Involvement of the Interferon Signaling Pathways in Pancreatic Cancer Cells

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Abstract. *Background/Aim:* To examine interferon (IFN) signaling pathways in human pancreatic cancer cells and their therapeutic application for pancreatic ductal adenocarcinoma (PDAC). *Materials and Methods:* We examined the effects of IFN α on cytotoxicity, migration, as well as on the levels of toll-like receptor (TLR) signaling pathway-associated genes expression in pancreatic cancer cells. We also examined the additive effects of IFN α and poly(I-C) on tyrosine kinase inhibitor (TKI)-induced cytotoxicity. We performed transcriptome analysis (RNA-Seq) of clinical samples and compared the profile between pancreatic intraepithelial neoplasias (PanINs) and PDACs. *Results:* IFN α suppressed cell viability and cell migration, and affected TLR signaling pathways, in pancreatic cancer cells. TLR3 is one of the potential genes involved in IFN-treated pancreatic cancer cells. Furthermore, similar to IFN, extracellular addition of poly(I-C) enhanced TKI-induced cytotoxicity in pancreatic cancer cells. RNA-Seq analysis demonstrated that IFN signaling is one of the potential pathways involved in the progression of PanIN to PDAC. *Conclusion:* IFN signaling may be involved in the development of PDAC. Treatments that target the IFN and TLR3 signaling pathways may be therapeutic options against PDAC.

The worldwide annual new cases of and deaths due to pancreatic ductal adenocarcinoma (PDAC) are estimated to

be at 458,918 and 432,242, respectively (1). Most patients with PDAC remain asymptomatic until the disease reaches an advanced stage (2). Despite the development of treatment methods, PDAC is still associated with a poor prognosis and a high case-fatality rate. Unfortunately, a specific diagnostic biomarker for the detection of early stage PDAC has not yet been developed (3).

Currently, it is well known that smoking, obesity, dietary factors (such as having a non-vegetarian diet), toxins, and excess alcohol intake are modifiable risk factors for PDAC. Additionally, aging, familial cancer syndromes, African-American race, a history of chronic pancreatitis, diabetes mellitus, and a non-O blood group are non-modifiable risk factors for PDAC (4). Further investigations of the pathogenesis and mechanism of PDAC are needed (5).

Immunotherapeutic approaches for PDAC may involve not only the stimulation of the immune system but also the precise control of tumor immunosuppressive effects through tumor microenvironments in PDAC patients (6-8). Type I interferons (IFNs), such as IFN α and IFN β , exert their effects via the type I IFN receptor, which results in the activation of the Janus kinases Jak1 and Tyk2, the phosphorylation and activation of the latent cytoplasmic signal transducers and activators of transcription (STAT1 and STAT2), the formation of a transcription complex in conjunction with IFN regulatory factor 9 (IRF9), and the activation of IFN-stimulated genes (ISGs) that are responsible for mediating the biological activities of type I IFNs (9). Thus, IFNs also affect innate immune signaling pathways.

Booy *et al.* reported that 91.5% of pancreatic cancers exhibited IFN α receptor-1 expression, and 23.4% of these cancers were strongly positive. Furthermore, 68.1% of the pancreatic cancers exhibited IFN α receptor-2c expression and 4.3% of these cancers exhibited strong expression as indicated by immunohistochemistry of the paraffin-embedded pancreatic cancer tissues (10).

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IFN α has potential antitumor effects on solid organ cancers (including PDAC), although the only randomized clinical trial focusing on the treatment of patients with pancreatic cancer did not demonstrate a significant increase in overall survival (11-15). However, it is not well known whether IFN has antitumor activities in PDAC and inhibitory effects on the pathogenesis of PDAC.

The innate immune response produces type I IFN and elicits apoptosis in susceptible cells, including pancreatic cancer cells (16). Constitutive toll-like receptor 3 (TLR3) expression is associated with constitutive Wingless (Wnt) family member 5A (Wnt5A) expression in PDAC (17). Phenylmethimazole (C10), which is a small TLR signaling inhibitor, has been observed to decrease constitutive Wnt5A and TLR3 expression levels together with the inhibition of cell growth and migration both *in vivo* and *in vitro* (17). In contrast, TLR3 and TLR7 agonists [poly(I-C) and imiquimod, respectively] enhance PDAC cell lysis *via* human gammadelta T cells (18).

