

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

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

**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'-difluorodeoxycytidine, Gemzar, Eli-Lilly, Indianapolis, IN), a novel pyrimidine nucleoside analogue, has become the standard chemothera-

peutic 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-x1 (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:** <sup>1</sup>Cancer Research UK Clinical Centre at Barts and London School of Medicine, London, United Kingdom and <sup>2</sup>Division of Gastroenterological Surgery, Department of Surgery, Tohoku University School of Medicine, Sendai, Japan

Received 9/2/04; revised 12/14/04; accepted 1/5/05.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**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.

©2005 American Association for Cancer Research.

showed that under hypoxic conditions pancreatic cancer cell lines become resistant to apoptosis primarily by activation of PI3K/Akt and nuclear factor  $\kappa$ B 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<sub>2</sub> 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 ( $\text{perm} = 10,000$ ) 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'-CGCC-TTCGGGTGTTAAAGA-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  $\mu$ g/mL aprotinin, and 10  $\mu$ g/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  $\mu$ g 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  $\mu$ g/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

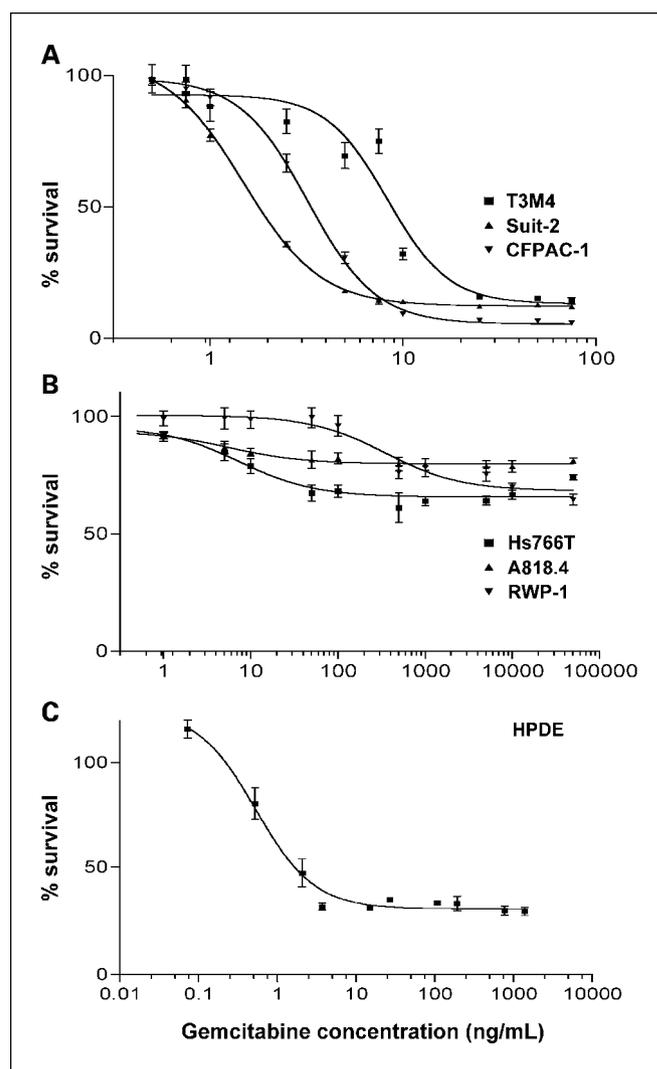
**Efficacy of cytotoxicity induced in pancreatic cancer cell lines by gemcitabine exposure.** The responses of 15 pancreatic cancer cell lines to gemcitabine treatment were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Representative dose-response curves are 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- $\beta$  (TGF- $\beta$ ) 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	dynamain 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, $\beta$ 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

