

# The Role of HuR in Gemcitabine Efficacy in Pancreatic Cancer: HuR Up-regulates the Expression of the Gemcitabine Metabolizing Enzyme Deoxycytidine Kinase

Christina L. Costantino,<sup>1</sup> Agnieszka K. Witkiewicz,<sup>2</sup> Yuki Kuwano,<sup>4</sup> Joseph A. Cozzitorto,<sup>1</sup> Eugene P. Kennedy,<sup>1</sup> Abhijit Dasgupta,<sup>3</sup> Judith C. Keen,<sup>5</sup> Charles J. Yeo,<sup>1</sup> Myriam Gorospe,<sup>4</sup> and Jonathan R. Brody<sup>1</sup>

<sup>1</sup>Department of Surgery, Jefferson Center for Pancreatic, Biliary and Related Cancers, Departments of <sup>2</sup>Pathology and <sup>3</sup>Pharmacology and Experimental Therapeutics, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania; <sup>4</sup>LCMB, National Institute on Aging-Intramural Research Program, NIH, Baltimore, Maryland; and <sup>5</sup>Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Camden, New Jersey

## Abstract

RNA-binding protein HuR binds U- or AU-rich sequences in the 3'-untranslated regions of target mRNAs, stabilizing them and/or modulating their translation. Given the links of HuR with cancer, we studied the consequences of modulating HuR levels in pancreatic cancer cells. HuR-overexpressing cancer cells, in some instances, are roughly up to 30-fold more sensitive to treatment with gemcitabine, the main chemotherapeutic component of treatment regimens for pancreatic ductal adenocarcinoma (PDA), compared with control cells. In pancreatic cancer cells, HuR associates with deoxycytidine kinase (dCK) mRNA, which encodes the enzyme that metabolizes and thereby activates gemcitabine. Gemcitabine exposure to pancreatic cancer cells enriches the association between HuR and dCK mRNA and increases cytoplasmic HuR levels. Accordingly, HuR overexpression elevates, whereas HuR silencing reduces, dCK protein expression in pancreatic cancer cells. In a clinical correlate study of gemcitabine treatment, we found a 7-fold increase in risk of mortality in PDA patients with low cytoplasmic HuR levels compared with patients with high HuR levels, after adjusting for other treatments and demographic variables. These data support the notion that HuR is a key mediator of gemcitabine efficacy in cancer cells, at least in part through its ability to regulate dCK levels posttranscriptionally. We propose that HuR levels in PDA modulate the therapeutic efficacy of gemcitabine, thus serving as a marker of the clinical utility of this common chemotherapeutic agent and a potential target for intervention in pancreatic cancer. [Cancer Res 2009;69(11):4567-72]

## Introduction

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer-related deaths in the United States (1). Currently, two therapeutic options that provide the best clinical benefit are surgical resection and chemotherapy regimens that include gemcitabine (2',2'-difluorodeoxycytidine). For over 10 years, gemci-

tabine has been the reference drug for the treatment of this often fatal disease (2). Gemcitabine is also used to treat other malignancies including non-small cell lung, breast, gastric, and ovarian cancers. Gemcitabine uses the same key metabolic enzyme for activation within the cell, deoxycytidine kinase (dCK), as does a previously developed and related nucleoside analogue cytarabine (Ara-C; ref. 3). dCK phosphorylates the prodrug, gemcitabine, generating the active metabolites gemcitabine diphosphates and triphosphates that inhibit DNA chain elongation and cause cellular death (4). The levels of dCK correlate with overall patient survival after gemcitabine-based therapy in PDA specimens ( $P = 0.0425$ ; ref. 4).

The stress-response protein Hu antigen R (HuR) is an RNA-binding protein that regulates gene expression posttranscriptionally. Like other related Hu/elav proteins, HuR harbors three conserved RNA recognition motifs through which it binds to target mRNAs (5) that frequently have AU- or U-rich stretches in their 3'-untranslated regions (UTR). HuR is predominantly nuclear, but in response to various stimuli, it is mobilized to the cytoplasm, prolongs target mRNA half-life, and can modulate target mRNA translation (5). Many HuR target mRNAs encode stress-response, immune-response, cell cycle regulatory proteins, oncogenes, and tumor suppressor genes (5). HuR modulates these transcripts in response to stimuli such as therapeutic agents (i.e., tamoxifen and prostaglandins), nutrient depletion (polyamines, amino acid starvation), heat shock, immune stimuli, short-wavelength UV irradiation, oxidants, and transcriptional inhibitors (actinomycin D; refs. 5-7).

