1↑, IRF2↓) could potentiate the antitumor activity of type I IFNs and may lead to the reduction of effective doses and the decrease of the rate and degree of side effects. (Page21, L13 – Page22, L7)
3. Type I IFN signals activate not only STAT-1 and STAT-2, but also STAT-3. Considering the application of type I IFN signals to the treatment of cancer, we have to overcome the survival pathways of cancer. As kindly pointed out, STAT-3 may play a crucial role in this context through the modulation of IRF1 and IRF2. This is a very interesting hypothesis and we have to continue and extend our studies. We added the description at this point in “Discussion”. (Page22, L7 – Page23, L10)
4. We have recreated the figures in Figure 1B and 1C to increase the quality. We also repeated the immunohistochemical staining experiments on some samples in Figure 1A and Supplementary Figure 1. First, we prepared Figure 1 in 1200 dpi resolution but the file size became too large (more than 500MB). Then, we attached Figure 1 in 300 dpi resolution. We believe that this version is also clear enough. If we need to upload the file in 1200 dpi format, please let us know.

The roles of interferon regulatory factor (IRF) 1 and IRF2 in the progression of human pancreatic cancer

Toshitaka Sakai, MD[1], Hirosato Mashima, MD, PhD[1], Yumi Yamada, MD[1], Takashi Goto, MD, PhD [1], Wataru Sato, MD, PhD [1], Takahiro Dohmen, MD, PhD [1], Kentaro Kamada, MD, PhD [1], Masato Yoshioka, MD, PhD [2], Hiroshi Uchinami, MD, PhD [2], Yuzo Yamamoto, MD, PhD [2], and Hirohide Ohnishi, MD, PhD [1].

[1]Department of Gastroenterology and [2]Department of Gastroenterological Surgery, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

Running Title: IRF1 and IRF2 in human pancreatic cancer

Disclosure: The authors declare no conflict of interest.

Address correspondence and reprint requests to:

Hirosato Mashima, MD, PhD.

Department of Gastroenterology, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan.

Tel: +81-18-884-6104, Fax: +81-18-836-2611,

E-mail: hmashima1-tky@umin.ac.jp

Abstract

Objectives: Pancreatic cancer is one of the most malignant diseases worldwide. Interferon regulatory factor (IRF) 1 and IRF2 function as a tumor suppressor and oncoprotein, respectively, in several types of cancers. We investigated whether IRF1 and IRF2 are involved in the progression of pancreatic cancer. **Methods**: We examined the expressions of IRF1 and IRF2 in pancreatic cancer specimens and analyzed the association with clinicopathological features. We evaluated the biological effects of IRF1 and IRF2 using a pancreatic cancer cell line. **Results**: The expression levels of IRF1 and IRF2 were decreased and increased, respectively, in the pancreatic cancer cells compared to those observed in the paired normal areas. A higher expression of IRF1 was associated with better features of tumor differentiation, infiltration depth, tumor size and survival, while that of IRF2 was associated with a worse feature of tumor infiltration depth. IRF2-overexpressing PANC-1 cells exhibited an increase in cell growth, less apoptotic features and chemoresistance to gemcitabine treatment. In contrast, IRF1-overexpressing cells exhibited the opposite characteristics. **Conclusions**: IRF1 and IRF2 may regulate the progression of pancreatic

cancer by functioning as an anti-oncoprotein and oncoprotein, respectively. These molecules may serve as potential targets of therapy.

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States and the fifth in Japan [1]. Worldwide, pancreatic cancer is 13th in overall incidence and 8th in the number of cancer deaths [2]. Despite the considerable progress in diagnosis and treatment of pancreatic cancer over the past few decades, the disease remains fatal, with a mean survival time of less than six months [3]. The poor prognosis of pancreatic cancer is primarily due to a locally advanced or metastatic stage at diagnosis, which precludes a curative resection, resulting in a lack of effective treatment options [3]. Gemcitabine (Gem) has been used as a standard treatment for advanced pancreatic cancer over the past decade and remains a key drug [4]. However, intrinsic and acquired resistance to gemcitabine is often encountered clinically. Therefore, there is an urgent need to develop novel effective therapeutic approaches.

Pancreatic cancer primarily originates from ductal epithelial cells. The progression of pancreatic cancer is attributed to dysregulation of multiple

genes, including oncogenes (KRAS) and tumor suppressor genes (p53, DPC4/SMAD4, p16/INK4A)[5-7]. Recent advances in clarifying the underlying molecular mechanisms of pancreatic cancer have permitted the development of new agents that target components of specific pathways [8]. Erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor, is a drug that has demonstrated modest survival benefits in combination with gemcitabine [9]. But the effect is not satisfactory.

Interferons (IFNs) comprise a family of multifunctional cytokines that mediate cellular resistance against viral infection and orchestrate numerous biological and cellular processes [10]. Moreover, IFN-α has been widely used in the treatment of several neoplasms,[11] and type I IFNs (IFN-α, IFN-β) have been shown to have antitumor activity against pancreatic cancer in both *in vitro* and *in vivo* studies [12-14]. IFN regulatory factors (IRFs) are DNA-binding proteins, nine members (IRF1-9) of which are present in humans [10]. These IRF molecules play pivotal roles in antiviral defense, immune response and cell growth by regulating the expressions of type I IFNs and IFN-inducible genes as well as the induction of cytokines and chemokines [10].

