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Short Communications

Expression of the Antiapoptotic Protein BAG3 Is a Feature of Pancreatic Adenocarcinoma and Its Overexpression Is Associated With Poorer Survival

Alessandra Rosati,*† Samantha Bersani,‡ Francesca Tavano,§ Elisa Dalla Pozza,¶ Margot De Marco,*† Marta Palmieri,¶ Vincenzo De Laurenzi,†‖ Renato Franco,** Giosuè Scognamiglio,** Raffaele Palaia,†† Andrea Fontana,‡‡ Pierluigi di Sebastiano,§ Massimo Donadelli,¶ Ilaria Dando,¶ Jan Paul Medema,§§ Frederike Dijk,¶¶ Lieke Welling,‖‖ Fabio Francesco di Mola,§ Raffaele Pezzilli,*** Maria Caterina Turco,*† and Aldo Scarpa‡

From the Department of Pharmaceutical and Biomedical Sciences and BIOUNIVERSA SRL,† University of Salerno, Fisciano (SA), Italy; the ARC-NET Center for Applied Research on Cancer and Department of Pathology and Diagnostics,‡ Hospital and University Trust, Verona, Italy; the Department of Surgery§ and Unit of Biostatistics,‡‡ "Casa Sollievo della Sofferenza" Hospital, IRCCS, San Giovanni Rotondo, Italy; the Biochemistry Section,¶ Department of Life and Reproduction Sciences, University of Verona, Verona, Italy; the Department of Biomedical Sciences,‖ University "G. d'Annunzio" Chieti-Pescara and "G. d'Annunzio" Foundation, Research Centre on Aging, Ce.S.I., Chieti, Italy; U.O.C. Pathological Anatomy,** and the Abdominal Surgery Unit,†† National Tumor Institute "G. Pascale" Foundation, Naples, Italy; the Laboratory for Experimental Oncology and Radiobiology,§§ Center for Experimental Molecular Medicine, and the Departments of Pathology¶¶ and Surgery,‖‖ Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and the Pancreas Unit,*** Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, Bologna, Italy*

Pancreatic ductal adenocarcinoma (PDAC) is one of the most deadly cancers, being the fourth leading cause of cancer-related deaths. Long-term survival reaching 15% is achieved in less than 5% of patients who undergo surgery, and median survival is only 6 months in those with inoperable lesions. A deeper understanding of PDAC biologic characteristics as well as novel prognostic markers are therefore required to improve outcomes. Herein we report that BAG3, a protein with recognized anti-apoptotic activity, was expressed in 346 PDACs analyzed, but was not expressed in the surrounding nonneoplastic tissue. In a cohort of 66 patients who underwent radical resection (R0), survival was significantly shorter in patients with high BAG3 expression (median, 12 months) than in those with low BAG3 expression (median, 23 months) ($P = 0.001$). Furthermore, we report that BAG3 expression in PDAC-derived cell lines protects from apoptosis and confers resistance to gemcitabine, offering a partial explanation for the survival data. Our results indicate that BAG3 has a relevant role in PDAC biology, and suggest that BAG3 expression level might be a potential marker for prediction of patient outcome. ***(Am J Pathol 2012, 181: 1524–1529; http://dx.doi.org/10.1016/j.ajpath.2012.07.016)***

Pancreatic cancer is the fourth leading cause of cancer-related deaths worldwide. Early symptoms of the disease are extremely rare, and therefore it is diagnosed in more than two-thirds of patients only when it is locally advanced and/or has metastasized. Consequently, the overall 5-year survival rate among patients is >5%.[1,2] At present, the only chance for cure is radical surgical resection. The median survival in patients who have undergone resection and in whom the tumor is limited to the pancreas (T1 and T2) is about 22.3 months, and in patients with cancer that extends outside the pancreas (T3) is 15.5 months.[3] Factors used to assess prognosis in-

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Address reprint requests to Maria Caterina Turco, M.D., Ph.D., Dipartimento di Scienze Farmaceutiche, DiFarma, University of Salerno, Via Ponte don Melillo, 84084 Fisciano, Italy. E-mail: mcturco@unisa.it.