Noninvasive precursors of invasive PDAC include pancreatic intraepithelial neoplasias (PanINs), intraductal papillary mucinous neoplasms (IPMNs), and mucinous cystic neoplasms (19). Low-grade PanINs (PanIN-1) are common with increasing age and high-grade PanINs (PanIN-3) are usually present in pancreas with invasive cancer. Although PanINs can harbor the somatic genetic alterations seen in invasive pancreatic cancers, the differences in gene expression between PanIN and PDAC are not well known (20).

In the present study, we investigated the TLR signaling pathway following IFN α treatment of pancreatic cancer cell lines. We observed that IFN α up-regulated TLR3 mRNA expression in human pancreatic cancer cell lines. We demonstrated that IFN α and poly(I-C) enhanced the cytotoxicity induced by tyrosine kinase inhibitors (TKIs) in human pancreatic cancer cell lines. We also examined the difference of PDACs and intermediate-grade PanINs (PanIN-2) using laser microdissection (LMD) and transcriptome analysis (RNA-Seq). Our results demonstrate that IFN signaling pathways may be involved in the development of PDAC and that modulation of the IFN and TLR signaling pathways may be a new therapeutic option for the treatment of PDAC.

Materials and Methods

Patients and clinical specimens. The present study included 5 patients with PDAC who underwent surgical treatment at the Department of Digestive Surgery, Nihon University School of Medicine between May 2016 and December 2017. The study protocol was approved by the Ethics Committee of Nihon University School of Medicine (241-1) and conformed to the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained from all patients. All pancreas tissues in the present study had cancerous lesion, pancreatic intraepithelial neoplasia (PanIN), and background non-cancerous parts (pancreatic ductal

epithelial cells) which were pathologically diagnosed by three authors (MF, MS and MM), according to the WHO Classification of Tumors of the Digestive System (21).

Laser microdissection (LMD). Formalin-fixed paraffin-embedded (FFPE) tissue samples were cut into 5 μ m sections and mounted on slides covered with polyethylene-naphthalate-membrane (Leica, Wetzlar, Hessen, Germany) for LMD. After deparaffinization performed directly on the slides, the slides were stained with hematoxylin and eosin (HE). By the LMD method that was conducted using LMD6000 (Leica), target lesions (pancreatic ductal epithelial cells, PanIN and PDAC) were dissected and directly conformed to RNA extraction. The total cut-out area was summed to more than 300,000 μ m².

RNA extraction and RNA-Seq by next generation sequencing. RNA extraction of FFPE samples was performed using RNeasy FFPE Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA quantity and quality were assessed by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

According to Illumina platform, each cDNA library was constructed by TruSeqTM RNA Exome Kit (Illumina, San Diego, CA, USA). One hundred and fifty bp paired-end sequencing protocol providing approximately 15 gigabases per sample was performed on Illumina HiSeq X10 (Illumina) with using Illumina NovaSeq reagent kit. Raw data obtained from sequencing was processed by filtering of index adapter sequences and low-quality reads. These RNA-Seq and data processing were outsourced to BGI Hong Kong Tech Solution GS Lab (BGI, Hong Kong, PR China). The analysis of potential target genes was carried out using Ingenuity pathways analysis (IPA) (Qiagen).

Cell lines and reagents. The human pancreatic cancer cell lines Panc-1 and SUIT-2 have been previously described (22) and were grown in Roswell Park Memorial Institute media (RPMI)-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g streptomycin at 37°C under 5% CO₂. IFN α -2a, poly(I-C), sorafenib, and regorafenib were purchased from Sigma-Aldrich, Imgenex (San Diego, CA, USA), AdooQ Bioscience (Irvine, CA, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. Small interfering RNA (siRNA) against TLR3 (si-TLR3) [sc-36685] and control siRNA (si-C) [sc-37007] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RNA extraction, cDNA synthesis and PCR array for TLR-associated signaling pathway-associated pathways. Human pancreatic cancer cell lines (Panc-1 or SUIT-2) were treated either with or without 0.1 μ g/ml IFN α -2a for 24 h. Cellular RNA was extracted by using the RNeasy Mini kit (Qiagen, Hilden, Germany), and RNA concentration was determined by using a NanoDrop (Thermo Scientific, Tokyo Japan) (23). A RT2 First Strand kit (Qiagen) was used on a GeneAmp PCR system 5700 (Applied Biosystems, Foster, CA, USA) to synthesize cDNA from 1.0 μ g RNA each for one plate (23).

