# Intrinsic Chemoresistance to Gemcitabine Is Associated with Decreased Expression of BNIP3 in Pancreatic Cancer

Masanori Akada,[1,2] Tatjana Crnogorac-Jurcevic,[1] Samuel Lattimore,[1] Patrick Mahon,[1] Rita Lopes,[1] Makoto Sunamura,[1,2] Seiki Matsuno,[2] and Nicholas R. Lemoine[1]

**Abstract**

**Purpose:** Although chemotherapy with gemcitabine is a common mode of treatment of pancreatic cancer, 75% of patients do not benefit from this therapy. It is likely that the sensitivity of cancer cells to gemcitabine is determined by a number of different factors.

**Experimental Design:** To identify genes that might contribute to resistance to gemcitabine, 15 pancreatic cancer cell lines were subjected to gemcitabine treatment. Simultaneously, gene expression profiling using a cDNA microarray to identify genes responsible for gemcitabine sensitivity was performed.

**Results:** The pancreatic cancer cell lines could be classified into three groups: a gemcitabine "sensitive," an "intermediate sensitive," and a "resistant" group. Microarray analysis identified 71 genes that show differential expression between gemcitabine-sensitive and -resistant cell lines including 27 genes relatively overexpressed in sensitive cell lines whereas 44 genes are relatively overexpressed in resistant cell lines. Among these genes, 7 genes are potentially involved in the phosphatidylinositol 3-kinase/Akt pathway. In addition to this major signaling pathway, Bcl2/adenovirus E1B 19 kDa protein interacting protein (BNIP3), a Bcl-2 family proapoptotic protein, was identified as being expressed at lower levels in drug-resistant pancreatic cancer cell lines. In an analysis of 21 pancreatic cancer tissue specimens, more than 90% showed down-regulated expression of BNIP3. When expression of BNIP3 was suppressed using small interfering RNA, gemcitabine-induced cytotoxicity *in vitro* was much reduced.

**Conclusions:** These results suggest that BNIP3 and the phosphatidylinositol 3-kinase/Akt pathway may play an important role in the poor response to gemcitabine treatment in pancreatic cancer patients.



Pancreatic adenocarcinoma is a common cancer with an extremely poor prognosis. It is the fourth leading cause of cancer death in the United States (1). Despite an enormous amount of effort spent in the development of cancer chemotherapies for pancreatic cancer, these are effective only in a small proportion of patients. Along with a lack of early diagnostic tests that might allow surgical intervention at a potentially curable stage, this is one of the major problems in the management of pancreatic cancer.

In the past few years, gemcitabine [2′,2′-difluorodeoxycitidine, Gemzar, Eli-Lilly, Indianapolis, IN), a novel pyrimidine nucleoside analogue, has become the standard chemotherapeutic agent used in patients with pancreatic cancer. A phase II randomized trial in advanced pancreatic cancer showed that gemcitabine was more effective than 5-fluorouracil (2, 3).

However, not more than 25% of patients with pancreatic cancer will benefit from gemcitabine, a proportion that is slightly less than in patients with other cancers (4). It has long been recognized that the effectiveness of anticancer drugs can vary significantly between individual patients. Several attempts have already been undertaken in both cell lines and clinical samples to detect molecular markers of gemcitabine chemosensitivity. Such markers can be categorized into two groups. The majority of genes are related to nucleoside transport and metabolism, which may be involved in the intracellular handling of gemcitabine in cancer cells. In this category, nucleoside transporter (5, 6), M1 or M2 subunit of ribonucleoside reductase (7–11), and deoxycytidine kinase (12) have all been described as being related to gemcitabine chemosensitivity. Another group comprises the genes involved in cell cycle regulation, proliferation, or apoptosis. Mutated p53 (13) and Bcl-xl (14, 15) have been identified as possible molecular markers for gemcitabine chemoresistance, and both are directly involved in apoptosis. In addition, c-Src (16, 17) and focal adhesion kinase (FAK; ref. 18) were also described as gemcitabine resistance–related genes. These genes may be involved in resistance of pancreatic cells to gemcitabine by activating the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Furthermore, another study

**Authors' Affiliations:** [1]Cancer Research UK Clinical Centre at Barts and London School of Medicine, London, United Kingdom and [2]Division of Gastroenterological Surgery, Department of Surgery, Tohoku University School of Medicine, Sendai, Japan

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**Requests for reprints:** Nicholas R. Lemoine, Molecular Oncology Unit, Cancer Research UK Clinical Centre at Barts and London School of Medicine, John Vane Science Building, Charterhouse Square, London EC1M 6BQ, United Kingdom. Phone: 44-20-7014-0420; Fax: 44-20-7014-0431; E-mail: nick.lemoine@cancer.org.uk.

showed that under hypoxic conditions pancreatic cancer cell lines become resistant to apoptosis primarily by activation of PI3K/Akt and nuclear factor κB pathways, as well as partially through the mitogen-activated protein (MAP) kinase signaling pathway (19).

It is clear that the sensitivity/resistance of cancer cells to gemcitabine cannot be predicted by a single factor but may be determined by the balance of many factors. Therefore, to establish the baseline for prediction of chemosensitivity, a comprehensive analysis of the sets of genes that characterize the response of cancer cells to gemcitabine treatment is needed.

During the past few years, cDNA/oligonucleotide microarray analysis has become a key tool for characterizing gene expression in a variety of experimental systems, and it has also been used for detecting gemcitabine chemosensitivity markers. So far, two studies have been reported (9, 10). In both of them, cell clones that had acquired resistance *in vitro* were compared with their chemosensitive parental cell lines. However, it is important to use nontreated cell lines to identify genetic factors that determine intrinsic (as opposed to acquired) chemoresistance, as this more closely represents the clinical situation at presentation of a cancer patient. In this study, by analyzing 15 different pancreatic cancer cell lines with a range of gemcitabine sensitivity, we attempted to identify novel genes associated with intrinsic gemcitabine resistance using a cDNA microarray system consisting of 9,464 human gene elements.