clude tumor grade, status of resection margins, and presence of lymph node metastases. High levels of the carbohydrate antigen 19–9 (CA 19–9) and its persistence after surgery are negative prognostic indicators,[2] and additional markers have been proposed recently.[4–6]

BAG3 has previously been reported to be strongly expressed in pancreatic tumor cells.[7] The protein is a 74-kDa member of the co-chaperone BAG family and a partner of heat shock protein 70, of which it modulates the activities either positively or negatively, depending on the context.[8] Furthermore, through a WW domain and a proline-rich repeat (PXXP), BAG3 can interact with other proteins.[8] Although its expression is constitutive in only a few cell types, it can be induced in leukocytes and other normal cells in response to stress. It is constitutively expressed in several tumors including leukemia, lymphoma, myeloma, thyroid carcinomas, glioblastomas, and melanomas,[9–15] in which several lines of evidence suggest that it can sustain tumor cell survival. Indeed, down-modulation of BAG3 in primary samples of B-cell chronic lymphocytic leukemia and acute lymphoblastic leukemia results in an increase in both basal and drug-induced apoptosis.[12–13] Recently, we have also reported that BAG3 is strongly expressed in glioblastomas when compared with the less aggressive astrocytomas and that in these tumors it exerts an anti-apoptotic activity by retaining BAX protein in the cytosol, thus preventing its mitochondrial translocation.[14] Furthermore, BAG3 overexpression was observed in a subset of melanomas in humans, with a significant increase in tumor cells in lymph nodes and distant organ metastases.[15] In the present study, we investigated the expression of BAG3 in a large cohort of PDACs, its correlation with survival, and its potential role in response to gemcitabine.

Materials and Methods

Tumor Samples

Tumor samples were obtained from a series of unselected patients who underwent surgical resection of PDAC between January 10, 1992 and September 30, 2010 at the University of Verona (Verona, Italy), the NCI "Fondazione G. Pascale" (Naples, Italy), the Department of Surgery, Academic Medical Center (University of Amsterdam, the Netherlands), and IRCCS "Casa Sollievo della Sofferenza" (San Giovanni Rotondo, Foggia, Italy). Furthermore, we analyzed tissue samples from two tissue microarrays, TMA No. 79560448 pancreatic cancer prognosis (Cambridge Bioscience Ltd., Cambridge, UK) and TMA PA2082 (US-Biomax, Inc., Rockville, MD), that contained also normal pancreas tissue samples from 11 donors (6 men and 5 women; mean ± SD age, 44.6 ± 19.3 years). Altogether, 346 tissue samples with histologically proved PDAC (184 men and 162 women; age, 63 ± 10.5 years) were available for immunohistochemical analysis. For PCR analysis, 25 fresh-frozen tissue samples obtained from patients who underwent surgery because of pancreatic cancer were available. PDAC was ascertained at histologic analysis in all 25 patients (21 men and 4 women; age, 65.4 ± 11.9 years). Clinical characteristics of patients are given in Table 1.

Immunohistochemistry

The immunohistochemistry protocol included deparaffination in xylene, rehydration via decreasing concentrations of alcohol down to pure water, non-enzymatic antigen retrieval in citrate buffer (pH 6.0) for 30 minutes at 95°C, and endogenous peroxidase quenching with H_2O_2 in methanol for 20 minutes. After rinsing with PBS, the samples were blocked using 5% normal horse serum in 0.1% PBS or bovine serum albumin. To detect BAG3, samples were incubated for 1 hour at room temperature with the monoclonal antibody AC-1 (BIOUNIVERSA SRL, Salerno, Italy) at a concentration of 3 μg/mL. After washing thoroughly with PBS, sections were incubated using a biotinylated secondary anti-mouse IgG for 20 minutes, then rinsed, incubated using avidin-biotin-peroxidase complex (Novocastra; Leica Microsystems, Milan, Italy) and developed using diaminobenzidine (Sigma-Aldrich Corp., St. Louis, MO). Finally, the sections were counterstained using hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted using Permount (Fisher Scientific, Inc., Milan, Italy).