TLR-associated signaling pathways were analyzed by using a RT2 profiler PCR array, which was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). To normalize the raw data for analysis, we automatically selected an optimal set of reference genes by using automatic selection from the full panel method, according to the manufacturer's instructions (23).

Figure 1. Viability of human pancreatic cancer cell lines treated with or without IFN α . (A) Panc-1, (B) SUI-2. Cells were treated with 0.05 μ g/ml IFN α for the indicated times. Cell viability was evaluated with MTS assays (Promega, Madison, WI, USA). * p <0.05, compared with the untreated control (open bar).

Figure 2. IFN α inhibits the migration of pancreatic cancer cell lines. (A) Panc-1, (B) SUI-2. Wound-healing scratch assays were performed. The observed migration is presented as a ratio of migration, considering the migration in the control at 0 hours as 0.01. * p <0.05, compared with the untreated control at 48 h (open bar).

Cell proliferation and cytotoxic assay. To evaluate cell proliferation and viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed with a CellTiter 96 Aqueous One-Solution cell proliferation kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. For each well, the absorbance at 490 nm was measured with the iMark Microplate Absorbance Reader (Bio-Rad, Tokyo, Japan) (23).

Wound-healing scratch assay. Cells were grown on 60-mm cell culture dishes until confluence. A p-200 pipette tip was used to scratch the dish, and the serum-free medium either included or did not include the treatment (24). Up to 48 h after scratching, the cells were observed *via* microscopy (BIOREVIEW BZ-9000, Keyence, Osaka, Japan). The migration activity was presented as a ratio, and the migration in the untreated control was considered to be 1 (24).

Transfection of siRNA. Twenty-four hours before transfection, cells were seeded at 1.5×10^5 cells per well in 6-well plates. Cells were transfected with 50 nM si-TLR3 or si-C using the Effectene transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions (22). After 48 h of transfection, cells were treated with IFN α (0.05 μ g/ml), either with or without regorafenib (1 μ M). After 96 h of transfection, MTS assays were performed to evaluate cell viability.

Statistical analysis. Data are indicated as the mean $\pm$ standard deviation (SD). Statistical analyses were performed by using a 2-tailed Student *t*-test or a Chi-square test. All the experiments were independently performed at least three times. Values of p <0.05 were considered to indicate a statistically significant difference. Statistical analyses were performed with the DA Stats software version PAF01644 (NIFTY Corp., Tokyo, Japan).

Results

IFN α inhibits viability of human pancreatic cancer cell lines.

In general, IFN has been demonstrated to have antitumor potential against human solid cancers (11-15). PDAC also expresses type I IFN receptors (10). These facts prompted us to investigate the effects of IFN α on the viability of human pancreatic cancer cell lines. As shown in Figure 1, the results of the MTS assay demonstrated that IFN α treatment suppressed the viability of both Panc-1 and SUI-2 cells, compared with the cells that were treated without IFN α .

IFN α inhibits migration of human pancreatic cancer cell lines. We also performed a wound-healing scratch assay in human pancreatic cells that were treated with or without

Table I. Up-regulation of Toll-like receptor-associated genes by interferon in human pancreatic cell line Panc-1.

Genes	Fold regulation	p-Value
TLR3	23.73	0.000496
CXCL10	6.04	0.000088
MYD88	4.40	0.000091
EIF2AK2	4.39	0.000282
CSF3	3.76	0.009503
IL6	3.61	0.125579
IRF1	3.38	0.013580
IFNB1	2.91	0.176126
TLR2	2.49	0.000956
B2M	2.48	0.000117
IL12A	2.33	0.000829
IL1B	2.20	0.020446
TNF	2.15	0.001948

TLR3: Toll like receptor 3; CXCL10: C-X-C motif chemokine ligand 10; MYD88: MYD88 innate immune signal transduction adaptor; EIF2AK2: eukaryotic translation initiation factor 2 alpha kinase 2; CSF3: colony stimulating factor 3; IL6: interleukin 6; IFNB1: interferon beta 1; IRF1: interferon regulatory factor 1; TLR2: toll like receptor 2; B2M: beta-2-microglobulin; IL12A: interleukin 12A; IL1B: interleukin 1 beta; TNF: tumor necrosis factor. Bold genes, threshold of fold regulation and *p*-values are 2 and 0.05, respectively.