Although HuR has never been reported to be mutated in cancer, it has been proposed to contribute to the tumorigenesis process (8-10). Elevated cytoplasmic accumulation of HuR both correlates with high-grade malignancy and serves as a prognostic factor of poor clinical outcome in some cancers (11-14). Given the extensive research examining the role of HuR in cancer and stress response over the past decade (5), we explored the role of HuR in PDA and the relationship of HuR to chemotherapeutic efficacy.

## Materials and Methods

**Transfection of pancreatic cancer cell lines.** HuR cDNA sequence was cloned into the pcDNA 3.1.Zeo vector (Invitrogen) for stable transfection of pancreatic cancer cell lines MiaPaca2, PL-5, and Hs766t (15). Pooled cells remained under selection media containing Zeocin (Invitrogen) for several months after transfection. Mia.HuR, PL5.HuR, and Hs766t.HuR denote HuR overexpressing lines; Mia.EV, PL5.EV, and Hs766t

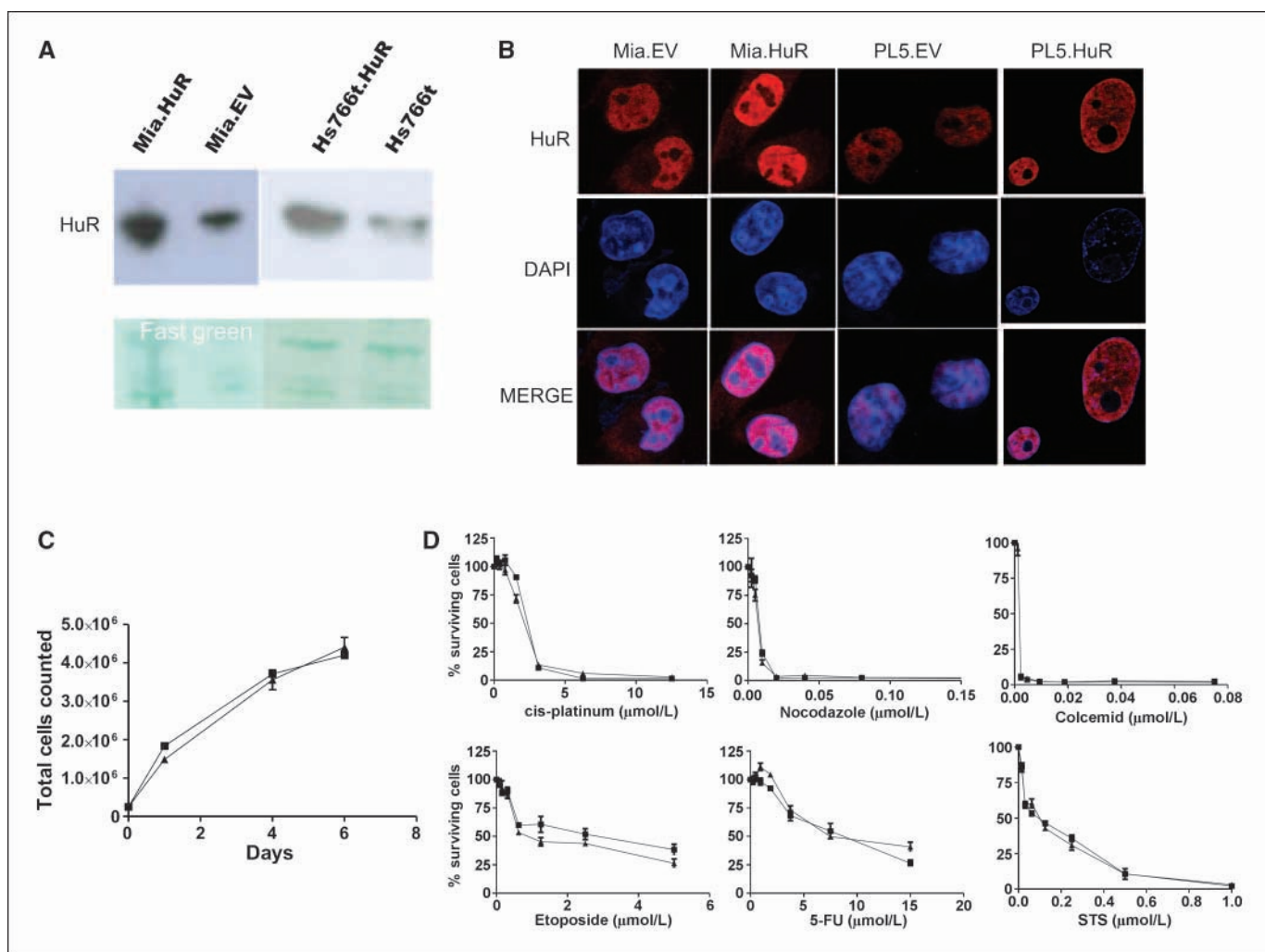
**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Note:** C.L. Costantino and A.K. Witkiewicz contributed equally.