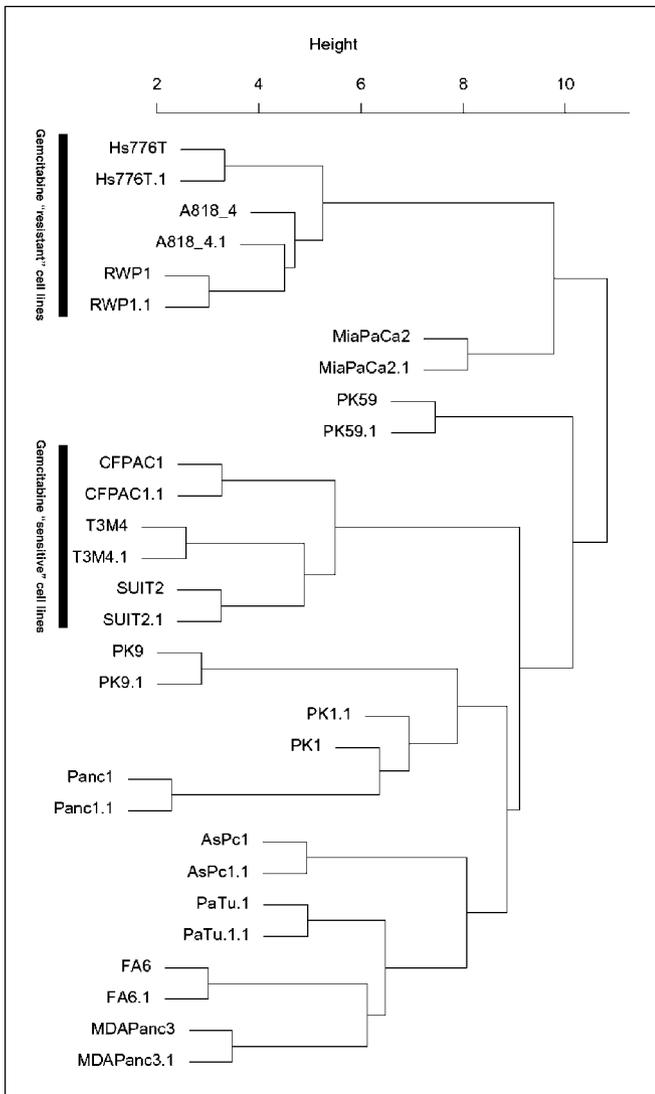
CFPAC-1. Especially in resistant but also in intermediate sensitive cell lines, BNIP3 expression levels were down-regulated more than 90% (Fig. 3A). Only the PANC-1 cell line in the intermediate sensitive group showed an expression level comparable to that of the immortalized normal cell line HPDE.