Table II. Up-regulation of Toll-like receptor-associated genes by interferon in human pancreatic cell line SUIT-2.

Genes	Fold regulation	p-Value
CXCL10	108.59	0.003353
IFNB1	4.17	0.143835
MYD88	3.32	0.002349
SIGIRR	3.09	0.008038
TLR3	2.95	0.003039
NFKB1A	2.41	0.035116
EIF2AK2	2.03	0.199155

SIGIRR: Single Ig and TIR domain containing; NFKB1A: nuclear factor kappa B subunit 1. Bold genes, threshold of fold regulation and *p*-values are 2 and 0.05, respectively.

IFN α to examine the effects of IFN α on the migration of these cells. As shown in Figure 2, the results of the wound-healing scratch assay demonstrated that IFN α treatment suppressed migration of both Panc-1 and SUIT-2 cells, compared with the cells that were treated without IFN α . Thus, because IFN α inhibited viability and migration of human pancreatic cancer cell lines, IFN signaling pathways seem to be involved in pancreatic carcinogenesis. Therefore, the molecular mechanisms were further examined.

Up-regulation of TLR3, CXCL10, and MYD88 in human pancreatic cancer cell lines treated with IFN α . Ida-Hosonuma *et al.* created poliovirus receptor-transgenic mice

Table III. Down-regulation of toll-like receptor-associated genes by interferon in human pancreatic cell line Panc-1.

Genes	Fold regulation	p-Value
CLEC4E	-2.06	0.385352
TLR5	-2.49	0.098818

CLEC4E: C-type lectin domain family 4 member E; TLR5: toll-like receptor 5. Bold genes, threshold of fold regulation and *p*-values are 2 and 0.05, respectively.

Table IV. Down-regulation of toll-like receptor-associated genes by interferon in human pancreatic cell line SUIT-2.

Genes	Fold regulation	p-Value
SARM1	-2.09	0.214029
TICAM2	-2.10	0.064764
MAP3K7	-2.11	0.235700
MAP4K4	-2.15	0.150279
ELK1	-2.23	0.190234
IRAK1	-2.24	0.225715
HSPD1	-2.43	0.332232
RIPK2	-2.50	0.028921
IFNA1	-4.64	0.013978

SARM1: Sterile alpha and TIR motif containing 1; TICAM2: toll like receptor adaptor molecule 2; MAP3K7: mitogen-activated protein kinase 7; MAP4k4: mitogen-activated protein kinase 4; ELK1: ETS transcription factor ELK1; IRAK1: interleukin 1 receptor associated kinase 1; HSPD1: heat shock protein family D (Hsp60) member 1; RIPK2: receptor interacting serine/threonine kinase 2; IFNA1: interferon alpha 1; Bold genes, threshold of fold regulation and *p*-values are 2 and 0.05, respectively.

that were crossed with IFN α / β receptor knockout mice, and they observed very high titers of poliovirus in the pancreatic tissues on day 3 after poliovirus infection, suggesting that the IFN signaling system is also an important pathway in the pancreas (25). Subsequently, we examined the effects of IFN α on innate immunity (including TLR signaling pathways) in human pancreatic cancer cells. We extracted total RNAs from Panc-1 and SUIT-2 cells that were treated with or without IFN α to examine the influence of IFN α on TLR signaling pathways by using a real-time RT-PCR-based array (Tables I-IV; Figure 3).

Among the 90 genes that were examined, 11 and 5 genes were significantly up-regulated by IFN α in Panc-1 and SUIT-2 cells, respectively. We observed that IFN α induced the significant up-regulation of TLR3, CXCL10, and MYD88 mRNA in both cell lines (Tables I, II). However, no genes were significantly down-regulated by IFN α in the Panc-1 cells (Table III), although IFNA1 and RIPK2 mRNAs were significantly down-regulated in SUIT-2 cells (Table IV).

Table V. Characteristics of patients with pancreatic cancer in the present study.