## Materials and Methods

***Pancreatic cancer tissues and cell lines.*** Sixteen human pancreatic cancer cell lines were used in this study: A818.4, AsPc-1, CFPAC-1, FA6, Hs766T, MDAPanc-3, MiaPaCa-2, PANC-1, PaTu-I, RWP-1, Suit-2, and T3M4 were obtained from Cancer Research UK cell production services. PK1, PK9, and PK59 were established and maintained at Tohoku University (20). All cell lines were kept in a humidified incubator at 37°C with 5% $CO_2$ and cultured in E4 complete medium, supplemented with 10% fetal bovine serum, penicillin (0.1 μg/mL), and streptomycin (100 units/mL). The human pancreatic ductal epithelial cell line HPDE was a kind gift from Dr. Ming-Sound Tsao (University of Toronto, Canada) and was grown in keratinocyte medium as described before (21).

Pancreatic cancer tissues were obtained from the Human Biomaterials Resource Centre (Hammersmith Hospital, London, United Kingdom) and Tohoku University Hospital (Sendai, Japan) with full ethical approval from the host institutions. All tissues used were enriched for the tumor cellular component (60-80%) by trimming freshly frozen blocks whereas performing H&E sections at frequent levels as described previously (22).

Total RNA extraction from cell lines and tissues was done using Trizol reagent (Invitrogen, Renfrew, Renfrewshire, United Kingdom) according to the protocol of the manufacturer.

***3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.*** Cells were resuspended in fresh medium at a concentration of $1 \times 10^4$ cells/well and seeded in a 96-well plate. Cells were incubated for 24 hours at 37°C, and then gemcitabine at various concentrations was added to each well. The plate was incubated at 37°C for a further 72 hours. For the assay, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) were added to each well and the plate was incubated for an additional 3 hours at room temperature. The absorbance was measured at 560 nm using a microplate reader (Dynex, Worthing, United Kingdom).

***Microarray hybridization.*** The 10K cDNA Sanger Human Arrays (version 1.2.1) obtained through the Cancer Research-UK/Ludwig Institute/Wellcome Trust consortium were used in this study. They contain 9,464 human gene elements. The glass arrays were manufactured and quality controlled at the Sanger Centre (Cambridge, United Kingdom). The spotting pattern and the complete annotated list of these cDNAs are available at the CRUK Microarray web site (http://www.sanger.ac.uk/Projects/Microarrays/informatics/hver1.2.1.shtml).

Labeling of 50 μg of total RNA was achieved by direct incorporation of Cy5-dCTP or Cy3-dCTP (Amersham Pharmacia Biotech, Amersham, United Kingdom) in a reverse transcription reaction using anchored oligodeoxythymidylate primers (Cancer Research-UK Oligonucleotide Service, London Research Institute, Clare Hall Laboratories, United Kingdom) and Superscript II reverse transcriptase (Invitrogen). The details of the hybridization and washing protocols are available online (http://www.cgal.icnet.uk/exprotocols/protocols.html).

The cDNA derived from the HPDE cell line was used as the control sample in all hybridizations. In each experiment, Cy5-dCTP–tagged cDNA from an individual pancreatic cancer cell line was mixed with Cy3-dCTP–tagged cDNA from HPDE cells and subsequently cohybridized to a microarray. All the experiments were done in duplicate.

Following hybridization, arrays were scanned using an Affymetrix 428 dual-laser microarray (Affymetrix, Santa Clara, CA) and separate images were acquired for Cy3 and Cy5 fluorescence.

***Image and data analysis.*** The signal intensity values of each element were extracted using the ImaGene 5 software program (BioDiscovery, Los Angeles, CA). Normalization of the resulting spot intensities was achieved through the VSN package as part of the Bioconductor software within R (23). Differentially expressed genes were isolated by permutation testing using the *t* statistic (perm = 10,000) and subsequent *P* value correction using the false discovery rate method of Benjamini et al. (24). Differentially expressed genes were those that had a corrected *P* value of $<0.05$. Sample-wise agglomerative hierarchical clustering was carried out by first selecting the top 1,000 genes based on variance, then using Euclidean distance to generate the distance matrix, and average linkage to group the samples. All of these were done within the R environment.

***Quantitative real-time reverse transcription-PCR.*** Primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The primer sequences for Bcl2/adenovirus E1B 19 kDa protein interacting protein (BNIP3; 61 bp amplicon) are as follows: forward, 5′-GTGGTCAAGTCGGCCGG-3′; reverse; 5′-GCGC-TTCGGGTGTTTAAAGA-3′.

Template cDNAs were synthesized from 1.5 μg of total RNA using the Taqman reverse transcription reaction kit (Applied Biosystems).

The real-time reverse transcription-PCR (RT-PCR) reactions were set up in a total volume of 20 μL using 3 μL of cDNA and 10 μL of SYBR Green Master Mix (Applied Biosystems). The final primer concentration was 300 mmol/L for both forward and reverse primers. For every target gene a set of triplicate reactions using five dilutions of reverse-transcribed Universal Human reference RNA (Stratagene, LA Jolla, CA) was included to construct a standard curve. No-template control reactions were also included. Real-time RT-PCR was done using the ABI 7700 sequence detector (Applied Biosystems).

***RNA interference.*** Custom-made oligonucleotide small interfering RNA (siRNA; SMARTpool) for BNIP3 was obtained from Dharmacon (Lafayette, CO), lamin siRNA was obtained from Qiagen GmbH (Hilden, Germany), and nonsilencing negative control siRNA was obtained from Ambion (Austin, TX). The $2 \times 10^5$ cells were seeded into a six-well plate and allowed to adhere for 24 hours. Aliquots of 150 pmol of siRNA, 4 μL of Enhancer R (Qiagen), and 93 μL of Buffer EC-R (Qiagen) were mixed and vortexed. After 5 minutes of incubation at room temperature, 8 μL of TransMessenger transfection reagent (Qiagen) were mixed together, then incubated for 10 minutes at room temperature. This siRNA/agent mixture was added into the wells with 800 μL of serum-free/antibiotic-free E4 medium and incubated for 3 hours, after which the medium was changed to 1 mL DMEM with 10%

fetal bovine serum. Twenty-four hours posttransfection, cells were trypsinized, seeded into a 96-well plate, and used for the cell growth inhibition assay.