Quantitative Real-Time RT-PCR

Tissue specimens of resected pancreatic cancer were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was isolated from frozen tissues and from pancreatic cancer cell lines via phenol extraction (TRIzol Reagent; Invitrogen Corp., Carlsbad, CA). In tissue samples, cancer cellularity was enriched via cryostat sectioning and dissection of most cellular areas. RNA concentration and purity (A260:A280 >2.0; A260:A230 >1.8) were validated using a spectrophotometer (NanoDrop; Thermo Fisher, Inc., Waltham, MA). One microgram total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. A quantitative real-time PCR assay was used to assess the differential expression of BAG3 in tumor tissue samples. Primers for the human BAG3 mRNA were synthesized by Primm SRL (Milan, Italy): forward primer, 5′-CCTGTTAGCTGTGGTTG-3′; and reverse primer, 5′-AACATACAGATATTCCTATGGC-3′. All quantitative PCRs were performed in a final volume of 25 μL, in 3 replicates per sample, using the QuantiFast SYBR Green PCR kit (QIAGEN, Hamburg, Germany), and run in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) according to the following conditions: 95°C for 5 minutes, 40 cycles at 95°C for 10 seconds, and 60°C for 30 seconds. Data were acquired as threshold cycle value using SDS software version 2.1. In each sample, the *BAG3* mRNA relative expression level was obtained using the comparative method, after normalizing for the expression of endogenous GAPDH (glyceraldehyde-3-phosphate dehydrogenase).[16]

Table 1. Analysis of Tumor Samples

Variable	No. of patients	Age mean ± SD, year	Sex		Local tumor stage no. (%)			
			Male	Female	T1	T2	T3	T4
Immunochemistry								
Patients with PDAC	346	63.0 ± 10.05	181	165	5 (1.4)	59 (17.1)	264 (76.3)	18 (5.2)
Patient survival	66	61.9 ± 11.3	36	30	0	1 (1.5)	65 (98.5)	0
RT-PCR								
Patients with PDAC	25*	65.4 ± 11.9	21	4	2 (8.0)	3 (12.0)	20 (80.0)	ND

(table continues)

*At RT-PCR, data for tumor grade differentiation were not available for 1 of 25 patients.
ND, no data; PDAC, pancreatic ductal adenocarcinoma.

Cell Cultures

The pancreatic cancer cell lines (MIA PaCa-2, AsPC-1, PSN1, Capan-1, and PANC-1) were obtained from the ATCC (Manassas, VA) cell bank. MIA PaCa-2 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) and supplemented with 10% fetal bovine serum (FBS) and 2.5% horse serum. AsPC-1 and PSN1 cells were grown in RPMI 1640 medium supplemented with 10% FBS. Capan-1 cells were cultured in RPMI 1640 medium containing 20% FBS, and PANC-1 were cultured in DMEM supplemented with 10% FBS. All media for the above cell lines were purchased from Lonza (Bergamo, Italy) or MediaTech (Manassas, VA), and were supplemented with 100 U/mL penicillin and 2 μg/mL streptomycin (Sigma-Aldrich Corp). Cells were incubated at 37°C in a 5% CO_2 environment, and were treated with gemcitabine [2′,2′-difluorodeoxycytidine (Gemzar; Eli Lilly Italia SPA, Sesto Fiorentino, Italy)] at the indicated concentrations.

Immunoblot Analysis

Cells were collected, washed in PBS, and resuspended in RIPA buffer (pH 8) (150 mmol/L NaCl [pH 8], 50 mmol/L Tris-HCl, 1% NP-40, 0.5% Na-Doc, and 0.1% SDS) and 1X protease inhibitor cocktail (Roche Diagnostics, Milan, Italy) for 30 minutes on ice. The lysates were centrifuged at 10,000 × *g* for 10 minutes at 4°C, and the supernatant was used for Western blot analysis. Protein concentration was measured using the Bradford protein assay reagent (Pierce, Celbio, Milan, Italy) using bovine serum albumin as standard. Fifteen micrograms protein extracts was subjected to electrophoresis via 10% SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes (Millipore SPA, Milan, Italy). Membranes were then incubated for 1 hour at room temperature with blocking solution consisting of 5% low-fat milk in Tris-buffered saline solution and Tween 20 [TBST; 100 mmol/L Tris (pH 7.5), 0.9% NaCl, and 0.1% Tween 20], and probed overnight at 4°C using the rabbit polyclonal antibody TOS-2 (1:5000 in blocking solution) or with anti-GAPDH antibody (mouse monoclonal antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Horseradish peroxidase–conjugated IgG (1:8000 in blocking solution; Millipore) was used to detect specific proteins. Immunodetection was performed using chemiluminescent substrates, and was recorded using Hyperfilm ECL (both from Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Apoptosis