Case No.	Age (years)/gender	Site	Size of tumor (cm)	Histological type	Staging	Type of surgery
1	77/male	Head	1	Tub2	T3N0M0	Pancreaticoduodenectomy
2	72/male	Head	2	Tub2	T2N1M0	Pancreaticoduodenectomy
3	64/female	Body	1	Tub2	T1N0M0	Distal pancreatectomy
4	77/male	Body	1	Tub2	T1N0M0	Distal pancreatectomy
5	50/female	Head	3	Tub1	T3N1M0	Pancreaticoduodenectomy

Tub1: Well differentiated tubular adenocarcinoma; Tub2: moderately differentiated tubular adenocarcinoma.

IFN α enhances the cytotoxicity of TKIs in human pancreatic cancer cell lines. Because limited clinical activity was observed with either of the TKI monotherapies in patients with advanced pancreatic cancer (26), we next examined whether IFN α enhances the cytotoxicity of TKIs *in vitro* (Figure 4). We confirmed that the combination of TKIs and IFN α significantly enhanced cytotoxicity, compared with monotherapies with either TKIs or IFN α , in Panc-1 (Figure 4A) and SUI-2 cells (Figure 4B).

TLR3 ligand poly(I-C) enhances the cytotoxicity of TKIs in human pancreatic cancer cell lines. We also examined whether the TLR3 ligand poly(I-C) enhances the cytotoxicity of TKIs *in vitro* (Figure 4). We confirmed that the combination of TKIs and extracellular addition of poly(I-C) significantly enhanced cytotoxicity, compared with those of TKIs or extracellular addition of poly(I-C) in Panc-1 (Figure 4A) and SUI-2 (Figure 4B). Of interest, there were no differences in the effects between the TKIs/IFN α combination and the TKIs/poly(I-C) combination groups (Figure 4).

Small interfering RNA (siRNA) for TLR3 attenuates the cytotoxicity induced by IFN α and regorafenib in human pancreatic cancer cell lines. In Panc-1 cells, cytotoxicity in the presence of IFN α did not differ between cells that were transfected with si-C (1.0 ± 0.019 , $n=6$) and cells that were transfected with si-TLR3 (1.0 ± 0.037 , $n=6$). However, the cytotoxicity of IFN α plus regorafenib was attenuated in Panc-1 cells that were transfected with si-TLR3 (1.0 ± 0.032 , $n=6$), compared with those cells that were transfected with si-C (0.89 ± 0.023 , $n=6$; $p < 0.05$).

In SUI-2 cells, although cytotoxicity in the presence of IFN α was not attenuated in cells that were transfected with si-TLR3 (1.1 ± 0.027 , $n=6$), compared to cells that were transfected with si-C (1.0 ± 0.060 , $n=6$), cytotoxicity of IFN α plus regorafenib was attenuated in SUI-2 cells that were transfected with si-TLR3 (1.1 ± 0.042 , $n=6$), compared with cells that were transfected with si-C (1.0 ± 0.024 , $n=6$; $p < 0.05$).

In the case of the knockdown of endogenous TLR3, cytotoxicity was not changed with the sole administration of IFN α . However, the knockdown of endogenous TLR3

attenuated the cytotoxicity induced by the combination of IFN α plus regorafenib.

IPA between PanIN and PDAC from 5 clinical samples showed the importance of IFN signaling pathway. We further performed RNA-Seq in 5 patients with PDAC (Table V), to study the molecular mechanism of the development of PDAC. Of interest, IPA between PanIN and PDAC from 5 clinical samples showed the importance of IFN signaling pathway (Tables VI, VII). Four of the 5 top canonical pathways were associated with immune signaling pathways including IFN and innate immunity-related signaling pathways (Table VI). IFN α , IFN lambda 1 (IFNL1/IL29) and IRF7 are extracted as 5 top upstream regulators (Table VII). Activation of IFN α receptor-mediated signaling induced the oncogene PDAC up-regulated factor (PAUF) and made PDAC resistant to oncolytic parvovirus H-1 infection (27). Swayden demonstrated an alteration of PML protein sumoylation was associated with both gemcitabine and oxaliplatin resistance in pancreatic cancer MIAPaCa cells (28). TLRs play a role in the induction of the immune response against tumor development. The TLR7 agonist gardiquimod up-regulated expression levels of IFNL1 and matrix metalloproteinase 9 (MMP-9) (29). IFNL1 also exhibits anti-tumor effects in pancreatic cancer Pan-48 cells through the up-regulation of cyclin-dependent kinase inhibitor 1 (p21) and Bcl-2-associated X protein (Bax) (30). Repressive MYC/MIZ1 complexes bind directly to the promoters of type I IFN regulators IRF5, IRF7, STAT1 and STAT2, resulting in the suppression of type I IFN pathway in mouse PDAC models (31). De-repression of IFN regulators allows pancreatic tumor infiltration by B cells and natural killer (NK) cells, resulting in increased survival (32). Restoration of IFN signaling might improve outcomes for PDAC patients.