*Western blot analysis.* The $2 \times 10^6$ cells were harvested and rinsed twice with PBS, at pH 7.4. Cell extracts were prepared with lysis buffer [20 mmol/L Tris (pH 7.5), 0.1% Triton X-100, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin]. Total protein concentration was measured using the Protein assay kit with bovine serum albumin as a standard, according to the instructions of the manufacturer (Bio-Rad, Hercules, CA). Cell extracts containing 40 μg of total protein were subjected to electrophoresis in 10% SDS/PAGE gels and after transfer and blocking with PBS containing 0.2% bovine serum albumin for 16 hours at 4°C, the membrane was incubated with 2 μg/mL mouse monoclonal anti-BNIP3 antibody clone Ana 40 (Sigma-Aldrich, St. Louis, MO). The incubation was for 2 hours at room temperature, followed by washing with 0.1% Tween 20/PBS thrice, and then incubation with secondary antibody mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes followed by three washes.

Signals were detected by chemiluminescence using the enhanced chemiluminescence detection system (Amersham Biosciences).

## Results

*Efficacy of cytotoxicity induced in pancreatic cancer cell lines by gemcitabine exposure.* The responses of 15 pancreatic cancer cell lines to gemcitabine treatment were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Representative dose-response curves are shown in Fig. 1A and B. Three cell lines (CFPAC-1, Suit-2, and T3M4) showed high sensitivity to gemcitabine with less than 20% of those cells surviving in the presence of 25 ng/mL of gemcitabine for 72 hours (Fig. 1A) and their $IC_{50}$ values were 0.5, 0.7, and 3.0 ng/mL, respectively. Hence, these three cell lines were classified as the "sensitive" group. In contrast, three cell lines (Hs766T, RWP-1, and A818.4) showed low sensitivity, with more than 60% of those cells surviving even in the presence of more than $1 \times 10^4$ ng/mL of the drug for 72 hours (Fig. 1B). These three cell lines were therefore classified as "resistant." The remaining cell lines (AsPc-1, FA6, MDAPanc-3, MiaPaCa-2, PANC-1 PaTu-I, PK1, PK9, and PK59) showed moderate sensitivity ($IC_{50}$ values 5-1,000 ng/mL) and were classified as "intermediate sensitive." Figure 1C shows the dose-response curve for the immortalized pancreatic ductal cell line HPDE ($IC_{50}$ 0.8 ng/mL).

*Genes involved in gemcitabine chemosensitivity.* To identify genes differentially expressed between sensitive and resistant cell lines, cDNA microarray experiments were carried out using mRNA extracted from all cell lines. In a comparison between the sensitive and resistant groups, 71 genes were identified that show differential expression between the two groups (Tables 1 and 2).

Of these 71 genes, 27 genes are relatively overexpressed in gemcitabine-sensitive cell lines, and 44 are relatively overexpressed in gemcitabine-resistant cell lines. Genes with a wide variety of functions were identified among these genes, with a large proportion that could be categorized into two groups: 14 genes are related to gemcitabine metabolism and transport (genes 1-8 in Table 1 and genes 28-33 in Table 2) and 27 genes are related to signaling pathways for cell cycle regulation, proliferation, or apoptosis (genes 9-16 in Table 1 and genes 34-52 in Table 2). Among these, FAK was already described as a gemcitabine sensitivity–related gene (18). In contrast, although nucleoside transporter 1 (5, 6), ribonucleoside reductase M1 and M2 (7–11), and deoxycytidine kinase (12) were previously described as gemcitabine sensitivity–related genes, they showed no significant difference between gemcitabine-sensitive cell lines and gemcitabine-resistant cell lines in our experiments. Other genes in this class that were previously described as gemcitabine sensitivity related were not included in our microarray. Also, seven genes are potentially involved in the PI3K/Akt pathway: *PXN*, *p70S6K*, *FAK*, *PIK3C3*, *TSC1*, *IGFBP7*, and *ITGA9*. A further three genes are related to the transforming growth factor-β (TGF-β) signaling pathway: *RALBP1*, *SMAD2*, and *LTBP1*.

Interestingly, among the selected genes, several are located at the same chromosomal regions such as 6q (MAP3K7, *C6orf93*, and *HECA*), 10q (*BNIP3*, *PPP3CB*, *KIAA0261*, and *MGEA5*), 19q (*EBP*, *PPP1R15A*, and *LRP3*), and 22q (*CDC42EP*, *FLJ22582*, *SLC25A1*, and *TCN2*), and these loci are also previously reported as sites of frequent aberrations and amplification in pancreatic cancer (25–27). In addition,