**Requests for reprints:** Jonathan R. Brody, Department of Surgery, Thomas Jefferson University, 1015 Walnut Street, Curtis 611A, Philadelphia, PA 19107. Phone: 215-955-2693; E-mail: Jonathan.Brody@jefferson.edu.

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**Figure 1.** Characterization of HuR-overexpressing pancreatic cancer cell lines. *A*, immunoblot analysis of HuR expression in lysates from MiaPaCa2 (Mia.HuR and Mia.EV) and Hs766t (Hs766t.HuR and Hs766t) cells. Fast Green staining confirmed the equality of protein loading. *B*, immunofluorescence to detect HuR and nuclei (DAPI). *C*, Mia.HuR and Mia.EV cell proliferation rates, as determined by direct cell counts. *D*, cell survival was measured by PicoGreen after incubation of cells for 5 to 7 d with the indicated compounds. Points, means from three measurements in a single experiment; bars, SE; each experiment is representative of at least three individual experiments. ▲, Mia.HuR cells; ■, Mia.EV cells.

denote empty vector or control lines. HuR and control siRNA sequences and transfection conditions were as described (7).

**Immunofluorescence.** Cells were plated on LabTek II Chamber slides (Fisher Scientific) and fixed in 3% paraformaldehyde (20 min, room temperature). Cells were washed with PBS and permeabilized using 0.5% Triton X-100/1% normal goat serum (Vector Laboratories) in PBS (15 min). After washes in 1% goat serum/PBS, cells were incubated (1:50 dilution, 1 h, room temperature) with mouse anti-HuR (Santa Cruz) or anti-dCK (Abnova) primary antibodies. After washes in PBS, cells were incubated for 1 h with goat anti-mouse secondary antibody (1:400; Alexa Fluor 647; Molecular Probes). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and cells were evaluated under a Zeiss LSM-510 Confocal Laser Microscope.

**Drug sensitivity assay.** Mia.HuR, Mia.EV, Hs766t.HuR, Hs766t, and PL5.HuR, PL5.EV cells were seeded (1,000 cells per well) in 96-well plates and treated with Etoposide, 5-Fluorouracil, Cis-platin, Staurosporine, Nocodazole, Colcemid, Ara-C (Sigma), and gemcitabine (Gemzar; Eli-Lilly) for 6 to 7 d. After treatment, cells were washed with PBS and lysed with 100 μL of water/well; cell viability was quantified by staining of double-stranded DNA with Quant-iT PicoGreen (Invitrogen) and analyzed with a TECAN SpectraFluor.

**Immunoblot.** Whole-cell, cytoplasmic, and nuclear lysates were prepared as described (7), and protein was size-fractionated by SDS-PAGE

(10% acrylamide). Membranes were blocked for 1 h in 5% Milk/TBS-T and incubated overnight with monoclonal antibodies (Santa Cruz) recognizing HuR, dCK, the cytoplasmic marker  $\alpha$ -Tubulin, or the nuclear marker hnRNP. Membranes were washed with TBS-T and incubated with secondary antibodies; and the resulting signals were visualized by chemiluminescence (Millipore). Total protein was visualized with Fast Green (USB).

**Cell cycle analysis and apoptosis assay.** Mia.EV and Mia.HuR cell lines were either left untreated or treated with 0.03 μmol/L gemcitabine for 48 h. For cell cycle analysis, cells were then fixed in 100% ethanol, and stained with a propidium iodide solution containing RNase A (Sigma Aldrich). For apoptosis assays, cells were resuspended at 10<sup>6</sup> cells/mL and incubated in Annexin V and Propidium Iodide, following the manufacturers' protocol (FITC Annexin V; BD Pharmigen). Both assays were analyzed by flow cytometry.