Discussion

The present study showed that IFN signaling plays a role in human cancer cell lines and that the IFN and TLR3 signaling pathways may be therapeutic targets for human PDAC. We observed that IFN α suppressed the viability and migration of human pancreatic cancer cell lines. We also observed that

Figure 3. Continued

Figure 3. Gene expression analysis of toll like receptor associated genes with or without interferon alpha (IFN- α) treatment of human pancreatic cancer cells. (A)-(C) Panc-1, (D)-(F) SUIT-2; (A), (D) Clustergram; (B), (E) Scatter plot comparing the normalized expressions of every gene on the PCR array between the 2 groups by plotting them against one another to quickly visualize large gene expression changes. (C), (F) Volcano plot identifying significant gene expression changes by plotting the $\log_2$ of the fold changes in gene expression on the x-axis versus their statistical significance on the y-axis. Yellow circle, upregulated; blue circle, downregulated; black circle, unchanged.

Figure 4. Additive effects of IFN α , or poly(I-C) on the effects of tyrosine kinase inhibitors on the viability of pancreatic cancer cell lines. (A) Panc-1, (B) SUIT-2. Cell were treated with or without IFN (0.05 μ g/ml), poly(I-C) (5 μ g/ml), tyrosine kinase inhibitors [sorafenib (1 μ M), or regorafenib (1 μ M)], or their combination for 48 h. * p <0.05, compared with the control (open bar); # p <0.05, compared with the sorafenib monotreatment; ## p <0.05, compared with the regorafenib monotreatment.

IFN α affects TLR signaling pathways in human pancreatic cancer cells and that TLR3 is one of the up-regulated TLR-associated genes after IFN treatment. We also observed a novel finding that, similar to IFN, poly(I-C) enhances the cytotoxicity that is induced by TKIs in human pancreatic cancer cell lines. In addition, siRNA for TLR3 attenuated the cytotoxicity that was induced by IFN α and regorafenib in human pancreatic cancer cells.

There is a long history of the use of IFN treatment against PDAC. Patients with PDAC exhibit deficiencies in NK cell activity and IFN production by leukocytes and these defects may contribute to the rapidly invasive and metastatic growth of PDAC (33). Previous studies have demonstrated antiproliferative effects of IFN α -2b in human pancreatic cancer cells *in vivo* and *in vitro* (14, 34). Certain patients with advanced PDAC have exhibited clinical responses to

Table VI. Ingenuity pathway analysis between PanIN and PDAC. Top canonical pathways.

Pathways	p-Value
Interferon (IFN) signaling	p <0.05
Activation of IRF by cytosolic pattern recognition receptors	p <0.05
Oleate biosynthesis II (animals)	p <0.05
Antigen presentation pathway	p <0.05
1,25-dihydroxyvitamin D3 biosynthesis	p <0.05

Table VII. Ingenuity pathway analysis between PanIN and PDAC. Top upstream regulators.

Regulators	p-Value	Predicted activation
IFNA2	p <0.05	Activated
PML	p <0.05	Activated
IFN α	p <0.05	Activated
IFNL1	p <0.05	Activated
IRF7	p <0.05	Activated

IFNA2: Interferon α -2; PML: promyelocytic leukemia; IFN α : interferon α ; IFNL1: interferon lambda 1; IRF7: interferon regulatory factor 7.

the treatment combination of IFN α -2b and doxorubicin (34). The biochemical modulation of 5-fluorouracil (5-FU) with folic acid and IFN α is effective in the treatment of PDAC with moderate toxicity (35, 36). The greatest value of the use of IFN may be in prolonging the disease-free interval when IFN is used in combination with other treatment modalities, although these combinations have displayed significant toxicity (37-39). Natural human IFN α has been observed to exhibit mild or marked growth inhibition of human pancreatic cancer cells including HuP-T3, HuP-T4, MIA-PaCa-2, and BxPC-3 cells (40), which supports our current results (Figure 1). Of note, we also observed that IFN α inhibits pancreatic cancer cell migration (Figure 2).