Cells were seeded in 24-well plates (1×10^4 cells per well) and treated with various concentrations of gemcitabine and/or transfected with BAG3 small-interfering RNA (siRNA) or nontargeted siRNA (NTsiRNA) (see figure legends). At the end of treatment, the percentage of sub-G0/G1 cells was analyzed via propidium iodide incorporation into permeabilized cells, and flow cytometry was performed as described previously.[9] Data were analyzed using Student's *t*-test using the Prism statistical program (GraphPad Software, Inc., San Diego, CA). Error bars depict SD. $P = 0.01$ to 0.05 was considered significant; 0.001 to 0.01, very significant; and <0.001, highly significant.

siRNAs and Transfections

A specific siRNA (5′-AAGGUUCAGACCAUCUUGGAA-3′) targeting *BAG3* mRNA and a control NTsiRNA (5′-CAGUCGCGUUUGCGACUGG-3′) were purchased from Dharmacon, Inc. (Lafayette, CO). Cells were transfected with siRNAs at a final concentration of 200 nmol/L using TransFectin (Bio-Rad Laboratories, Inc., Hercules, CA).

Statistical Analysis

Baseline characteristics of patients were reported as mean ± SD or as frequency and percentage for continuous and categorical variables, respectively. Comparisons between low-positive and high-positive results were made using Fisher's exact test and the Mann-Whitney *U* test for categorical and continuous variables, respectively. *BAG3* mRNA expression levels were reported as median, with interquartiles (Q1 and Q3), and comparison between different histologic types of pancreatic cancer was assessed using the Mann-Whitney *U*-test. Association analyses between *BAG3* mRNA, expression and categorical and continuous clinical variables were performed using the Mann-Whitney *U* test or Kruskal-Wallis one-way analysis of variance and Spearman's correlation coefficient, respectively. Survival analyses were performed using the Kaplan-Meier curve and Cox proportional hazards models using commercially available software (release 13; SPSS, Inc., Chicago, IL).

Table 1. *Continued*

Node stage no. (%)		Tumor grade no. (%)		
N0	N1	G1	G2	G3
130 (37.6)	216 (62.4)	16 (4.6)	177 (51.2)	153 (44.2)
13 (19.7)	53 (80.3)	3 (4.5)	37 (56.1)	26 (39.4)
6 (24.0)	19 (76.0)	3 (12.5)	13 (54.2)	8 (33.3)

Results

We analyzed PDAC samples from 346 patients (Table 1). BAG3 staining revealed moderate positivity of the islets of Langerhans, whereas normal pancreatic ducts and pancreatic acinar cells exhibited no BAG3 expression. This was true in both normal pancreas and non-neoplastic pancreatic tissue adjacent to the tumor mass. BAG3 staining was observed predominantly in the cytoplasm of tumor cells. The intensity of staining of BAG3 was variable, as was the number of positive cancer cells.

We assigned a score based on the percentage of positive cancer cells in the sample by counting the number of positive cells over the total cancer cells in 10 non-overlapping fields using ×400 magnification. The median percentage of BAG3-positive cells, calculated as described, was 40%, and this value was used as the cut-off to differentiate low- and high-positive samples. On the basis of this classification, 190 patient samples (55%) were classified as low positive (≤40% of positive cells), and 156 (45%) were classified as high positive (>40% of positive cells) (Figure 1A). More than 75% and 90% of analyzed tumors were, respectively, stage T3 and grade G2/G3 (Table 1). There was no significant correlation between high or low BAG3 positivity and tumor stage or grade (data not shown). Survival analysis was performed in 66 patients, and all lesions examined had resection margins free of tumor cells (R0), and only 3.7% had metastasized to distant organs (Table 1). In patients with high BAG3 expression, survival was significantly shorter (median, 12.0 months) than in those with low BAG3 expression (median, 23.0 months) ($P = 0.001$) (Figure 1B). On the basis of Cox proportional analysis, high BAG3 expression was associated with a greater than twofold higher risk of death (Table 2).