**RNA-binding: biotin pulldown and RNP IP assays.** MiaPaCa2 cells were treated with 4 μmol/L gemcitabine and collected 48 h later. For biotin pulldown analysis (7), cytoplasmic extracts were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology). Probes for biotin pull-down analysis were synthesized as described (7) using the following PCR primers (sense and antisense, respectively) containing the T7 RNA polymerase promoter sequence

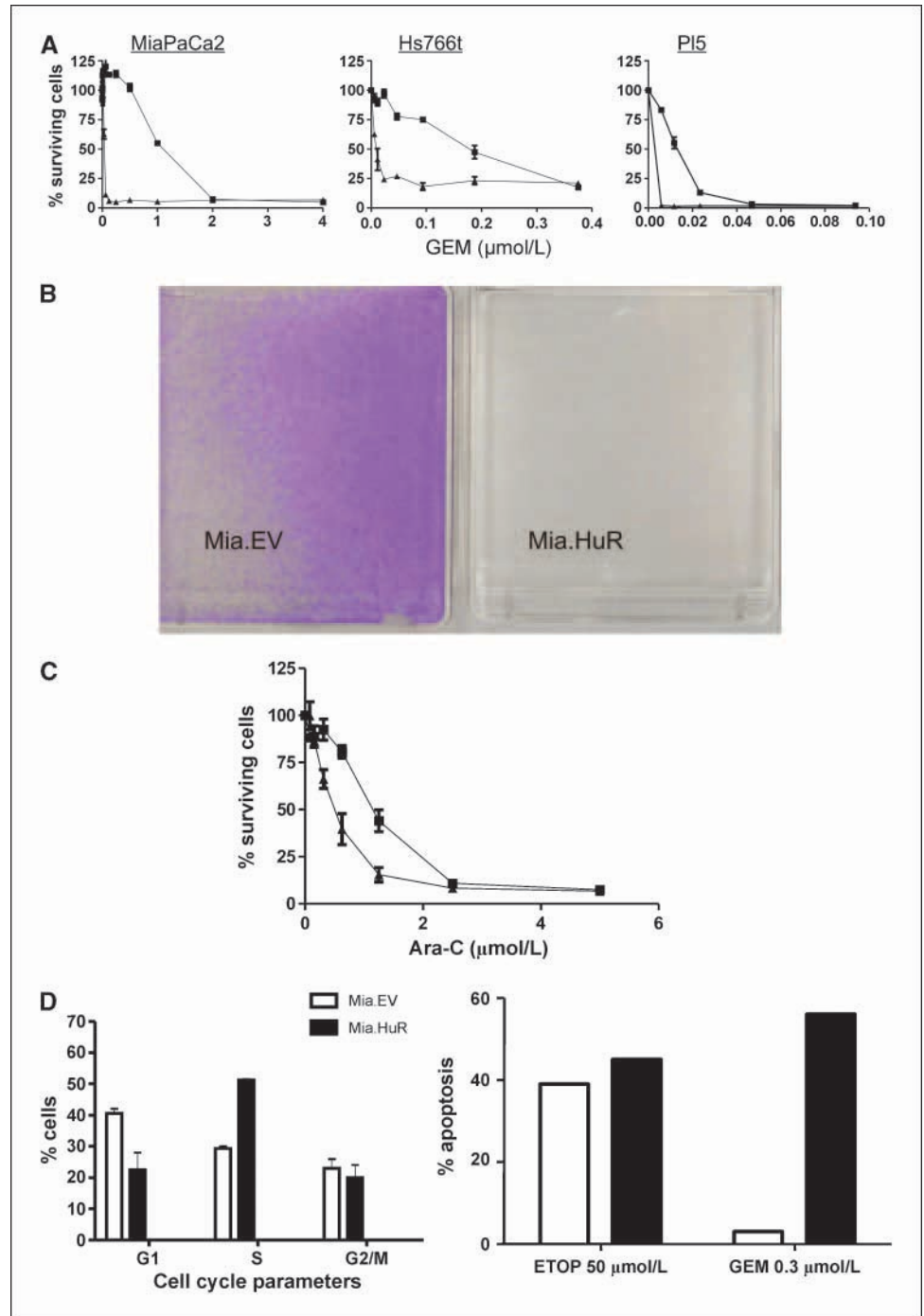
CCAAGCTTCTAATACGACTCACTATAGGGAGA (T7):(T7)GATCTTGCT-GAAGACTACAGGC and TTATTAGCGTCTTTTCAATTCTACAAA for dCK 3'UTR; (T7)CTCAACGACCACCTTTGTC AAGC and CACAGGGTACTTTATT-GATGGTACAT for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 3'UTR (see Supplementary Figure for depiction of UTR positions; ref. 16). Biotinylated probes were synthesized using the MAXIscript T7 kit (Ambion) and Biotinylated dCTP (Enzo Life Sciences).