IRF1 was initially characterized as a transcriptional activator. Although

IRF2 is considered to be a transcriptional repressor and to act as an antagonist to IRF1, IRF2 is also known to act as a positive regulator of some genes [15]. Accumulating evidence also shows that IRF1 and IRF2 play important roles in the regulation of cell growth and possess anti-oncogenic and oncogenic potential, respectively[16-18]. NIH3T3 cells undergo transformation in response to IRF2 overexpression and this transformation is reversed by the overexpression of IRF1 [16]. IRF1 maps to chromosome 5q31.1, a genomic region frequently affected by cytogenetic abnormalities. A number of clinical studies have shown a correlation between the loss of IRF1 expression or function and human malignancies, including myelodysplastic syndrome, leukemia, esophageal cancer, gastric cancer and breast cancer [19]. Downregulated expression of IRF1 and upregulated expression of IRF2 have been reported to be correlated with malignant phenotypes in human melanoma and breast cancer cells[20,21]. Relative amounts of IRF1 to IRF2 have been shown to be important for the development and progression of esophageal cancer and leukemia, and a reduction in the IRF1/IRF2 ratio may be a determining factor of tumorigenicity [22,23]. An overexpression of IRF1 enhances chemosensitivity

to 5-fluorouracil in gastric cancer cells [24]. Meanwhile, the oncosuppressive role of IRF2 was recently reported in patients with hepatitis B virus-related hepatocellular carcinoma evaluated with whole-exome sequencing [25]. Functional analyses of hepatoma cells have demonstrated a tumor suppressing property of IRF2 and a function of this molecule as a regulator of p53 pathway [25]. Therefore, the functions of IRF1 and IRF2 may be cell type- and context-dependent.

An elevated expression of IRF2 has been reported in pancreatic cancer patients, and IRF2 has been shown to promote the growth of pancreatic cancer cells [26]. We recently demonstrated that IRF2 is involved in the pathogenesis of acute pancreatitis [27] and pancreatitis is a risk factor for the development of pancreatic cancer [2]. In this study, we evaluated and analyzed the clinical features of the expressions of IRF1 and IRF2 in pancreatic cancer specimens as well as paired normal areas of the pancreas. We also manipulated the expression levels of IRF1 and IRF2 in a pancreatic cancer cell line and assessed the biological responses.

Materials and Methods

Materials

Rabbit polyclonal anti-human IRF1 (for immunohistochemistry (IHC)), mouse monoclonal anti-human IRF2 (for IHC) and rabbit monoclonal anti-human cyclin D1 antibodies (Abs) were purchased from Abcam (CB, UK). Rabbit monoclonal anti-human IRF1 Ab (for Western blotting (WB)) was purchased from Cell Signaling (MA, USA). Mouse monoclonal anti-human IRF2 (for WB) and goat polyclonal anti-human actin Abs were obtained from Santa Cruz Biotechnology (CA, USA). Mouse monoclonal anti-rat PCNA and rabbit polyclonal anti-human Bax Abs were obtained from DAKO (Glostrup, Denmark). Horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG and HRP-conjugated donkey anti-goat IgG were purchased from Jackson Immuno Research (West Grove, PA).

Human pancreatic cancer tissues

Consecutive pancreatic cancer tissues, which were macroscopically successfully resected at Akita University Hospital between 2003 and 2011, were evaluated in this study. The accompanying normal areas of the pancreas that were at least 3 cm away from the margin of the cancer were

also used. The clinical information of the patients is summarized in Table 1. None of the patients received any neoadjuvant therapy. This study was reviewed and approved by the ethics committee of Akita University Faculty of Medicine. All patients gave their written consent for the use of their tissue specimens.

Immunohistochemistry and grading system of the expressions of IRF1 and IRF2

Following deparaffinization, heat-induced epitope retrieval and quenching the endogenous peroxidase, the samples were immunostained sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), anti-IRF1 Ab (1:225) or anti-IRF2 Ab (1:200) and secondary Abs. Specific immunostaining was developed with 3,3'-diaminobenzidine tetrahydrochloride substrate (DAKO). All sections were counterstained with hematoxylin. As a negative control, the primary Ab was replaced with a species-specific IgG isotype control at the same concentration (DAKO).

The immunohistochemical evaluation of the expressions of IRF1 and IRF2 was performed independently by two gastroenterologists who were instructed by a pathologist and were blinded to the patients' clinical

information. The scoring system was developed as follows: cells bearing obvious brown signals compared to the negative controls were considered to be positive, and the percentage of positively-stained tumor cells was graded as 0 (none), 1 (1–33% of the total number of tumor cells), 2 (34–66%) or 3 (67–100%).

Cell culture

The human pancreatic cancer cell line PANC-1 (RBRC-RCB2095) was purchased from RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humidified environment of 95% air and 5% CO_2.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was obtained from PANC-1 cells using an RNeasy Mini kit (QIAGEN, Valencia, CA). First-strand complementary DNA was synthesized from total RNA using the Superscript™ First-stranded Synthesis System for Reverse-Transcription Polymerase Chain Reaction (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PCR primers used in the study were as follows: human IRF1 (GenBank Accession No.

NM_002198), 5'-GCCTTAAGAACCCGGCAACCT-3' (sense), 5'-GGGTCTCATGCGCATCCGAG-3' (antisense); human IRF2 (GenBank Accession No. NM_002199), 5'-CTGAGAGCGACGAGCAGCGG-3' (sense), 5'-GTCTCCCGGTCTGGCCGACT-3' (antisense).

Construction of retroviruses expressing IRF1, IRF2 and a dominant-negative IRF2 mutant

The construction of retroviruses expressing IRF2 and dominant-negative IRF2 has been previously described elsewhere[27]. A retrovirus expressing IRF1 was constructed in a similar manner. The human full-length IRF1 was cloned using PCR with total RNA obtained from the human colon cancer cell line CaCO2 (RBRC-RCB0988) as a template. The primers used were as follows: 5'-CTCGAGCCAACATGCCCATCACTCGG-3' (sense) and 5'-GCGGCCGCTACTACGGTGCACAGGGAATGGC-3' (antisense). The PCR products were digested with XhoI and NotI restriction enzymes and inserted into the XhoI/NotI sites of the pMXs-IRES-EGFP vector, a generous gift from Dr. Tetsuya Nosaki at the University of Tokyo. The whole nucleotide sequences of the constructs were confirmed using sequencing.