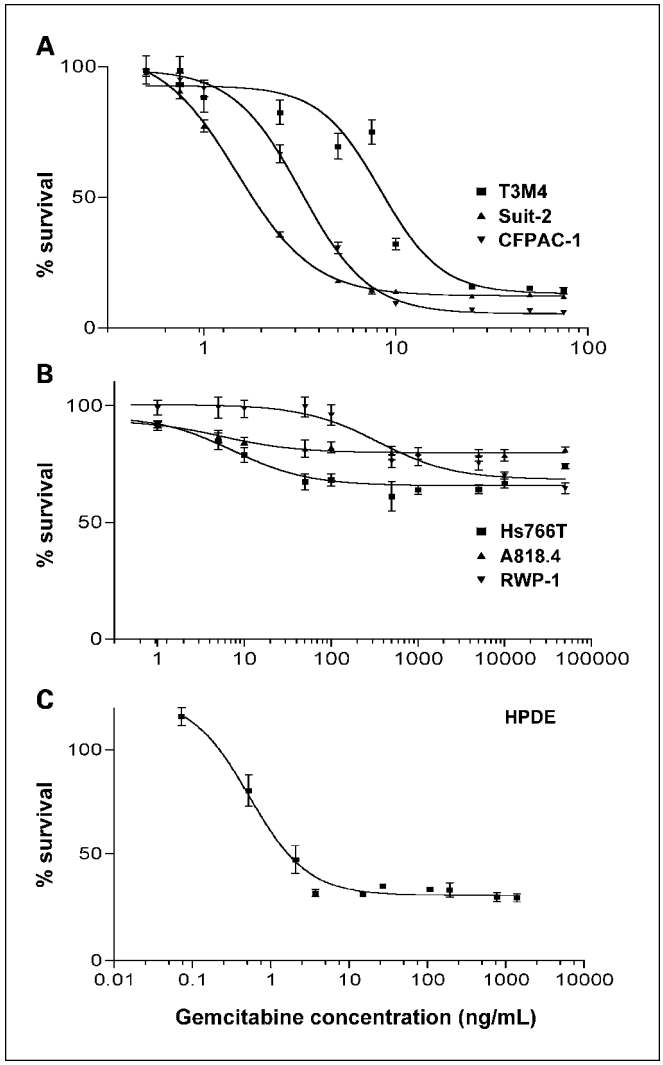


**Fig. 1.** Dose-response curves for gemcitabine in pancreatic cancer cell lines. *A*, gemcitabine-sensitive cell lines: CFPAC-1, Suit-2, and T3M4. *B*, gemcitabine-resistant cell lines: RWP-1, A818.4, and Hs766T. *C*, HPDE. Points, mean; bars, SD.

**Table 1.** Genes relatively overexpressed in gemcitabine-sensitive pancreatic cancer cell lines

| No. | Common name | Unigene ID | Description | Chromosomal location | *P* | *t* |
|---|---|---|---|---|---|---|
| Genes involved in nucleoside metabolism and transportation | | | | | | |
| 1 | *PAICS* | Hs.444439 | phosphoribosylaminoimidazole carboxylase | 4q12 | 1.65e-06 | −9.9914 |
| 2 | *CDC42EP1* | Hs.148101 | CDC42 effector protein (Rho GTPase binding) 1 | 22q13.1 | 5.95e-05 | −6.65 |
| 3 | *TNNI3* | Hs.512709 | troponin I, cardiac | 19q13.42 | 0.00071 | −5.8469 |
| 4 | *DNM1* | Hs.436132 | dynamin 1 | 9q34.11 | 0.0002 | −5.778 |
| 5 | *EBP* | Hs.196669 | emopamil binding protein (sterol isomerase) | Xp11.23 | 0.0006 | −5.6273 |
| 6 | *SMARCA3* | Hs.3068 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3 | 3q24 | 0.00058 | −5.4641 |
| 7 | *P2RX4* | Hs.321709 | purinergic receptor P2X, ligand-gated ion channel, 4 | 12q24.31 | 0.00042 | −5.3659 |
| 8 | *CDA* | Hs.72924 | cytidine deaminase | 1p36.12 | 0.00092 | −4.822 |
| Genes involved in signaling pathways for cell cycle regulation, proliferation, or apoptosis. | | | | | | |
| 9 | *IGFBP7* | Hs.435795 | insulin-like growth factor binding protein 7 | 4q12 | 4.00e-08 | −17.251 |
| 10 | *p70S6K* | Hs.86858 | Ribosomal protein S6 kinase, 70kDa | 17q11.2 | 0.00012 | −8.0003 |
| 11 | *BNIP3* | Hs.79428 | Bcl2/adenovirus E1B 19 kDa protein interacting protein | 10q26.3 | 0.00014 | −7.5318 |
| 12 | *PXN* | Hs.446336 | paxillin | 12q24.23 | 0.00017 | −6.2075 |
| 13 | *CHAF1B* | Hs.75238 | chromatin assembly factor 1, subunit B (p60) | 21q22.13 | 0.00039 | −5.3061 |
| 14 | *RBMS2* | Hs.438778 | RNA binding motif, single stranded interacting protein 2 | 12q23.2 | 0.00045 | −5.267 |
| 15 | *RGS2* | Hs.78944 | regulator of G-protein signaling 2, 24 kDa | 1q31.2 | 0.00048 | −5.0793 |
| 16 | *CREM* | Hs.231975 | cyclic AMP responsive element modulator | 10p11.21 | 0.00059 | −4.9462 |
| Unknown and other function | | | | | | |
| 17 | *PCCB* | Hs.63788 | propionyl CoA carboxylase, β polypeptide | 3q22.1 | 1.83e-05 | −8.037 |
| 18 | *MPDZ* | Hs.169378 | multiple PDZ domain protein | 9p23 | 0.00012 | −7.0767 |
| 19 | *SGCE* | Hs.409798 | sarcoglycan, epsilon | 7q21.3 | 0.00048 | −7.0147 |
| 20 | *GPSN2* | Hs.306122 | glycoprotein, synaptic 2 | 4q22.1 | 9.46e-05 | −6.6151 |
| 21 | *LMNB1* | Hs.89497 | lamin B1 | 5q23.1 | 7.21e-05 | −6.4772 |
| 22 | *FLJ22582* | Hs.126783 | hypothetical protein FLJ22582 | 22q13.1 | 0.00019 | −5.7262 |
| 23 | *DHCR7* | Hs.11806 | 7-dehydrocholesterol reductase | 11q13.1 | 0.00039 | −5.4782 |
| 24 | *SERPINF2* | Hs.159509 | serine proteinase inhibitor, clade F, member 2 | 17p13.3 | 0.00079 | −5.3929 |
| 25 | *SLC25A1* | Hs.111024 | solute carrier family 25 , member 1 | 22q11 | 0.00069 | −5.0347 |
| 26 | *TCN2* | Hs.417948 | transcobalamin II; macrocytic anemia | 22q12.2 | 0.00071 | −5.0308 |
| 27 | *RDX* | Hs.263671 | radixin | 11q22.3 | 0.00092 | −4.6462 |

several other genes were also located at frequently aberrant sites such as 3p, 3q, 5p, 8p, 8q, 9p, 17p, 17q, 18p, 19p, and 20q.