**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	<i>DYT1</i>	Hs.278429	<i>Homo sapiens</i> dystonia 1, torsion	9q34.11	5.76e-05	7.10491
29	<i>VIPR1</i>	Hs.348500	vasoactive intestinal peptide receptor 1	3p22.1	0.00014	6.50633
30	<i>ATP11C</i>	Hs.88252	ATPase, Class VI, type 11C	Xq27.1	0.00055	5.55326
31	<i>MTSS1</i>	Hs.77694	metastasis suppressor 1	8q24.13	0.00051	5.52812
32	<i>SAFB</i>	Hs.23978	scaffold attachment factor B	19p13.2	0.00068	5.13198
33	<i>RALBP1</i>	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	<i>SMAD2</i>	Hs.110741	SMAD, mothers against DPP homologue 2	18q21.1	4.3e-05	7.82148
35	<i>FAK</i>	Hs.434281	focal adhesion kinase	8q24	0.00029	7.03583
36	<i>TRAF6</i>	Hs.444172	Tumor necrosis factor receptor – associated factor 6	11p13	0.0001	6.71903
37	<i>MADD</i>	Hs.82548	MAP-kinase activating death domain	11p11.2	6.30e-05	6.60535
38	<i>PPP3CB</i>	Hs.187543	protein phosphatase 3 catalytic subunit, $\beta$ isoform	10q22.2	0.00012	6.10191
39	<i>TSC1</i>	Hs.69429	tuberous sclerosis 1	9q34.13	0.00039	5.92062
40	<i>STAT5A</i>	Hs.437058	signal transducer and activator of transcription 5A	17q11.2	0.00015	5.91317
41	<i>SOCS5</i>	Hs.169836	suppressor of cytokine signaling 5	2p21	0.00027	5.88452
42	<i>PHF16</i>	Hs.82292	PHD finger protein 16	Xp11.3	0.00054	5.84577
43	<i>PIK3C3</i>	Hs.418150	phosphoinositide-3-kinase, class 3	18q12.3	0.00035	5.73027
44	<i>UNC13B</i>	Hs.155001	unc-13 homologue B	9p13.3	0.00023	5.70029
45	<i>MAP3K7</i>	Hs.290346	MAP kinase kinase 7	6q15	0.00045	5.49991
46	<i>LTBP1</i>	Hs.241257	latent TGF- $\beta$ binding protein 1	2p22.3	0.0008	5.38788
47	<i>ITGA9</i>	Hs.222	integrin, 9	3p22.3	0.00033	5.35081
48	<i>GNAQ</i>	Hs.469951	guanine nucleotide binding protein (G protein), q polypeptide	2q21.1	0.00066	5.30905
49	<i>MYST3</i>	Hs.93231	MYST histone acetyltransferase (monocytic leukemia) 3	8p11.21	0.00044	5.16659
50	<i>SKIP</i>	Hs.178347	skeletal muscle and kidney enriched inositol phosphatase	17p13.3	0.00052	5.13827
51	<i>C5</i>	Hs.1281	complement component 5	9q33.2	0.00092	4.97948
52	<i>PPP1R15A</i>	Hs.76556	protein phosphatase 1, regulatory (inhibitor) subunit 15A	19q13.33	0.00075	4.81317
Unknown and other function						
53	<i>LRP3</i>	Hs.143641	low density lipoprotein receptor-related protein 3	19q12	6.02e-06	10.2416
54	<i>SLC9A3</i>	Hs.123044	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3	5p15.33	0.0002	6.85467
55	<i>HSU79266</i>	Hs.23642	protein predicted by clone 23627	11q13.1	0.00037	6.59899
56	<i>MCART1</i>	Hs.46791	mitochondrial carrier triple repeat 1	9p13.3	0.00014	6.03726
57	<i>SEMA5A</i>	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	<i>ELN</i>	Hs.252418	elastin	7q11.23	0.00025	5.53682
59	<i>KIAA0663</i>	Hs.17969	KIAA0663 gene product	1q32.1	0.0003	5.45804
60	<i>OGT</i>	Hs.405410	O-linked N-acetylglucosamine (GlcNAc) transferase	Xq13.1	0.00035	5.29243
61	<i>KIAA0261</i>	Hs.439188	KIAA0261	10q23.2	0.00041	5.26712
62	<i>UAP1</i>	Hs.21293	UDP-N-acetylglucosamine pyrophosphorylase 1	1q24.2	0.00049	5.22761
63	<i>INPP5E</i>	Hs.25156	inositol polyphosphate-5-phosphatase, 72 kDa	9q34.3	0.00039	5.22321
64	<i>HECA</i>	Hs.6679	headcase homologue ( <i>Drosophila</i> )	6q24.1	0.00095	5.16452
65	<i>RPS21</i>	Hs.372960	ribosomal protein S21	20q13.33	0.00071	5.03318
66	<i>PCSK7</i>	Hs.443752	proprotein convertase subtilisin/kexin type 7	11q23.2	0.0006	4.94516
67	<i>SEC23B</i>	Hs.173497	Sec23 homologue B	20p11.23	0.00063	4.92033
68	<i>MGEA5</i>	Hs.5734	meningioma expressed antigen 5	10q24.32	0.00096	4.79081
69	<i>C6orf93</i>	Hs.33944	chromosome 6 open reading frame 93	6q24.2	0.00095	4.73743
70	<i>HAGH</i>	Hs.155482	hydroxyacylglutathione hydrolase	16p13.3	0.00082	4.72254
71	<i>LAMP2</i>	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.