Construction of IRF1-, IRF2- and dominant-negative IRF2-overexpressing

PANC-1 cells

The expression vectors (pMXs-IRF1-IRES-EGFP, pMXs-IRF2-IRES-EGFP, pMXs-dominant-negative IRF2-IRES-EGFP) and the mock vector (pMXs-IRES-EGFP) used as a negative control were transfected with FuGENE 6 Transfection Reagent (Roche Applied Science, Basel, Switzerland) into PLAT-E cells to obtain the viruses. The PANC-1 cells were infected with the viruses, and the cells expressing EGFP were sorted into the medium using FACS Vantage (Beckton Dickinson, NJ, USA) 48–72 hours after infection. The cells were named PANC-1/IRF1, PANC-1/IRF2, PANC-1/dnIRF2 and PANC-1/cont cells, respectively.

Measurement of cell growth and DNA synthesis

To measure cell growth, the cells were seeded at a density of 2 x 10^4 cells/ml in plastic 12-well plates and cultured. After 48, 96 and 144 hours, the cells were detached using incubation of 0.05% trypsin/EDTA, and the number of cells was counted using a Cell Counter Plate (Watson, Kobe, Japan).

To evaluate DNA synthesis, PANC-1 cells were seeded at a density of 1 x 10^4 cells/ml in 96-well culture plates and cultured. Following serum starvation for 24 hours, the cells were cultured for an additional 48 hours. BrdU was

added for the last two hours of incubation. The DNA synthesis was evaluated using a BrdU incorporation assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Western blotting

Briefly, 10–20 μg of protein was loaded on each lane of 5–15% sodium dodecyl sulphate-polyacrylamide gels and run at 200 V. The proteins were then transferred onto nitrocellulose membranes at 60 V for four hours. The membranes were incubated sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), primary Abs (cyclin D1 (1:200), PCNA (1:200), Bax (1:200), actin (1:1000)) and secondary Abs, and then proteins were detected using an enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ) to visualize the secondary Abs.

TUNEL assay

PANC-1 cells were seeded at a density of 1 x 10^5 cells/ml onto BD Falcon™ culture slides and cultured for 24 hours. Following serum starvation for 48 hours, apoptotic cells were detected using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) according to the

manufacturer's instructions. The nuclei were counterstained with DAPI. The number of positive cells in five fields was counted at x200 magnification under an Olympus IX70 fluorescence microscope. The apoptotic index (AI) was calculated as follows: (the number of apoptotic cells)/(total number of the cells) x 100 (%).

Analysis of drug sensitivity

Chemosensitivity to gemcitabine (Gem) treatment was analyzed using an MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Cell Growth Assay Kit (Millipore, MA, USA) according to the manufacturer's instructions. PANC-1 cells were seeded at a density of $1x10^4$ cells/ml in 96-well culture plates and cultured overnight. Next, the cells were serum starved and exposed to Gem (Sigma Aldrich, MO, USA) at a concentration ranging from 0 to 100 μM. The cells were cultured for 48 hours. A total of 10 μl of MTT was added for the last four hours of incubation. The absorbance at 570 nm was measured using a multiwell plate reader.

Statistical analysis

The relationships between the expressions of IRF1 and IRF2 and the clinicopathologic features were explored with Spearman's rank correlation

coefficients using the statcel3 program for Windows. Univariate survival analysis was performed by using the Kaplan-Meier method and then analyzed by the log-rank test. In the experiments using PANC-1 cells, all of the data were presented as the mean ± standard deviation (SD), and the statistical significance of the values obtained was evaluated using Student's *t* test. A value of $p < 0.05$ was considered to be significant.

Results

Changes in the expressions of IRF1 and IRF2 in human pancreatic cancer and clinicopathological features.

Surgically resected human pancreatic cancer specimens obtained from 45 patients (male: 24, female: 21; age 42–89 (68.1 ± 10.2)) were analyzed for the expressions of IRF1 and IRF2 using immunohistochemistry. Immunoreactivity was recorded on a scale of 0–3 according to the grading system described in the Materials and Methods (Figure 1, Supplementary Figure 1). Representative images of higher magnification showed that staining of IRF1 was primarily concentrated in the nuclei in the normal areas of the pancreas. In the cancerous counterparts, however, the

intensity of IRF1 was much decreased in the nuclei. In contrast to IRF1, IRF2 was faintly stained in the nuclei of the normal areas of the pancreas and was densely stained in the nuclei of the corresponding pancreatic cancer tissues. IRF2 was highly expressed in all of the tumors examined. When we compared the expressions between the cancerous regions and the matched normal areas of the pancreas, the expression of IRF1 was higher in the normal areas ($p < 0.001$), whereas that of IRF2 was higher in the cancerous regions ($p < 0.001$) (Figure 1B, Table 1). These results suggested that the scores of IRF1 and IRF2 shifted from high to low and from low to high, respectively, in association with the tumor progression.

We next examined the expression levels of IRF1 and IRF2 according to the clinicopathological features. As shown in Table 1, a higher expression of IRF1 was associated with a higher grade of differentiation ($p = 0.037$), a less infiltration depth ($p = 0.027$) and a smaller tumor size ($p = 0.001$). In contrast, a higher expression of IRF2 was associated with a greater degree of tumor infiltration ($p = 0.022$). Gender, ages, metastasis to other organs and/or lymph node, and tumor-node-metastasis (TNM) stage showed no correlation with the expressions of IRF1 and IRF2.

The median overall survival (OS) of the patients was 27.6 months. A Kaplan-Meier univariate analysis using the log-rank test revealed that the patients with a higher expression of IRF1 had a significantly increased median OS compared to the patients with a lower expression of IRF1 (Figure 1C left panel: score 2-3, median OS: 51.2 months; score 0-1, median OS: 21.9 months, $p = 0.046$). The patients with a higher expression of IRF2 exhibited a slightly reduced median OS compared to the patients with a lower expression of IRF2, although there was no statistical significance (Figure 1C right panel: score 3, median OS: 22.5 months; score 0-2, median OS: 33.7 months, $p = 0.348$).