***Expression profiling and clustering.*** To investigate whether cell lines grouped as either sensitive or resistant are also genetically similar, hierarchical clustering was done as described in Materials and Methods, and this was able clearly to separate gemcitabine-resistant and gemcitabine-sensitive cell lines.

It is also evident that all the replicates for analyses of individual cell lines are situated close to each other, indicating the overall reproducibility of the array technique (Fig. 2).

***BNIP3 down-regulation is associated with chemoresistance of pancreatic cancer to gemcitabine.*** We have selected BNIP3 as a candidate gene involved in chemosensitivity for further analysis. The expression of BNIP3 mRNA was examined in all pancreatic cell lines by quantitative real-time RT-PCR, and the data obtained were in a complete agreement with our cDNA microarray results. It is evident that in comparison with HPDE, BNIP3 expression was down-regulated in all pancreatic cancer cell lines analyzed except PANC-1 and CFPAC-1. Especially in resistant but also in intermediate sensitive cell lines, BNIP3 expression levels were down-regulated more than 90% (Fig. 3A). Only the PANC-1 cell line in the intermediate sensitive group showed an expression level comparable to that of the immortalized normal cell line HPDE.

***BNIP3 expression is down-regulated in pancreatic cancer.*** To explore if the expression level of BNIP3 in clinical specimens corresponds to the data obtained in cell lines, quantitative real-time RT-PCR analysis was done using mRNA extracted from tissues from 21 different patients with pancreatic adenocarcinoma. Figure 3B shows the expression compared with a control pancreatic tissue from a healthy donor. In all but two (T1 and T20) of the cancer specimens, BNIP3 expression levels were reduced compared with the normal control, and in 14 of 21 samples more than 50% down-regulation of BNIP3 expression was observed.

***BNIP3 siRNA treatment increases chemoresistance of pancreatic cancer cells to gemcitabine.*** To verify that BNIP3 is involved in gemcitabine sensitivity, siRNA experiments were