For immunoprecipitation of endogenous RNA-protein complexes (RNP IP) from cytoplasmic (450 µg) extracts, reactions were carried out as described (7), using protein A-Sepharose beads (Sigma) that were precoated with 30 µg of either mouse IgG1 (BD Biosciences), or anti-HuR antibodies. After IP, the RNA in the IP materials was isolated and

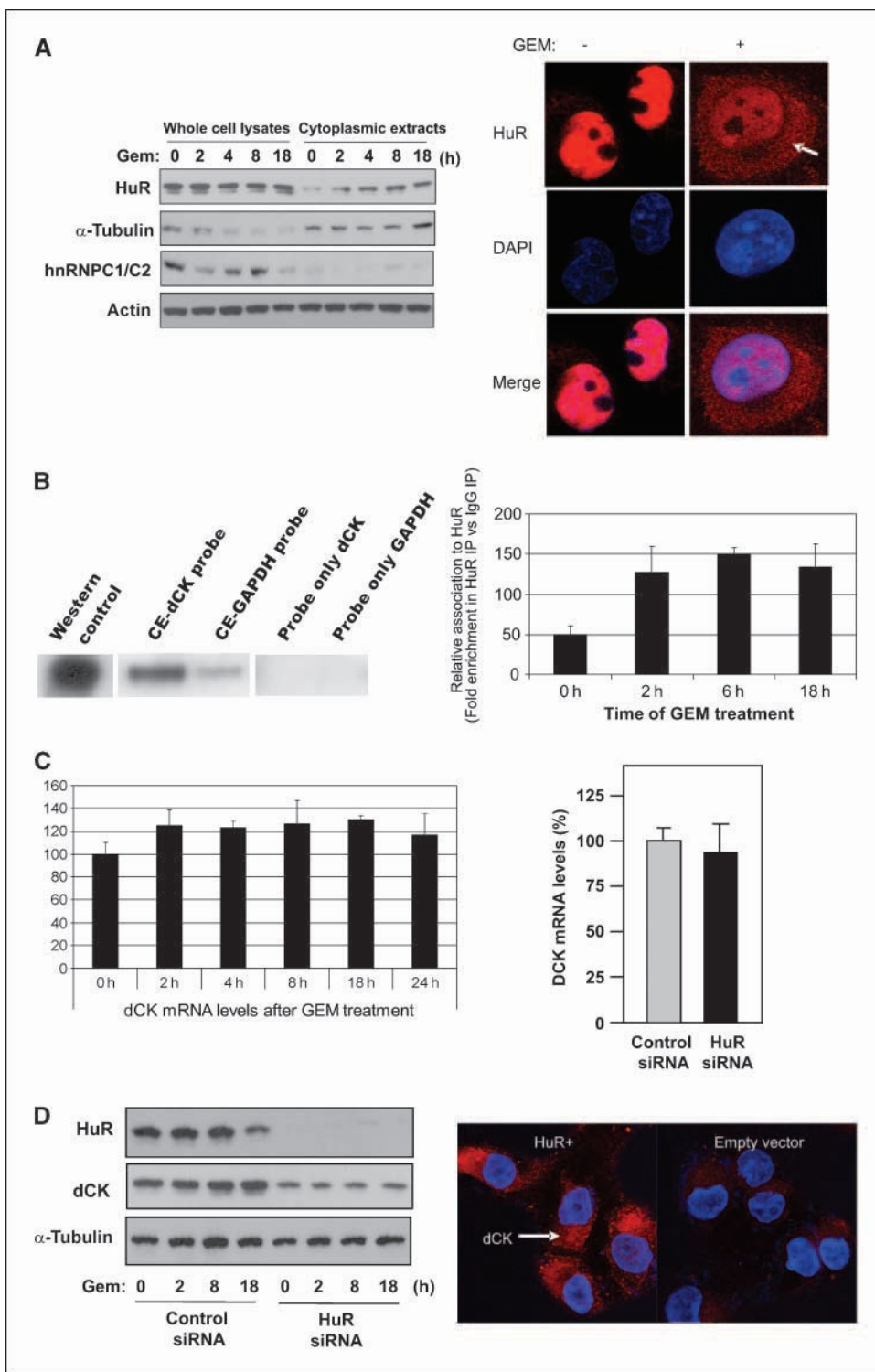
reverse-transcribed. GAPDH and dCK transcripts were quantified by real-time PCR analysis using each specific primers: AGCAAGGCATTCTCTT-GAA and CTACAGGCAGCCAAATGGTT for dCK, TGCACCACCAACTGCT-TAGC and CTCATGACCACAGTCCATGCC for GAPDH. The relative levels of dCK product were first normalized to GAPDH product in all IP samples, then fold enrichments in HuR IP were compared with IgG IP, as described (7).