Effects of IRF1 and IRF2 on the proliferation of pancreatic cancer cells

To investigate the functional roles of IRF1 and IRF2 in pancreatic cancer cells, we used a human pancreatic cancer cell line, PANC-1, in which we manipulated the IRF1 and IRF2 expression using a retroviral system. More than 90% of the cells in the PANC-1/cont, PANC-1/dnIRF2 and PANC-1/IRF1 lines expressed EGFP and approximately 70% of the cells in the PANC-1/IRF2 line expressed EGFP (data not shown). We confirmed the overexpression of IRF1 in the PANC-1/IRF1 line and that of IRF2 in the

PANC-1/IRF2 line using Western blotting (Figure 2A). Interestingly, the expression of IRF1 was lower in the PANC-1/IRF2 cells. The overexpression of the dominant-negative form of IRF2 was confirmed using semiquantitative competitive RT-PCR, as previously described (data not shown)[27].

The effects of IRF1 and IRF2 on cellular proliferation were studied by measuring the number of cells and the degree of BrdU incorporation. As shown in Figure 2B, the overexpression of IRF2 resulted in a marked increase in cell growth, while that of IRF1 resulted in a decrease. Similar results were obtained in the BrdU incorporation assay (Figure 2C). These results suggest that IRF2 enhances, while IRF1 inhibits, the proliferation of pancreatic cancer cells, consistent with the findings in our clinical samples showing that IRF2 is positively and IRF1 is negatively associated with pancreatic cancer progression.

Effects of IRF1 and IRF2 on apoptosis

We examined the population of apoptotic cells under serum deprivation for 48 hours using a TUNEL assay (Figure 2D). The number of apoptotic nuclei was counted and the apoptotic index (AI) was measured. The mean AI was

increased up to 45.9% in the PANC-1/IRF1 cell line and decreased to 16.3% in the PANC-1/IRF2 cell line. In contrast, it was 30.4% in the PANC-1/cont cell line and 30.8% in the PANC-1/dnIRF2 cell line. Apoptosis was significantly affected by the expressions of IRF1 and IRF2.

Effects of IRF1 and IRF2 on the expressions of genes related to proliferation and apoptosis

IRF2 has been reported to regulate the expressions of several genes implicated in cell proliferation and apoptosis in human esophageal cancer [23]. Therefore, we examined the expressions of proliferation-related genes (cyclin D1, proliferation cell nuclear antigen (PCNA)) and a pro-apoptotic gene (BAX). As shown in Figure 2E, the overexpression of IRF2 upregulated the expressions of cyclin D1 and PCNA. In contrast, the overexpression of IRF1 downregulated the expressions of these genes and upregulated the expression of BAX.

Effects of the IRF1 and IRF2 expression on chemosensitivity to gemcitabine

PANC-1 cells were exposed to various concentrations of Gem and the drug sensitivity was assessed using an MTT assay. As shown in Figure 2F, the PANC-1/IRF2 cells were resistant to Gem treatment, while the

responsiveness to Gem increased in the PANC-1/IRF1 and PANC-1/dnIRF2 cells in a dose-dependent manner. Cell viability was significantly decreased at 0.1 and 1 μM of Gem treatment in these cells. These results suggest that IRF2 works as a resistant factor of chemosensitivity, while IRF1 works as a promoting factor of chemosensitivity, in pancreatic cancer cells.

Discussion

Pancreatic cancer has a high propensity for local invasion and distal metastases. Despite recent advances in clarifying the molecular abnormalities observed in pancreatic cancer, the incidence rate is approximately the same as mortality rate and the survival has not improved dramatically. Therefore, identifying additional molecular mechanisms underlying the pathogenesis of pancreatic cancer and applying a better therapeutic strategy to the treatment are extremely important.

We herein reported that the IRF1 and IRF2 expressions are associated with the progression of pancreatic cancer. Immunohistochemical analysis showed a reduction in the IRF1 expression and an elevation in the IRF2 expression in the pancreatic cancer tissues compared to the paired normal

areas of pancreas. A higher expression of IRF1 was associated with better features of tumor differentiation, infiltration depth, tumor size and survival, while that of IRF2 was associated with a worse feature of tumor infiltration depth (Table 1, Figure 1). The reciprocal expression of IRF1 and IRF2 (IRF1↓, IRF2↑) was shown to contribute to the progression of pancreatic cancer as previously reported in melanoma, leukemia, breast cancer and esophageal cancer, but not in hepatoma [20-23,25]. Cui L et al. recently reported that IRF2 was upregulated in pancreatic cancer samples and associated with tumor size, differentiation, TNM stage, and survival of the patients [26]. For a better understanding, we have to add more patients for the analysis and enlarge the field to pre-cancerous lesions, such as pancreatic intraepithelial neoplasia (PanIN).

In the current study, we used an overexpression system of IRF1 and IRF2 and a dominant-negative form of IRF2 to examine biological responses in pancreatic cancer cells *in vitro*, while Cui L et al. used an siRNA knockdown system of IRF2 [26]. We demonstrated that upregulation of IRF2 increased cell proliferation, inhibited apoptosis and induced chemoresistance to Gem treatment. In contrast, upregulation of IRF1 decreased proliferation,

induced apoptosis and increased chemosensitivity (Figure 2). These results suggest that IRF1 and IRF2 play important roles in pancreatic cancer as an anti-oncoprotein and oncoprotein, respectively, in accordance with our findings in clinical pancreatic cancers (Table1, Figure 1).

When we blocked the effects of IRF2 using a dominant-negative form of IRF2 (PANC-1/dnIRF2), the rate of proliferation and apoptosis was nearly equivalent to that observed in the control cells (PANC-1/cont), while chemosensitivity was increased to the level in the IRF1-overexpressing cells (PANC-1/IRF1). Considering that the expression of IRF1 was the almost same level in the PANC-1/cont and PANC-1/dnIRF2 cells (Figure 2A), these findings imply that chemosensitivity may primarily depend on the expression of IRF2.