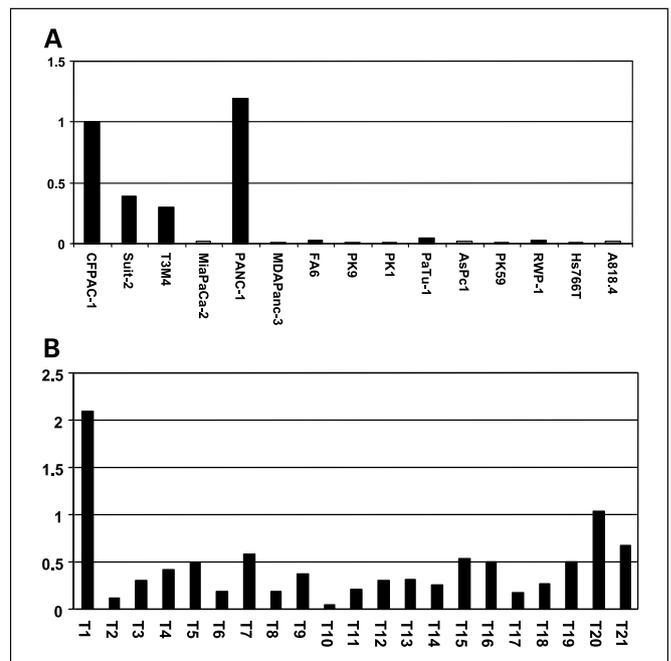
done on the gemcitabine-sensitive CFPAC-1 pancreatic cancer cell line. The ability of BNIP3 siRNA to suppress BNIP3 expression was confirmed by both quantitative real-time RT-PCR (Fig. 4A) and Western blot (Fig. 4B). After transfection with BNIP3 siRNA, up to 80% suppression of BNIP3 expression was observed. CFPAC-1 cells treated with BNIP3 siRNA also showed an increase in drug resistance with the IC<sub>50</sub> 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**

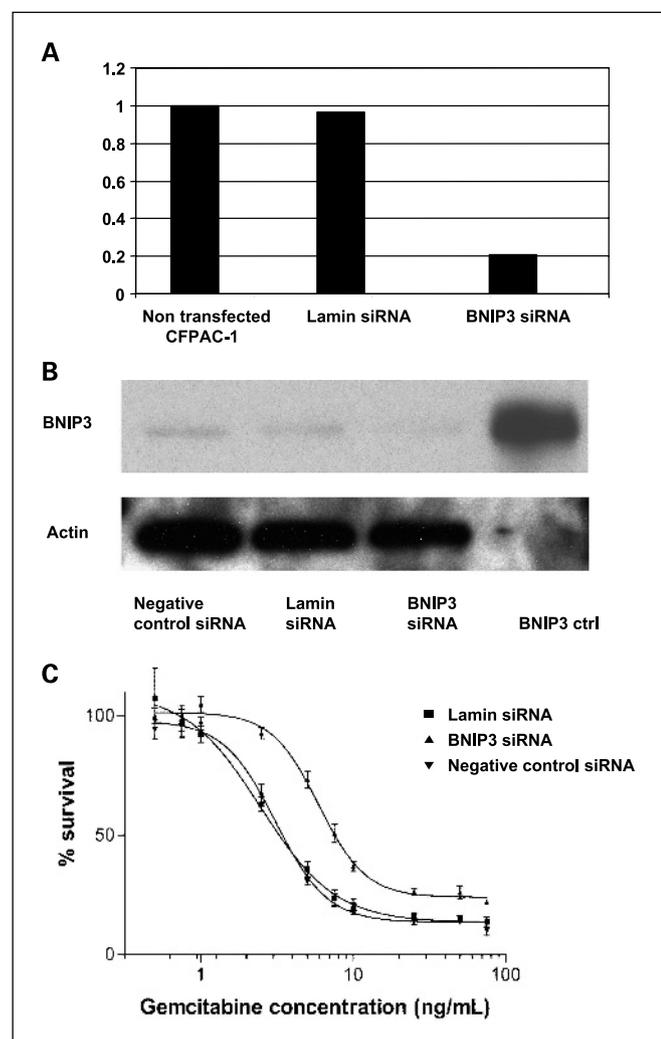
In the current study we were able to identify a number of genes which are potentially involved in intrinsic resistance to gemcitabine in pancreatic cancer cell lines. Some of these are involved in signaling pathways known from other investiga-

tions 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- $\beta$  pathway has also been reported to be involved in sensitivity to cisplatin chemotherapy in a leukemia model. Stimulation of TGF- $\beta$  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- $\beta$ -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- $\beta$  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.