**Table 2.** Genes relatively overexpressed in gemcitabine-resistant pancreatic cancer cell lines

| No. | Common name | Unigene ID | Description | Chromosomal location | *P* | *t* |
|---|---|---|---|---|---|---|
| Genes for nucleoside metabolism and transportation | | | | | | |
| 28 | *DYT1* | Hs.278429 | *Homo sapiens* dystonia 1, torsion | 9q34.11 | 5.76e-05 | 7.10491 |
| 29 | *VIPR1* | Hs.348500 | vasoactive intestinal peptide receptor 1 | 3p22.1 | 0.00014 | 6.50633 |
| 30 | *ATP11C* | Hs.88252 | ATPase, Class VI, type 11C | Xq27.1 | 0.00055 | 5.55326 |
| 31 | *MTSS1* | Hs.77694 | metastasis suppressor 1 | 8q24.13 | 0.00051 | 5.52812 |
| 32 | *SAFB* | Hs.23978 | scaffold attachment factor B | 19p13.2 | 0.00068 | 5.13198 |
| 33 | *RALBP1* | Hs.75447 | ralA binding protein 1 | 18p11.22 | 0.00075 | 4.7879 |
| Genes involved in signaling pathways for cell cycle regulation, proliferation, or apoptosis. | | | | | | |
| 34 | *SMAD2* | Hs.110741 | SMAD, mothers against DPP homologue 2 | 18q21.1 | 4.3e-05 | 7.82148 |
| 35 | *FAK* | Hs.434281 | focal adhesion kinase | 8q24 | 0.00029 | 7.03583 |
| 36 | *TRAF6* | Hs.444172 | Tumor necrosis factor receptor – associated factor 6 | 11p13 | 0.0001 | 6.71903 |
| 37 | *MADD* | Hs.82548 | MAP-kinase activating death domain | 11p11.2 | 6.30e-05 | 6.60535 |
| 38 | *PPP3CB* | Hs.187543 | protein phosphatase 3 catalytic subunit, β isoform | 10q22.2 | 0.00012 | 6.10191 |
| 39 | *TSC1* | Hs.69429 | tuberous sclerosis 1 | 9q34.13 | 0.00039 | 5.92062 |
| 40 | *STAT5A* | Hs.437058 | signal transducer and activator of transcription 5A | 17q11.2 | 0.00015 | 5.91317 |
| 41 | *SOCS5* | Hs.169836 | suppressor of cytokine signaling 5 | 2p21 | 0.00027 | 5.88452 |
| 42 | *PHF16* | Hs.82292 | PHD finger protein 16 | Xp11.3 | 0.00054 | 5.84577 |
| 43 | *PIK3C3* | Hs.418150 | phosphoinositide-3-kinase, class 3 | 18q12.3 | 0.00035 | 5.73027 |
| 44 | *UNC13B* | Hs.155001 | unc-13 homologue B | 9p13.3 | 0.00023 | 5.70029 |
| 45 | *MAP3K7* | Hs.290346 | MAP kinase kinase 7 | 6q15 | 0.00045 | 5.49991 |
| 46 | *LTBP1* | Hs.241257 | latent TGF-β binding protein 1 | 2p22.3 | 0.0008 | 5.38788 |
| 47 | *ITGA9* | Hs.222 | integrin, 9 | 3p22.3 | 0.00033 | 5.35081 |
| 48 | *GNAQ* | Hs.469951 | guanine nucleotide binding protein (G protein), q polypeptide | 2q21.1 | 0.00066 | 5.30905 |
| 49 | *MYST3* | Hs.93231 | MYST histone acetyltransferase (monocytic leukemia) 3 | 8p11.21 | 0.00044 | 5.16659 |
| 50 | *SKIP* | Hs.178347 | skeletal muscle and kidney enriched inositol phosphatase | 17p13.3 | 0.00052 | 5.13827 |
| 51 | *C5* | Hs.1281 | complement component 5 | 9q33.2 | 0.00092 | 4.97948 |
| 52 | *PPP1R15A* | Hs.76556 | protein phosphatase 1, regulatory (inhibitor) subunit 15A | 19q13.33 | 0.00075 | 4.81317 |
| Unknown and other function | | | | | | |
| 53 | *LRP3* | Hs.143641 | low density lipoprotein receptor-related protein 3 | 19q12 | 6.02e-06 | 10.2416 |
| 54 | *SLC9A3* | Hs.123044 | solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 | 5p15.33 | 0.0002 | 6.85467 |
| 55 | *HSU79266* | Hs.23642 | protein predicted by clone 23627 | 11q13.1 | 0.00037 | 6.59899 |
| 56 | *MCART1* | Hs.46791 | mitochondrial carrier triple repeat 1 | 9p13.3 | 0.00014 | 6.03726 |
| 57 | *SEMA5A* | Hs.528707 | sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain, and short cytoplasmic domain (semaphorin) 5A | 5p15.31 | 0.00027 | 5.69344 |
| 58 | *ELN* | Hs.252418 | elastin | 7q11.23 | 0.00025 | 5.53682 |
| 59 | *KIAA0663* | Hs.17969 | KIAA0663 gene product | 1q32.1 | 0.0003 | 5.45804 |
| 60 | *OGT* | Hs.405410 | *O*-linked *N*-acetylglucosamine (GlcNAc) transferase | Xq13.1 | 0.00035 | 5.29243 |
| 61 | *KIAA0261* | Hs.439188 | KIAA0261 | 10q23.2 | 0.00041 | 5.26712 |
| 62 | *UAP1* | Hs.21293 | UDP-N-acteylglucosamine pyrophosphorylase 1 | 1q24.2 | 0.00049 | 5.22761 |
| 63 | *INPP5E* | Hs.25156 | inositol polyphosphate-5-phosphatase, 72 kDa | 9q34.3 | 0.00039 | 5.22321 |
| 64 | *HECA* | Hs.6679 | headcase homologue (*Drosophila*) | 6q24.1 | 0.00095 | 5.16452 |
| 65 | *RPS21* | Hs.372960 | ribosomal protein S21 | 20q13.33 | 0.00071 | 5.03318 |
| 66 | *PCSK7* | Hs.443752 | proprotein convertase subtilisin/kexin type 7 | 11q23.2 | 0.0006 | 4.94516 |
| 67 | *SEC23B* | Hs.173497 | Sec23 homologue B | 20p11.23 | 0.00063 | 4.92033 |
| 68 | *MGEA5* | Hs.5734 | meningioma expressed antigen 5 | 10q24.32 | 0.00096 | 4.79081 |
| 69 | *C6orf93* | Hs.33944 | chromosome 6 open reading frame 93 | 6q24.2 | 0.00095 | 4.73743 |
| 70 | *HAGH* | Hs.155482 | hydroxyacylglutathione hydrolase | 16p13.3 | 0.00082 | 4.72254 |
| 71 | *LAMP2* | Hs.232432 | lysosomal-associated membrane protein 2 | Xq24 | 0.00086 | 4.69221 |

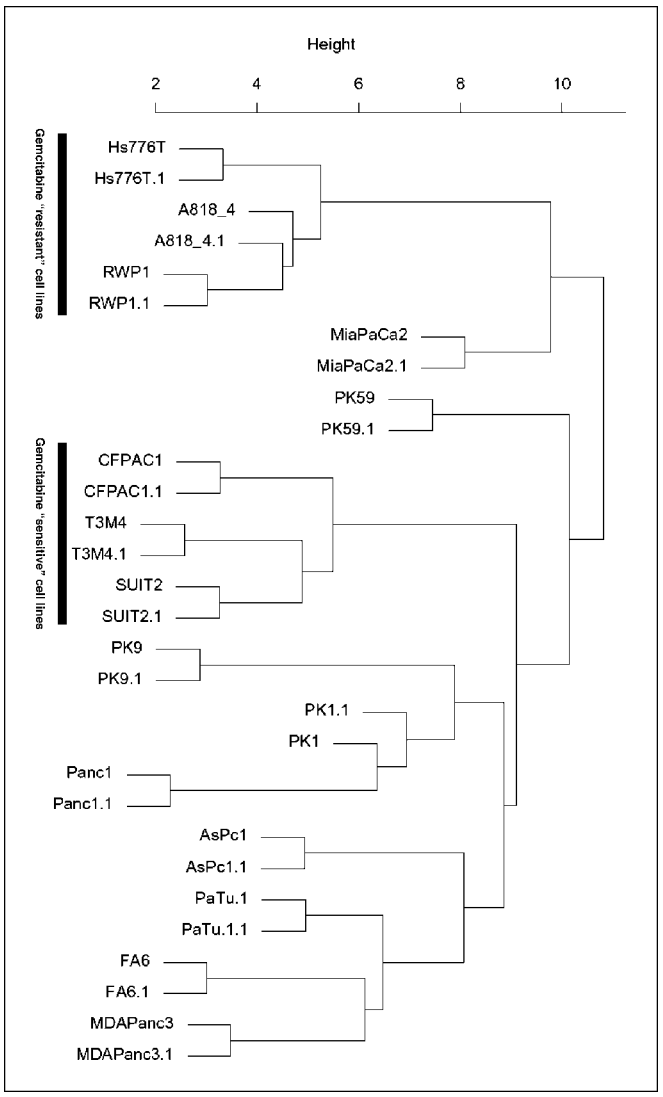


**Fig. 2.** Dendrogram of hierarchical clustering. The dendrogram shows that gemcitabine-sensitive cell lines CFPAC-1, Suit-2, and T3M4 reside on a separate branch from the gemcitabine-resistant cell lines Hs766T, A818.4, and RWP-1.