IFN α affected TLR signaling pathways in human pancreatic cancer cell lines (Tables I-IV). We also observed that IFN α up-regulated TLR3 expression in human pancreatic cancer cell lines. In human hepatocytes, independent pathways involving retinoic acid-inducible gene I (RIG-I), IFN induced with helicase C domain 1 (IFIH1/MDA5), and TLR-3 signaling comprise major pathways of host defenses that are triggered by double-stranded RNA, such as poly(I-C) (9). RIG-I and IFIH1, which are two members of the RIG-I-like receptor (RLR) family, may be able to induce growth inhibition or apoptosis of PDAC cells upon activation *via* RNA ligands in IFN-dependent or IFN-independent approaches (41). The enhancement of RIG-I or the enhancement that results from

IFIH1 signaling can confer potent antitumor efficacies against PDAC (16, 42-45), although we did not examine these two molecules.

IFN α partially confers its antitumor activity through its antiangiogenic activity, which results from Sp1/Sp3-mediated inhibition of VEGF gene transcription (46). The combination of IFN α with gemcitabine can induce apoptosis in tumor-associated endothelial cells and can reduce the growth of human pancreatic cancer cells that were orthotopically implanted in nude mice (47). In PDAC, epidermal growth factor receptor (EGFR) and the tyrosine-protein kinase Met (cMET) have been demonstrated to be overexpressed in ~60% and 27-60% of cases, respectively (48). Some TKIs may be used as second-line treatments, following the use of chemotherapy. Interestingly, IFN α and poly(I-C) enhanced TKI-induced cytotoxicity in human pancreatic cancer cell lines (Figure 4).

In the present study, we also observed that the enhancement of TLR3 signaling confers potent antitumor efficacy against human PDAC (Figure 4), which supports the findings of a previous study (18). In general, IFN α induced a considerable amount of ISGs. After the knockdown of TLR3, we did not observe the attenuation of IFN α on cell cytotoxicity. The transcriptional regulation of TLR3 depends on IRF1 and IRF2 (49). Further studies are needed to verify these effects.

After patients with hepatitis C virus (HCV)-related chronic liver disease underwent surveillance for hepatocellular carcinoma (HCC), some patients were diagnosed as having PDAC at a relatively early stage (50). Direct-acting antivirals against HCV, which have recently been introduced as a treatment for HCV, can induce rapid HCV clearance and changes in immune status, including serum interferon levels (51). These effects may be associated with the occurrence of PDAC. Careful attention should be paid to the pancreas during HCC surveillance (50).

PanIN includes atypical hyperplasia, papillary duct lesion with atypia, low-grade dysplasia and some cases of moderate dysplasia, and has been recognized as one of the precancerous lesions (20, 21). IFN signaling was shown by IPA following RNA-Seq to be a prominent different signaling pathway between PanIN and PDAC from 5 patients with PDAC, suggesting that IFN signaling pathway plays a potential role in PDAC development. In conclusion, IFN plays an essential role in PDAC development, and treatment targeting IFN and TLR3 signaling pathways will be a therapeutic option against PDAC.

In conclusion, IFN plays an essential role in human pancreatic cancer progression and treatments of PDAC, and targeting IFN and TLR3 signaling pathways may be therapeutic options against PDAC.

Conflicts of Interest

There are no conflicts of interest that could be perceived as prejudicing the impartiality of the reported research.

Author's Contributions

Conception and design: Mariko Fujisawa, Tatsuo Kanda, and Mitsuhiko Moriyama; Experiments, Data analysis and Interpretation: Mariko Fujisawa, Tatsuo Kanda, Toshikatsu Shibata, Kazumichi Kuroda, Masahiko Sugitani, and Mitsuhiko Moriyama; Article Writing: All Authors; Final approval of article: All Authors.

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