Taken together, these findings indicate that upregulating the expression of IRF1 and downregulating the expression of IRF2 (IRF1↑, IRF2↓) in pancreatic cancer cells could be a potential therapeutic strategy. This may also strengthen the anti-tumor activity of type I IFNs. IFN-α has been used in the treatment of several neoplasms [11]. IFN-β binds to the same receptor system as IFN-α with high affinity (approximately 10-fold) [11]. While high

doses of IFNs, which produce beneficial anti-tumor effects, may inevitably produce dose-dependent systemic side effects, these are intolerable for the patients and cause the IFN therapy to fail. The systemic administration of low doses of IFNs is ineffective. Therefore, the present strategy (IRF1↑, IRF2↓) could potentiate the type I IFN signals, leading to a reduction of the dose needed and the rate and the degree of side effects. It may also strengthen the effects of chemotherapy. However, the main limitation of IFN-α for cancer therapy has been reported to be the occurrence of tumor resistance mediated by the alteration of type I IFN signaling pathways and the activation of survival pathways[28]. Type I IFNs classically lead to the phosphorylation of signal transducer and activator of transcription (STAT)-1 and STAT-2 proteins, inducing apoptosis and cell cycle arrest in cancer cells. It has been described that STAT-3 is also activated by type I IFNs and plays a crucial role to counteract the anti-tumor activity of type I IFNs. STAT-3 modifies the biological responses in pancreatic cancer cells through direct and/or indirect modulation of the phosphatidylinositol 3 kinase (PI3K) pathway, NF-κB activation and epidermal growth factor receptor (EGF-R)/Ras/Raf/mitogen activated protein kinase (MAPK) pathway,

resulting in the survival of cancer cells [29,30]. One of the downstream targets of STAT-3 is IRF1[31]. Therefore, the survival of pancreatic cancer may occur at least in part through the STAT-3-mediated modulation of IRF1 and/or IRF2. To overcome such survival pathways, the regulation of the IRF1 and IRF2 levels seems to be a promising strategy. Peroxisome proliferation-activated receptor (PPAR)-γ agonists functioned as negative modulators of STAT-3 in several normal and cancer models and seem to be potential candidates [29,30]. However, it will be necessary to accumulate a body of evidence regarding the effects of these agents in pancreatic cancer, especially their ability to overcome the survival pathways in future studies.

In summary, IRF1 and IRF2 may regulate the progression of pancreatic cancer as an anti-oncoprotein and oncoprotein, respectively. Therefore, increasing the level of IRF1 and decreasing the level of IRF2 could be a potential therapeutic strategy for treating pancreatic cancer.

Acknowledgements

We particularly thank Satoshi Eguchi (Department of Medical Biology, Akita University Graduate school of Medicine, Japan) and Taku Sato (Department

of Biodefense Research, Medical Research Institute, Tokyo Medical and Dental University, Japan) for their excellent sorting technique. We are grateful to Yuko Hiroshima (Department of Surgery, Honjo-Daiichi Hospital, Japan) for the instruction of pathological scoring. We also thank Chihiro Taira and Yukie Komatsu for their technical assistance. This work was supported by Grants-in-Aid from Ministry of Education, Culture, Sports, Science and Technology of Japan to H. M.

Abbreviations

Ab, antibody; AI, apoptotic index; EGF-R, epidermal growth factor receptor; Gem, gemcitabine; IHC, immunohistochemistry; MAPK, mitogen activated protein kinase; OS, overall survival; PCNA, proliferation cell nuclear antigen; PCR, polymerase chain reaction; PARP, poly ADP-ribose polymerase; PI3K, phosphatidylinositol 3 kinase; PPAR-γ, peroxisome proliferation-activated receptor γ; RT-PCR, reverse-transcription polymerase chain reaction; SD, standard deviation; STAT, signal transducer and activator of transcription; TNM, tumor-node-metastasis; WB, Western blotting

References

1. Klein AP. Identifying people at a high risk of developing pancreatic cancer. *Nat Rev Cancer.* 2013;13:66-74.
2. Bond-Smith G, Banga N, Hammond TM, et al. Pancreatic adenocarcinoma. *BMJ.* 2012;344.
3. Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. *Nat Rev Clin Oncol.* 2010;7:163-172.
4. Burris HA, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J. Clin. Oncol.* 1997;15:2403-2413.
5. Macgregor-Das AM, Iacobuzio-Donahue CA. Molecular pathways in pancreatic carcinogenesis. *J. Surg. Oncol.* 2013;107:8-14.
6. Saif MW, Karapanagiotou L, Syrigos K. Genetic alterations in pancreatic cancer. *World J Gastroenterol.* 2007;13:4423-4430.
7. Biankin AV, Kench JG, Morey AL, et al. Overexpression of p21WAF1/CIP1 is an Early Event in the Development of Pancreatic Intraepithelial Neoplasia. *Cancer Res.* 2001;61:8830-8837.
8. Wong HH, Lemoine NR. Pancreatic cancer: molecular pathogenesis and new therapeutic targets. *Nat Rev Gastroenterol Hepatol.* 2009;6:412-422.
9. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib Plus Gemcitabine Compared With Gemcitabine Alone in Patients With Advanced Pancreatic Cancer: A Phase III Trial of the National Cancer Institute of Canada Clinical Trials Group. *J. Clin. Oncol.* 2007;25:1960-1966.
10. Mamane Y, Heylbroeck C, Génin P, et al. Interferon regulatory factors: the next generation. *Gene.* 1999;237:1-14.
11. Borden EC, Sen GC, Uze G, et al. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov.* 2007;6:975-990.
12. Picozzi VJ, Kozarek RA, Traverso LW. Interferon-based adjuvant chemoradiation therapy after pancreaticoduodenectomy for pancreatic adenocarcinoma. *The American Journal of Surgery.* 2003;185:476-480.
13. Vitale G, van Eijck CH, van Koetsveld Ing PM, et al. Type I interferons in the treatment of pancreatic cancer: mechanisms of action and role of related receptors. *Ann. Surg.* 2007;246:259-268.