The immunohistochemical data on BAG3 expression was also confirmed measuring BAG3 mRNA levels in 25 PDAC tissue samples (Table 1). In particular, at the time

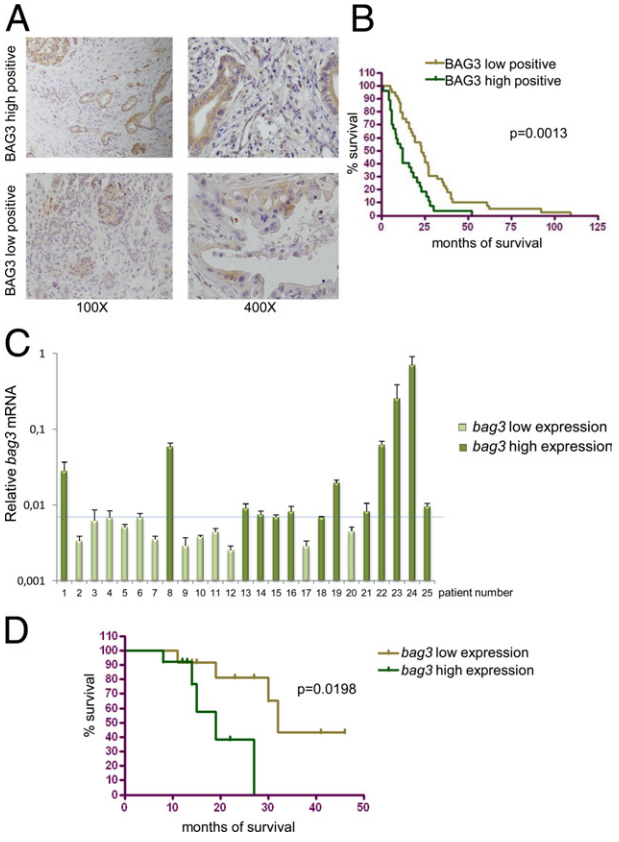

Figure 1. BAG3 expression correlates with patient survival. **A:** Representative images of BAG3 low-positive and BAG3 high-positive tumor samples stained using a monoclonal anti-BAG3 antibody revealed by using a biotinylated secondary antibody. Sections were counterstained with hematoxylin. Two different magnifications are shown: ×100 (**left panels**) and ×400 (**right panels**). **B:** Survival curves were made comparing 39 patients with low BAG3 staining (≤40% of positive cells) with 27 patients with high BAG3 staining (>40% of positive cells). Median survival increased from 12 months in the high-positive group to 23 months in the low-positive group. ($P = 0.001$, log rank test). **C:** *BAG3* mRNA relative expression was evaluated using quantitative RT-PCR. Values are given as mean ± SD. The blue line represents the median value calculated. **D:** Survival analysis was made for all patients analyzed using quantitative RT-PCR. In 13 patients with high *BAG3* expression, survival was shorter (median, 19.0 months) than in 12 patients with low *BAG3* expression (median, 32.0 months) ($P = 0.02$, log rank test).

Table 2. Multivariate Cox Regression Analysis

Variable	HR	95% CI	*P* value
Age, year	0.99	0.97–1.02	0.60
Sex, M/F	0.86	0.48–1.55	0.62
Tumor grade, G2 vs. G1	0.84	0.24–2.98	0.79
Tumor grade (G3 vs G1)	1.55	0.45–5.37	0.49
Local tumor stage, T3 vs T2	2.5	0.30–21.17	0.400
Node stage, N1 vs N0	1.17	0.58–2.37	0.67
BAG3 positivity, high vs low	**2.7**	**1.53–4.78**	**<0.001**
	death events = 66		
	n TOT = 66		

F, female; M, male; CI, confidence interval; HR, hazard ratio.
Bold indicates underlining results from different BAG3 positivity groups.