done on the gemcitabine-sensitive CFPAC-1 pancreatic cancer cell line. The ability of BNIP3 siRNA to suppress BNIP3 expression was confirmed by both quantitative real-time RT-PCR (Fig. 4A) and Western blot (Fig. 4B). After transfection with BNIP3 siRNA, up to 80% suppression of BNIP3 expression was observed. CFPAC-1 cells treated with BNIP3 siRNA also showed an increase in drug resistance with the $IC_{50}$ rising from 0.5 to 1.2 ng/mL. In comparison, CFPAC-1 cells treated with siRNA targeted against lamin and non-silencing control siRNA showed no change in sensitivity to gemcitabine (Fig. 4C).

## Discussion

In the current study we were able to identify a number of genes which are potentially involved in intrinsic resistance to gemcitabine in pancreatic cancer cell lines. Some of these are involved in signaling pathways known from other investigations to contribute to drug resistance (e.g., FAK), whereas in the list of 71 differentially expressed genes, more than half were related to nucleoside metabolism, proliferation, cell cycle regulation, and apoptosis.

The PI3K signaling cascade plays a crucial role in the regulation of apoptosis, acting in part via its downstream target Akt in several cancer cell types including pancreatic cancer (28–30). Activated Akt plays a role in apoptosis suppression by regulating critical factors such as Bcl-associated death promoter, caspase-9, and mammalian target of rapamycin (31). Several studies have already described the contribution of the PI3K/Akt pathway to gemcitabine sensitivity in pancreatic cancer cells. FAK and c-Src play a role in adhesion-dependent activation of the PI3K/Akt pathway and their suppression enhances gemcitabine chemosensitivity in pancreatic cancer (16–18). Moreover, hypoxic conditions also activate the PI3K/Akt pathway in pancreatic cancer (19). In the current study, we identified 71 genes associated with differential gemcitabine sensitivity and, of these, seven encode proteins that contribute to the PI3K/Akt pathway. As well as FAK, of which contribution to drug resistance is well known, integrin α4 may have the ability to stimulate PI3K through PXN as substrate (32, 33). TSC1 and p70S6K are downstream targets of Akt, and PIK3C3 is a member of the PI3K family (34). IGFBP7 can reduce PI3K signaling by binding to IGF and preventing its interaction with its membrane receptor. Two genes that are relatively underexpressed in gemcitabine-resistant cell lines are *p70S6K* and *PXN*. Interestingly, in a leukemia cell clone resistant to apoptosis, although p70S6K activation was increased by signaling through the PI3K/Akt pathway, its selective inhibition did not restore sensitivity to

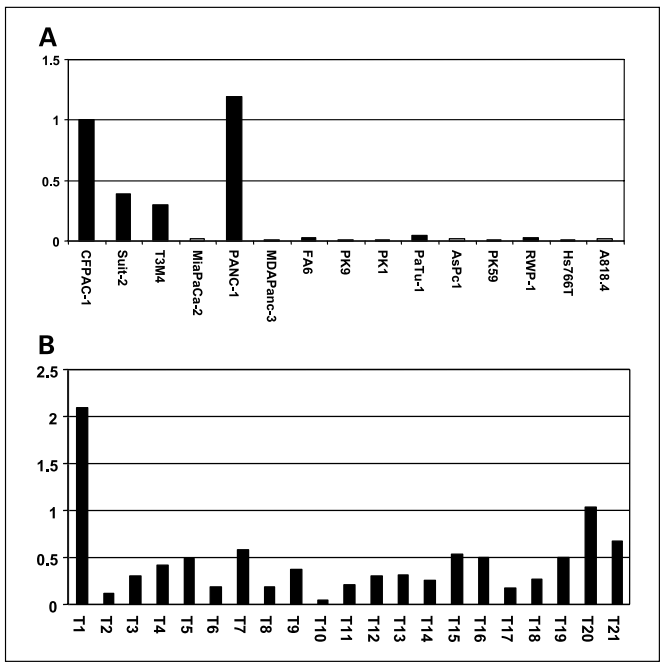


**Fig. 3.** *A*, BNIP3 expression in pancreatic cancer cell lines determined by quantitative real-time RT-PCR. BNIP3 expression is observed in the gemcitabine-sensitive cancer cell lines CFPAC-1, Suit-2, and T3M4 and only in PANC-1 among the intermediate sensitive group. None of the resistant cell lines express significant levels of this gene product. *B*, BNIP3 expression in pancreatic cancer tissues. Quantitative real-time PCR shows that in 19 of 22 cases BNIP3 expression was down-regulated.