**Case selection and immunohistochemistry.** HuR immunostaining was performed on 40 resected PDA specimens from the Thomas Jefferson University pathology archives after Institutional Review Board approval. All patients received gemcitabine, alone or in combination with Xeloda (two patients), radiation therapy (eight patients) or both (two patients). The

**Figure 2.** Stable expression of HuR renders cells hypersensitive to the nucleoside analogues gemcitabine (GEM) and Ara-C. *A*, survival of MiaPaCa2, Hs766t, and PL5 cell lines was measured by the PicoGreen assay after 5 to 7 d of incubation with the indicated gemcitabine doses. Graphs represent single experiments (SE); each experiment is representative of less than three individual experiments. ▲, HuR-expressing cells; ■, control cells. *B*, crystal violet-stained flasks of Mia.HuR and Mia.EV cultures after gemcitabine treatment (0.1 µmol/L, 7 d). *C*, sensitivity of MiaPaCa2 cells to Ara-C treatment was measured as explained in *A*. *D*, fluorescence-activated cell sorting analysis of cells treated with gemcitabine (0.03 µmol/L) for 48 h, depicting the percentages of cells in G<sub>1</sub>, S, and G<sub>2</sub>-M compartments (*left*). Measurement of apoptotic fractions (*right*) in treated cultures.



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experienced pancreatic pathologist (A.K.W.) reviewed all cases in a blinded fashion and classified the tumors as well-differentiated ( $n = 6$ ), moderately differentiated ( $n = 22$ ), or poorly differentiated ( $n = 12$ ). For each case, representative sections were selected for immunohistochemical analysis of HuR cytoplasmic and nuclear staining patterns, which were scored using the following scale: 0 for no staining, 1 for weak and/or focal (<10% of the cells) staining, 2 for moderate or strong staining (10–50% of the cells), and 3 for moderate or strong staining (>50% of the cells). Combined scores 0 and 1

represented low expression, whereas combined scores 2 and 3 represented high expression.

### Results and Discussion

**HuR overexpression preferentially sensitized pancreatic cancer cell lines to the nucleoside analogues gemcitabine and Ara-C.** Stable HuR overexpression in the indicated pancreatic

**Figure 3.** HuR associates with dCK mRNA and promotes dCK protein expression in MiaPaCa2 cells. *A*, Western blot analysis of HuR levels in whole-cell and cytoplasmic lysates after treatment of MiaPaCa2 cells with gemcitabine (Gem; 1  $\mu$ mol/L) for the indicated times (left). Immunofluorescence analysis of HuR levels and localization (red) in cells treated with 4  $\mu$ mol/L gemcitabine for 24 h; nuclei were distinguished by staining with DAPI (blue; right). *B*, biotin pull-down analysis of HuR RNP complexes. Cytoplasmic extracts were incubated with biotinylated transcripts spanning the dCK or GAPDH 3'UTRs. The association of HuR with biotinylated RNAs was tested by Western blot analysis. Positive control, HuR cytoplasmic lysate. Negative controls, "probe only" lanes contain only biotinylated RNAs that were not incubated with protein lysates. Shown is a representative blot (left). HuR binding to dCK mRNA was tested by RNP IP analysis (Materials and Methods) in MiaPaCa2 cells treated with gemcitabine for the times indicated; gemcitabine mRNA levels in HuR and IgG IP samples were first normalized to GAPDH mRNA levels in the same IP reactions, and plotted as fold enrichment in dCK mRNA in HuR IP compared with IgG IP. Columns, mean from three independent experiments (right); bars, SD. *C*, dCK mRNA levels were measured in cells that were left untransfected (left) or were transfected with either a control siRNA or HuR siRNA(7) and tested 48 h later (right). *D*, Western blot analysis of HuR, dCK, and  $\alpha$ -Tubulin in cells expressing normal or silenced HuR levels (left). Immunofluorescence analysis of dCK levels (arrow) and localization (red) in cells expressing normal or elevated HuR levels; nuclei were visualized by staining with DAPI (blue; right).