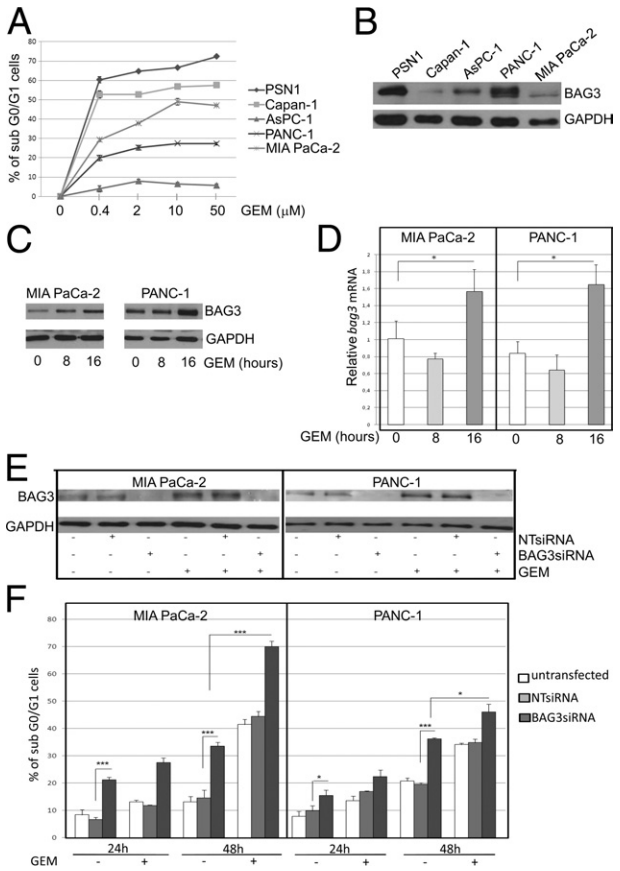

Figure 2. Down-regulation of BAG3 sensitizes PDAC cell lines to gemcitabine. **A:** Pancreatic cancer cell lines (PSN1, Capan-1, AsPC-1, PANC-1, and MIA PaCa-2) were treated using various concentrations of gemcitabine. After 48 hours, apoptotic cell death was analyzed (see *Materials and Methods*). Graph depicts mean ± SD percentage of sub-G0/G1 cells. Data are representative of three independent experiments. **B:** Western blot analysis of BAG3 in pancreatic cancer cell lines; GAPDH housekeeping protein contents were used to monitor equal loading conditions. **C:** MIA PaCa-2 and PANC-1 cell lines were treated using 2 μmol/L gemcitabine (GEM) for the indicated times. BAG3 protein expression levels were monitored using Western blot analysis, and *BAG3* mRNA levels were analyzed using RT-PCR **(D)**; the graph depicts relative *BAG3* mRNA levels (± SD), and data are representative of three independent experiments. **E:** MIA PaCa-2 and PANC-1 cell lines were transfected with BAG3 siRNA or a nontargeted siRNA (NTsiRNA) for 72 hours, then treated with 2 μmol/L gemcitabine (GEM) for 24 hours. BAG3 levels were analyzed using Western blotting, and GAPDH levels were detected to monitor equal loading conditions. **F:** MIA PaCa-2 and PANC-1 cells were transfected as described above, and treated with 2 μmol/L gemcitabine (GEM) for 24 or 48 hours. Apoptotic cell death was analyzed (see *Materials and Methods*). Graph depicts mean ± SD percentage of sub-G0/G1 cells. Data are representative of three independent experiments.

of analysis, 16 of 25 patients had survived and 9 patients had died of pancreatic cancer progression. The median of expression of BAG3 mRNA in tumors analyzed was set at 0.007 (Q1 = 0.004; Q3 = 0.01) (Figure 1C). All of the considered demographic and clinical features in patients with PDAC were unrelated to BAG3 mRNA levels. Thus, correlation with survival was evaluated, and the median BAG3 expression in PDAC samples was used as a cutoff to differentiate patients with low versus high BAG3 expression. Thirteen samples (52%) were thus classified as high BAG3 positive, and 12 samples (48%) as low *BAG3* positive. Patients with high BAG3 expression had shorter survival (median, 19.0 months) than did those with low BAG3 expression (median, 32.0 months) ($P = 0.02$) (Figure 1D). On the basis of Cox proportional analysis, high BAG3 expression was associated with a greater than sixfold higher risk of death (univariate analysis: hazard ratio, 6.09; 95% confidence interval, 1.11–33.60; $P = 0.04$).