## References

- Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA Cancer J Clin* 2003;53:5–26.
- Casper ES, Green MR, Kelsen DP, et al. Phase II trial of gemcitabine (2,2'-difluorodeoxycytidine) in patients with adenocarcinoma of the pancreas. *Invest New Drugs* 1994;12:29–34.
- Rothenberg ML, Moore MJ, Cripps MC, et al. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol* 1996;7:347–53.
- 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;15:2403–13.
- Rauchwerger DR, Firby PS, Hedley DW, Moore MJ. Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. *Cancer Res* 2000;60:6075–9.
- Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res* 1998;58:4349–57.
- Dumontet C, Bauchu EC, Fabianowska K, et al. Common resistance mechanisms to nucleoside analogues in variants of the human erythroleukemic line K562. *Adv Exp Med Biol* 1999;457:571–7.
- Goan YG, Zhou B, Hu E, Mi S, Yen Y. Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2'-difluorodeoxycytidine in the human KB cancer cell line. *Cancer Res* 1999;59:4204–7.
- Liu X, Zhou B, Xue L, et al. Nuclear factor Y regulation and promoter transactivation of human ribonucleotide reductase subunit M2 gene in a gemcitabine resistant KB clone. *Biochem Pharmacol* 2004;67:1499–511.
- Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA. An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. *Cancer Res* 2004;64:3761–6.
- Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. *Oncogene* 2004;23:1539–48.
- Galmarini CM, Clarke ML, Jordheim L, et al. Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene. *BMC Pharmacol* 2004;4:8.
- Galmarini CM, Clarke ML, Falette N, Puisieux A, Mackey JR, Dumontet C. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. *Int J Cancer* 2002;97:439–45.
- Shi X, Liu S, Kleeff J, Friess H, Buchler MW. Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. *Oncology* 2002;62:354–62.
- Schniewind B, Christgen M, Kurdow R, et al. Resistance of pancreatic cancer to gemcitabine treatment is dependent on mitochondria-mediated apoptosis. *Int J Cancer* 2004;109:182–8.
- Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Inhibition of SRC tyrosine kinase impairs inherent and acquired gemcitabine resistance in human pancreatic adenocarcinoma cells. *Clin Cancer Res* 2004;10:2307–18.
- Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. siRNA directed against c-Src enhances pancreatic adenocarcinoma cell gemcitabine chemosensitivity. *J Am Coll Surg* 2004;198:953–9.
- Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Focal adhesion kinase gene silencing promotes anoikis and suppresses metastasis of human pancreatic adenocarcinoma cells. *Surgery* 2004;135:555–62.
- Yokoi K, Fidler IJ. Hypoxia increases resistance of human pancreatic cancer cells to apoptosis induced by gemcitabine. *Clin Cancer Res* 2004;10:299–306.
- Kobari M, Matsuno S, Sato T, Kan M, Tachibana T. Establishment of a human pancreatic cancer cell line and detection of pancreatic cancer associated antigen. *Tohoku J Exp Med* 1984;143:33–46.
- Furukawa T, Duguid WP, Rosenberg L, Viallet J, Galloway DA, Tsao MS. Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. *Am J Pathol* 1996;148:1763–70.
- Terris B, Blaveri E, Crnogorac-Jurcovic T, et al. Characterization of gene expression profiles in intraductal papillary-mucinous tumors of the pancreas. *Am J Pathol* 2002;160:1745–54.
- Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 2002;18 Suppl 1:S96–104.
- Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* 2001;125:279–84.
- Iacobuzio-Donahue CA, van der Heijden MS, Baumgartner MR, et al. Large-scale allelotyping of pancreaticobiliary carcinoma provides quantitative estimates of genome-wide allelic loss. *Cancer Res* 2004;64:871–5.