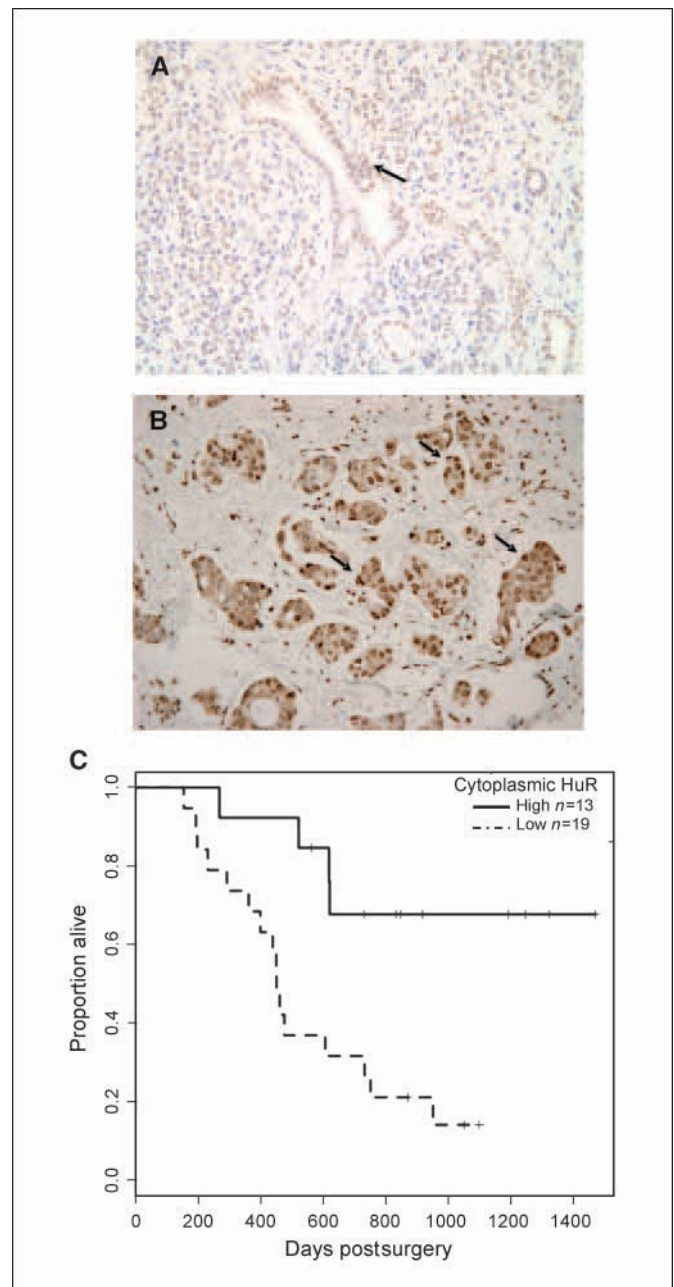
cancer cell lines was confirmed by immunoblot and immunofluorescence analyses (Fig. 1A and B). Contrary to previous studies in colon cancer cell lines (9), isogenic, transfected cell lines grew roughly at the same rates (Fig. 1C). Treatment with the indicated chemotherapeutic agents (but not gemcitabine) showed no difference in sensitivity in HuR-overexpressing cells (Fig. 1D).

By contrast, cell lines overexpressing HuR were found to be strikingly more sensitive to gemcitabine than were control lines, as assessed both by PicoGreen measurement (Fig. 2A) and by staining with crystal violet even when cells were treated with low concentrations of gemcitabine (Fig. 2B). HuR-overexpressing cell lines were similarly selectively more sensitive to Ara-C, another anticancer agent that uses dCK (Fig. 2C). After gemcitabine treatment, HuR-overexpressing cells showed selective enrichment in the S phase of the cell division cycle and increased apoptosis (Fig. 2D) compared with the control cells.