14. Wang B, Xiong Q, Shi Q, et al. Intact Nitric Oxide Synthase II Gene Is Required for Interferon-ß-mediated Suppression of Growth and Metastasis of Pancreatic Adenocarcinoma. *Cancer Res.* 2001;61:71-75.
15. Vaughan PS, Aziz F, van Wijnen AJ, et al. Activation of a cell-cycle-regulated histone gene by the oncogenic transcription factor IRF-2. *Nature.* 1995;377:362-365.
16. Harada H, Kitagawa M, Tanaka N, et al. Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. *Science.* 1993;259:971-974.
17. Taniguchi T, Ogasawara K, Takaoka A, et al. IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol.* 2001;19:623-655.
18. Ozato K, Tailor P, Kubota T. The Interferon Regulatory Factor Family in Host Defense: Mechanism of Action. *J. Biol. Chem.* 2007;282:20065-20069.
19. Yanai H, Negishi H, Taniguchi T. The IRF family of transcription factors: Inception, impact and implications in oncogenesis. *Oncoimmunology.* 2012;1:1376-1386.
20. Connett JM, Badri L, Giordano TJ, et al. Interferon regulatory factor 1 (IRF-1) and IRF-2 expression in breast cancer tissue microarrays. *J. Interferon Cytokine Res.* 2005;25:587-594.
21. Lowney J, Boucher L, Swanson P, et al. Interferon Regulatory Factor-1 and -2 Expression in Human Melanoma Specimens. *Ann. Surg. Oncol.* 1999;6:604-608.
22. Choo A, Palladinetti P, Holmes T, et al. siRNA targeting the IRF2 transcription factor inhibits leukaemic cell growth. *Int. J. Oncol.* 2008;33:175-183.
23. Wang Y, Liu D-P, Chen P-P, et al. Involvement of IFN Regulatory Factor (IRF)-1 and IRF-2 in the Formation and Progression of Human Esophageal Cancers. *Cancer Res.* 2007;67:2535-2543.
24. Gao J, Tian Y, Zhang J. Overexpression of interferon regulatory factor 1 enhances chemosensitivity to 5-fluorouracil in gastric cancer cells. *J Cancer Res Ther.* 2012;8:57-61.
25. Guichard C, Amaddeo G, Imbeaud S, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat. Genet.* 2012;44:694-698.
26. Cui L, Deng Y, Rong Y, et al. IRF-2 is over-expressed in pancreatic cancer and promotes the growth of pancreatic cancer cells. *Tumor Biol.* 2012;33:247-255.
27. Mashima H, Sato T, Horie Y, et al. Interferon regulatory factor-2 regulates exocytosis mechanisms mediated by SNAREs in pancreatic acinar cells. *Gastroenterology.* 2011;141:1102-1113 e1101-1108.
28. Caraglia M, Marra M, Tagliaferri P, et al. Emerging strategies to strengthen the anti-tumour activity of type I interferons: overcoming survival pathways. *Current*

cancer drug targets. 2009;9:690-704.

29. Dicitore A, Caraglia M, Colao A, et al. Combined treatment with PPAR-gamma agonists in pancreatic cancer: a glimmer of hope for cancer therapy? *Current cancer drug targets.* 2013;13:460-471.

30. Vitale G, Zappavigna S, Marra M, et al. The PPAR-γ agonist troglitazone antagonizes survival pathways induced by STAT-3 in recombinant interferon-ß treated pancreatic cancer cells. *Biotechnology Advances.* 2012;30:169-184.

31. Chapman RS, Duff EK, Lourenco PC, et al. A novel role for IRF-1 as a suppressor of apoptosis. *Oncogene.* 2000;19:6386-6391.

Table and Figure Legends

Table 1. Correlations between the expressions of IRF1 and IRF2 and the clinicopathological features of pancreatic cancer.

Figure 1. The expressions of IRF1 and IRF2 in the pancreatic cancer specimens and the associations between the levels of IRF1 and IRF2 and patient survival.

A. Representative images of immunohistochemistry of IRF1 and IRF2 in the human pancreatic cancer specimens and paired normal areas of the pancreas. The lower panels show the higher magnification of the boxed areas. IRF1 was concentrated in the nuclei in the normal areas and was much decreased in the cancerous regions. In contrast, IRF2 was faintly stained in the nuclei of the normal areas and densely concentrated in the nuclei in the cancerous

regions. Bars = 50 μm. The other images with scores are presented in Supplementary Figure 1. B. Histogram of the histological scores of IRF1 and IRF2 in the pancreatic cancer specimens and paired normal areas of the pancreas. C. Kaplan-Meier survival curves of the pancreatic cancer patients. The Kaplan-Meier curves are shown according to the expression levels of IRF1 (higher (score (2-3) vs. lower (score 0-1)) and IRF2 (higher (score 3) vs. lower (score 0-2)).

Figure 2. Effects of the IRF1 and IRF2 expressions in pancreatic cancer cells

A. Total cellular homogenates of PANC-1/cont, PANC-1/IRF2, PANC-1/dnIRF2 and PANC-1/IRF1 cells were prepared and Western blotting was performed to confirm the overexpression of IRF1 in the PANC-1/IRF1 cells and IRF2 (arrow) in the PANC-1/IRF2 cells. β-actin was used as an internal control. B. PANC-1 cells were seeded at a density of 2 x 10^4 cells/ml in plastic 12-well plates and cultured for the indicated amount of time. The cells were trypsinized and the number of cells was counted. C. PANC-1 cells were seeded at a density of 1 x 10^4 cells/ml in 96-well culture plates. The following day, the cells were deprived of serum for 24 hours then incubated in

the presence of serum for an additional 48 hours. BrdU was added for the last two hours of incubation, and the level of BrdU incorporation was measured. D. PANC-1 cells were seeded at a density of 1 x 10^5 cells/ml in a culture slide and serum deprived for 48 hours. The apoptotic cells were detected using a TUNEL assay. The apoptotic index (AI) was calculated as follows: (the number of apoptotic cells)/(total number of the cells) x 100 (%). E. Total cellular homogenates of PANC-1 cells were prepared and Western blotting was performed. The blots are representative of three independent experiments with similar results. F. PANC-1 cells were seeded at a density of 1x10^4 cells/ml in 96-well culture plates. After 24 hours, the cells were serum starved and exposed to various concentrations of gemcitabine (Gem). The cells were cultured for an additional 48 hours and the cell viability was measured using an MTT assay. The experiments in B, C, D and F were repeated three times independently with similar results, and the representative figures are shown. The values are presented as the mean ± SD (n=3 or 4). *, $p < 0.05$; **, $p < 0.01$, according to the analysis of variance.