- Schleger C, Arens N, Zentgraf H, Bleyl U, Verbeke C. Identification of frequent chromosomal aberrations in ductal adenocarcinoma of the pancreas by comparative genomic hybridization (CGH). *J Pathol* 2000;191:27–32.
- Ghadimi BM, Schrock E, Walker RL, et al. Specific chromosomal aberrations and amplification of the AIB1 nuclear receptor coactivator gene in pancreatic carcinomas. *Am J Pathol* 1999;154:525–36.
- Neri LM, Borgatti P, Tazzari PL, et al. The phosphoinositide 3-kinase/AKT1 pathway involvement in drug and all-trans-retinoic acid resistance of leukemia cells. *Mol Cancer Res* 2003;1:234–46.
- Franke TF, Hornik CP, Segel L, Shostak GA, Sugimoto C. PI3K/Akt and apoptosis: size matters. *Oncogene* 2003;22:8983–98.
- Seufferlein T. Novel protein kinases in pancreatic cell growth and cancer. *Int J Gastrointest Cancer* 2002;31:15–21.
- Asnaghi L, Calastretti A, Bevilacqua A, et al. Bcl-2 phosphorylation and apoptosis activated by damaged microtubules require mTOR and are regulated by Akt. *Oncogene* 2004;23:5781–91.
- Turner CE. Paxillin and focal adhesion signalling. *Nat Cell Biol* 2000;2:E231–6.
- Schaller MD. FAK and paxillin: regulators of N-cadherin adhesion and inhibitors of cell migration? *J Cell Biol* 2004;166:157–9.
- Stopkova P, Saito T, Papolos DF, et al. Identification of PIK3C3 promoter variant associated with bipolar disorder and schizophrenia. *Biol Psychiatry* 2004;55:981–8.
- Turner CE. Paxillin interactions. *J Cell Sci* 2000;113 Pt 23:4139–40.
- Nicolas FJ, Hill CS. Attenuation of the TGF- $\beta$ -Smad signaling pathway in pancreatic tumor cells confers resistance to TGF- $\beta$ -induced growth arrest. *Oncogene* 2003;22:3698–711.
- de Angelis PM, Fjell B, Kravik KL, et al. Molecular characterizations of derivatives of HCT116 colorectal cancer cells that are resistant to the chemotherapeutic agent 5-fluorouracil. *Int J Oncol* 2004;24:1279–88.
- Bani MR, Nicoletti MI, Alkharouf NW, et al. Gene expression correlating with response to paclitaxel in ovarian carcinoma xenografts. *Mol Cancer Ther* 2004;3:111–21.
- Ray R, Chen G, Vande Velde C, et al. BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. *J Biol Chem* 2000;275:1439–48.
- Vande Velde C, Cizeau J, Dubik D, et al. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* 2000;20:5454–68.
- Sowter HM, Ferguson M, Pym C, et al. Expression of the cell death genes BNIP3 and NIX in ductal carcinoma *in situ* of the breast; correlation of BNIP3 levels with necrosis and grade. *J Pathol* 2003;201:573–80.
- Kubasiak LA, Hernandez OM, Bishopric NH, Webster KA. Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. *Proc Natl Acad Sci U S A* 2002;99:12825–30.
- Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci U S A* 2000;97:9082–7.
- Koong AC, Mehta VK, Le QT, et al. Pancreatic tumors show high levels of hypoxia. *Int J Radiat Oncol Biol Phys* 2000;48:919–22.
- Duffy JP, Eibl G, Reber HA, Hines OJ. Influence of hypoxia and neovascularization on the growth of pancreatic cancer. *Mol Cancer* 2003;2:12.
- Okami J, Simeone DM, Logsdon CD. Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. *Cancer Res* 2004;64:5338–46.
- Neoptolemos JP, Stocken DD, Friess H, et al. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* 2004;350:1200–10.

# Clinical Cancer Research

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

Masanori Akada, Tatjana Crnogorac-Jurcevic, Samuel Lattimore, et al.

*Clin Cancer Res* 2005;11:3094-3101.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/11/8/3094>

**Cited articles** This article cites 47 articles, 16 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/11/8/3094.full#ref-list-1>

**Citing articles** This article has been cited by 17 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/11/8/3094.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://clincancerres.aacrjournals.org/content/11/8/3094>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.