**HuR localization and association with dCK mRNA upon gemcitabine treatment.** Gemcitabine treatment did not alter whole-cell HuR levels in parental MiaPaCa2 cells, but significantly increased the cytoplasmic HuR levels, as determined by immunoblot and immunofluorescence analyses (Fig. 3A). Given that the dCK 3'UTR region contained 8 putative hits of an HuR recognition motif (17), we tested if HuR associates with dCK mRNA using 2 different RNA-binding assays. First, MiaPaCa2 cytoplasmic extracts were incubated with equimolar amounts of biotinylated transcripts spanning the dCK 3'UTR and the GAPDH 3'UTR (a control RNA, not a target of HuR); the resulting complexes were analyzed by HuR immunoblot. As shown in Fig. 3B (left), HuR bound the dCK 3'UTR much more strongly than the GAPDH 3'UTR. Second, we tested the association of HuR with the endogenous dCK mRNA by using a ribonucleoprotein immunoprecipitation (RNP IP) assay. As shown, although gemcitabine did not alter overall dCK protein or mRNA levels (Fig. 3A and C), it significantly increased the association of HuR with dCK mRNA (Fig. 3B, right).

Inhibition of HuR expression using small interfering (si)RNA (7) did not alter dCK mRNA levels (Fig. 3C, right) but decreased dCK protein levels (Fig. 3D, left) regardless of gemcitabine treatment. Conversely, HuR-overexpressing cells displayed higher dCK signals (Fig. 3D, right). Together, these data show for the first time that (a) gemcitabine exposure to cancer cells increases cytoplasmic HuR levels (Fig. 3A), (b) HuR associates with dCK mRNA (Fig. 3B), and (c) HuR regulates dCK protein levels (Fig. 3D).

**HuR localization and expression in PDA specimens.** We detected primarily weak to moderate nuclear HuR expression in normal pancreatic ductal and acinar cells (Fig. 4A). Strong nuclear expression of HuR was found in well-differentiated, moderately, and poorly differentiated PDAs. Cytoplasmic HuR accumulation was associated with poorly differentiated PDAs (data not shown; Fig. 4B). Figure 4C shows the Kaplan-Meier overall survival curves of patients receiving gemcitabine, stratified by their HuR status. The median survival time for patients on gemcitabine was 619 days, with 21 deaths. Thirty-two of 40 patients received gemcitabine and therefore were included in this analysis. A univariate Cox regression model gives a hazard ratio of low to high HuR of 4.48, with a 95% confidence interval of (1.49–13.5). Adjusting for age, sex, Xeloda use, and radiation therapy in this patient group gives an adjusted hazard ratio of 7.34 ( $P = 0.0022$ ) with a 95% confidence interval of (2.05–26.22). These data indicate a >7-fold increased risk of mortality in patients with low cytoplasmic HuR levels (compared with high cytoplasmic HuR



**Figure 4.** HuR cytoplasmic expression correlates with gemcitabine response in pancreatic cancer patients. A, primarily nuclear staining of HuR in normal pancreas is shown (arrows;  $\times 200$ ). B, high cytoplasmic expression in PDA specimen is shown (arrows;  $\times 200$ ). C, Kaplan-Meier plot of overall survival among patients receiving gemcitabine ( $n = 32$ ), stratified by HuR levels. The curves are significantly different ( $P = 0.0036$  by log-rank).

levels) among patients receiving gemcitabine, after adjusting for variables as mentioned above.

**Perspective.** As elevated cytoplasmic HuR has been widely correlated with advanced malignancy, the finding that high cytoplasmic HuR levels were associated with an increased therapeutic efficacy of gemcitabine in pancreatic cancer was unexpected. Our results that HuR regulates dCK protein concentration and that cytoplasmic HuR levels predict gemcitabine response in our patient cohort lead us to hypothesize

that HuR could be a key molecule involved in gemcitabine efficacy in cancer. It is intriguing to postulate that part of the survival repertoire of HuR may be to increase dCK levels to process deoxyribonucleosides for survival; however, in the presence of nucleoside analogues (such as gemcitabine) HuR's augmentation of dCK may be deleterious. Further studies are warranted to investigate if HuR dictates gemcitabine effectiveness by regulating additional target mRNAs, and to assess if HuR is a suitable marker for gemcitabine response in larger cancer patient cohorts for which gemcitabine therapy is used.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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