Table

Table1. Correlations between the expressions of IRF1 and IRF2 and the clinicopathological features of pancreatic cancer.

Characteristics	total	Number of patients Expression of IRF1				*p*	Number of patients Expression of IRF2				*p*
		0	1	2	3		0	1	2	3	
Normal area	45	8	6	18	13	<0.001	11	11	17	6	<0.001
Pancreatic cancer tissue	45	11	22	10	2		0	3	12	30	
Gender						0.503					0.901
Male	24	5	12	6	1		0	1	7	16	
Female	21	6	10	4	1		0	2	5	14	
Age						0.590					0.855
<60	34	8	17	8	1		0	3	7	24	
≧60	11	3	5	2	1		0	0	5	6	
Tumor differentiation						0.037					0.425
Well	12	5	6	0	1		0	1	3	8	
Moderate	29	6	14	8	1		0	1	7	21	
Poor	4	0	2	2	0		0	1	2	1	
Tumor infiltration depth						0.027					0.022
T1	1	0	0	0	1		0	0	1	0	
T2	1	0	0	1	0		0	1	0	0	
T3	43	11	22	9	1		0	2	11	30	
T4	0	0	0	5	0		0	0	0	0	
Lymph node metastasis(LNM)						0.208					0.649
N0	13	2	6	4	1		0	1	4	8	
N1	32	9	16	6	1		0	2	8	22	
LMN number						0.680					0.144
0	13	2	6	4	1		0	1	4	8	
1	15	5	8	2	0		0	2	6	7	
2	12	3	6	2	1		0	0	0	12	
3	1	0	1	0	0		0	0	0	1	
≧4	4	1	1	2	0		0	0	2	2	
Metastasis to other organs						0.376					0.137
＋	3	1	2	0	0		0	1	1	1	
－	42	10	20	10	2		0	2	11	29	
TNM stage						0.103					0.912
IA	1	0	0	0	1		0	0	1	0	
IB	1	0	0	1	0		0	1	0	0	
IIA	10	2	6	2	0		0	0	2	8	
IIB	29	8	14	6	1		0	1	7	21	
III	0	0	0	0	0		0	0	0	0	
IV	3	1	2	0	0		0	1	1	1	
Tumor size (cm)						0.001					0.223
<4	18	7	11	0	0		0	1	3	14	
≧4	27	4	11	10	2		0	2	9	16	