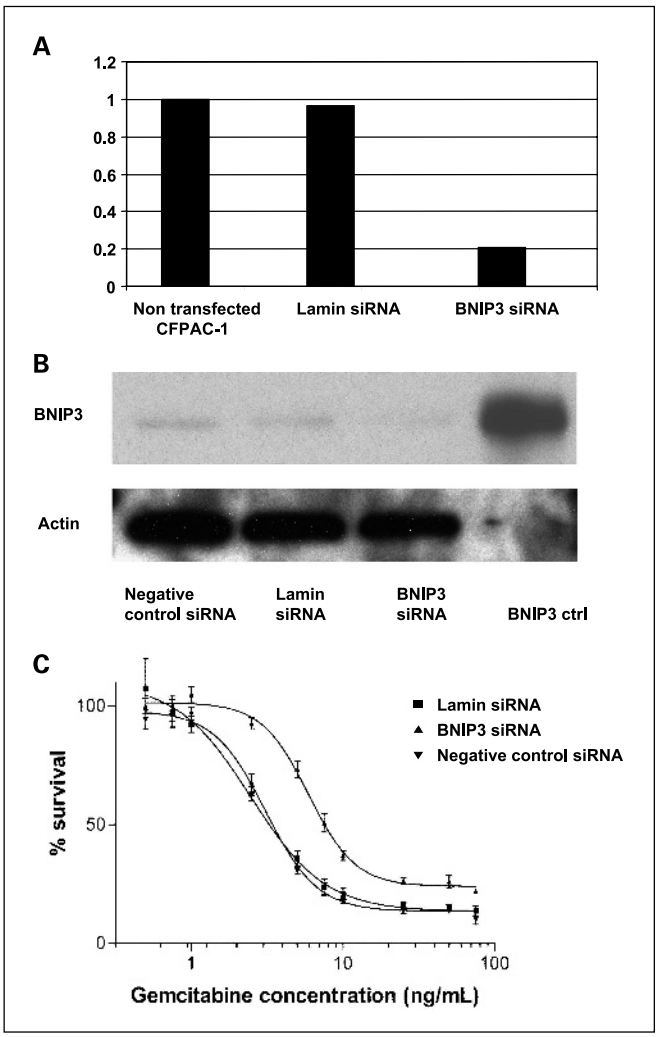


**Fig. 4.** *A*, representative quantitative real-time RT-PCR data for BNIP3 expression in CFPAC-1 pancreatic cancer cells at 96 hours following treatment with BNIP3 siRNA or lamin siRNA. *B*, Western blot for BNIP3 96 hours following treatment with nonsilencing negative control siRNA, lamin siRNA, and BNIP3 siRNA. Protein lysate extracted from BNIP3-transfected 293 cells was used for positive control. Neither control nor lamin siRNA significantly affected BNIP3 expression, whereas BNIP3 siRNA induced marked (up to 80%) suppression of BNIP3. *C*, dose-response curves for gemcitabine in the CFPAC-1 pancreatic cancer cell line treated with siRNA. ▼, CFPAC-1 treated with control siRNA; ■, CFPAC-1 treated with lamin siRNA; and ▲, CFPAC-1 treated with BNIP3 siRNA. CFPAC-1 treated with BNIP3 siRNA shows an increase in resistance to gemcitabine.

drugs (28). PXN is a substrate for FAK and SRC, whereas negative regulators of these also bind directly to it (35). Altogether, these results strongly support the importance of the PI3K/Akt pathway in gemcitabine sensitivity in pancreatic cancer.

The TGF-β pathway has also been reported to be involved in sensitivity to cisplatin chemotherapy in a leukemia model. Stimulation of TGF-β receptors leads to activation of Smad proteins that cause growth inhibition and induce apoptosis in normal cells. Several pancreatic cancer cell lines are resistant to TGF-β–induced growth arrest (36) and that might be another reason why pancreatic cancer is resistant to chemotherapeutic reagents. In our gene list, *RALBP1*, *SMAD2*, and *LTBP1* are all members of the TGF-β signaling pathway expressed in resistant cell lines and can also potentially contribute to gemcitabine sensitivity.

Interestingly, among our selected genes, several are located at the same chromosomal regions and these loci are also previously reported as frequent sites for aberrations and amplification in pancreatic cancer (25–27). This could be a possible reason for differential abundance of those gene transcripts in pancreatic cancer cells.

In this study, we identified *BNIP3* as a gene strongly associated with intrinsic resistance to gemcitabine and frequently down-regulated in pancreatic cancer. We also show that suppression of BNIP3 by siRNA reduced gemcitabine-induced cytotoxicity in pancreatic cancer cells *in vitro*. Previously, BNIP3 expression was shown to be down-regulated in clones with acquired resistance against 5-fluorouracil compared with their parental colorectal cancer cell line (37). Another study showed that BNIP3 expression was associated with paclitaxel response in an ovarian cancer model (38).

BNIP3, a member of the BH3-only subfamily of Bcl-2 family proteins, heterodimerizes and antagonizes the activity of prosurvival proteins such as Bcl-2 and Bcl-xl, thus promoting apoptosis. Overexpression of BNIP3 induces cell death characterized by its localization at the mitochondria, by opening of the permeability transition pore, and by loss of membrane potential and production of reactive oxygen species (39, 40).

BNIP3 expression is normally undetectable in most tissues, but it has been reported to be expressed in hypoxic regions (41, 42) and can be induced in cell lines by hypoxia *in vitro* (43). Despite the fact that pancreatic cancer usually grows under hypoxic conditions (44, 45), our study shows that BNIP3 expression levels in both cell lines and tissues from surgically resected specimens are low. Furthermore, we have determined that hypoxia does not induce expression of BNIP3 in cell lines that are intrinsically resistant to gemcitabine (supplementary data available at http://sci.cancerresearchuk.org/axp/mphh/ccr04/). Recently, Okami et al. (46) clearly showed a high prevalence of BNIP3 down-regulation in pancreatic cancer and showed that it is caused by methylation of its promoter site.

In our study of a large series of clinical specimens and cell lines, we were able to show that only a small proportion of cases continue to express BNIP3 at normal levels (those observed in normal pancreas which is composed predominantly of acinar tissue). It will be interesting to integrate analysis of BNIP3 status in biomarker studies for clinical trials of chemotherapeutic agents in pancreatic cancer, where responses are typically observed in a similarly small proportion of cases (47).

In conclusion, we have highlighted the potential importance of the PI3K/Akt pathway in gemcitabine resistance and have shown the effect of BNIP3 on gemcitabine sensitivity in a pancreatic cancer cell line model. This is the first report that targeting BNIP3 could increase tumor cell susceptibility to such a chemotherapeutic agent. BNIP3 could therefore be a promising candidate marker for gemcitabine chemosensitivity, and determining BNIP3 status could potentially aid in decision-making when treating patients with pancreatic adenocarcinoma, as well as representing a potential gene therapeutic target to increase gemcitabine sensitivity.

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