The first-line chemotherapy for treatment of pancreatic cancer is gemcitabine. [17] To investigate the role of BAG3 protein in response to gemcitabine, we analyzed the levels of BAG3 protein in pancreatic carcinoma cell lines with various degrees of sensitivity to the drug (Figure 2A).[18–21] The cell lines express very different levels of BAG3 (Figure 2B); however, no correlation between these and sensitivity to gemcitabine can be made. Gemcitabine significantly induced BAG3 expression in all cell lines tested, at both the protein and mRNA levels (Figure 2, C-D). We therefore tested two of the cell lines with intermediate sensitivity to gemcitabine and very different levels of BAG3 (MIA PaCa-2 and PANC-1) to determine whether down-regulation of BAG3 sensitized cells to gemcitabine. To this end, we transfected both cell lines with a specific siRNA that targeted BAG3 mRNA or with a nonspecific NTsiRNA, and treated cells with gemcitabine for the indicated times. Silencing of BAG3 alone induced cell death in both cell lines, and treatment with gemcitabine resulted in a significant increase in cell death that reached 70% in the MIA PaCa-2 cell line (Figure 2, E–F). These data clearly show that BAG3 is a survival factor for pancreatic cancer cells and that its down-regulation when combined with gemcitabine can contribute to their eradication.

Discussion

Pancreatic ductal adenocarcinoma is one of the most deadly cancers because of its aggressiveness and resistance to current therapies. Identification of novel prognostic markers that will enable better classification of patients and better knowledge of the tumor biology are mandatory if we hope to improve survival. BAG3 is overexpressed both at the protein and mRNA levels in pancreatic adenocarcinomas, and, more important, high expression levels are associated with a greater risk of death. Markers that can identify patients with resection margins free of tumor cells (R0), with a better prognosis, are required for assistance in making therapeutic choices. In this respect, it is interesting that in a homogeneous set of R0 samples, BAG3 expression inversely correlated with patient survival. Patients with tumors that expressed low positivity of BAG3 expression had significantly ($P = 0.001$) longer survival (median, 23 months) than did patients with tumors that expressed high levels of the protein (median, 12 months). Similarly, although in a smaller cohort of patients, survival was inversely correlated with BAG3 mRNA levels.

In addition, BAG3 down-modulation enhances apoptosis in PDAC cell lines, which supports the theory that BAG3 up-regulation is important to survival of pancreatic cancer cells and contributes to their pathogenesis. We and others have previously reported increased BAG3 expression in various tumors.[9,11–15] In all tumors studied to date, however, only some were positive for BAG3 expression, with the highest percentages reached in melanomas (71%)[15] and glioblastomas (88.9%).[14] In the pres-

ent study, all 346 PDACs studied were positive for BAG3, again suggesting that this alteration may have a role in the biology of this tumor. This is probably linked to the ability to act as an important survival factor for tumor cells. This is consistent with previous studies that found that BAG3 exerts an anti-apoptotic activity via multiple mechanisms. We have previously reported that in melanoma cells, BAG3 protects cells by increasing NF-κB activation,[9] whereas in glioblastomas, it does so by sequestering BAX protein in the cytosol, thus preventing activation of the intrinsic apoptotic pathway.[14] It therefore seems that BAG3, because of its multifaceted ability to complex with many proteins, can regulate different factors depending on the specific cell context, however always resulting in increased survival of the cell.[14] We also found that gemcitabine induces BAG3 up-regulation, and this can possibly contribute to survival of cells undergoing treatment. BAG3 regulation by gemcitabine seems to be at the mRNA level. This could depend on the reported ability of gemcitabine to up-regulate *WT1*,[22] which in turn binds and activates BAG3 promoter.[23]

In conclusion, our results show that BAG3 is overexpressed in PDAC samples and that in samples from patients who have undergone radical resection, low expression of the protein evaluated either via immunohistochemistry or PCR correlates with higher survival. Furthermore, since BAG3 down-modulation results in increased death of pancreatic cancer cells alone or in combination with gemcitabine *in vitro*, the relation of this marker with patient response to therapy seems worthy of investigation. Because of its wide expression in all of the lesions tested and its involvement in sustaining pancreatic cancer cell survival, BAG3 could represent a valuable target for innovative therapies in PDAC.

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