TNM:tumor-node-metastasis

Figure
Click here to download high resolution image

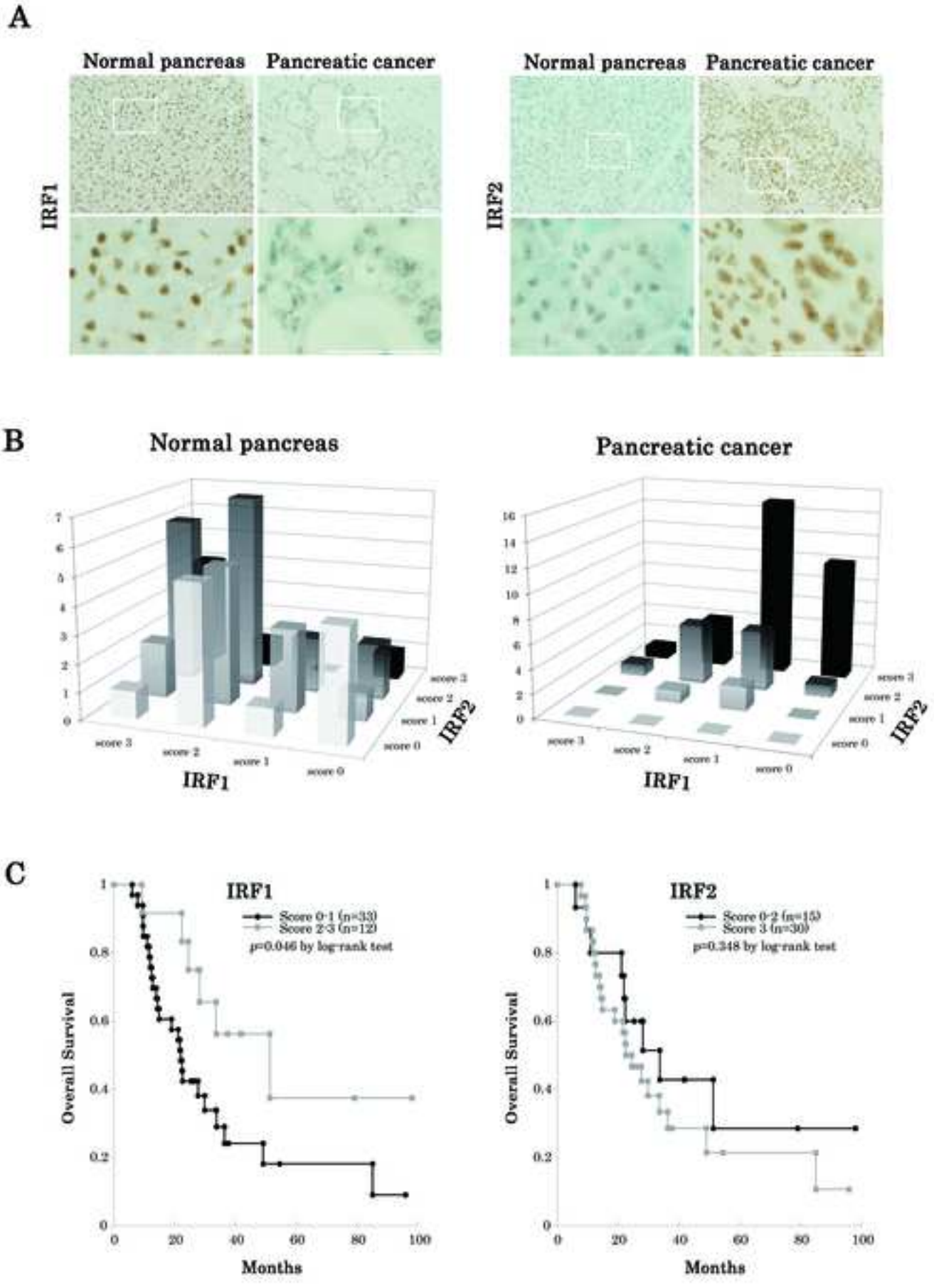
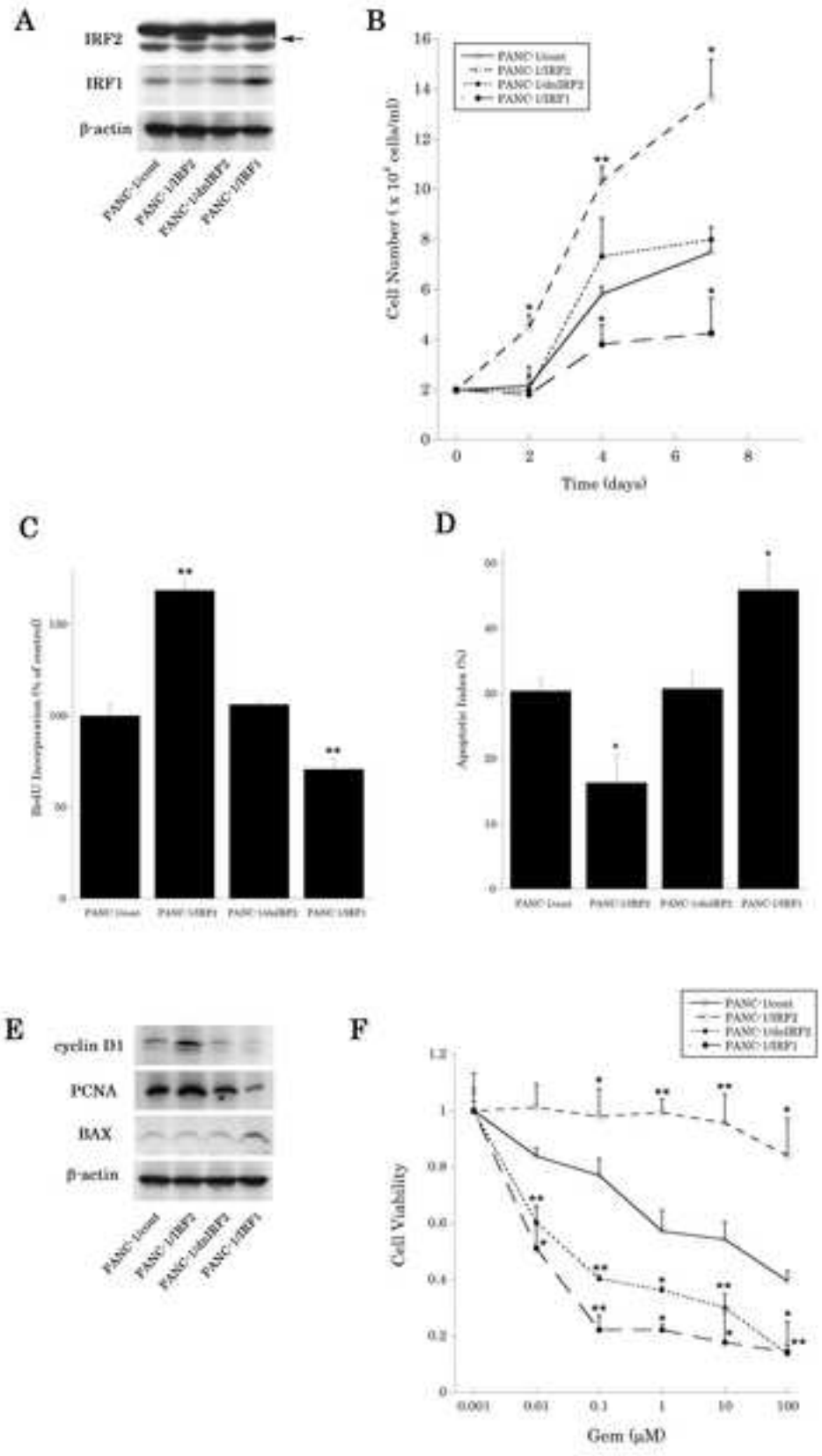


Figure
Click here to download high resolution image



Supplemental Data File (doc, pdf, etc.)
Click here to download Supplemental Data File (doc, pdf, etc.): Supplementary Figure Legend.doc

Supplemental Data File (doc, pdf, etc.)

Click here to download Supplemental Data File (doc, pdf, etc.): Supplementary Figure Sakia revision.tif

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Sakai T..pdf

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Mashima H..pdf

LWW Copyright Transfer and Disclosure Form

Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Yamada Y..pdf

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Goto T..pdf

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Sato W..pdf

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Dohmen T..pdf

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Kamada K..pdf

LWW Copyright Transfer and Disclosure Form

Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Yoshioka M..pdf

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Uchinami H.pdf

LWW Copyright Transfer and Disclosure Form

Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Yamamoto Y..pdf

LWW Copyright Transfer and Disclosure Form
Click here to download LWW Copyright Transfer and Disclosure Form: copyright 13362 Ohnishi H..pdf