HMGA1 Is a Molecular Determinant of Chemoresistance to Gemcitabine in Pancreatic Adenocarcinoma
Siong-Seng Liau and Edward Whang

Abstract

Purpose: HMGA1 proteins are architectural transcription factors that are overexpressed by pancreatic adenocarcinomas. We previously have shown that RNA interference targeting the HMGA1 gene may represent a potential chemosensitizing strategy in pancreatic adenocarcinoma cells. In this study, we tested the hypothesis that HMGA1 promotes chemoresistance to gemcitabine in pancreatic cancer cells.

Experimental Design and Results: Stable short hairpin RNA–mediated HMGA1 silencing in BxPC3 and MiaPaCa2 cells promoted chemosensitivity to gemcitabine, with reductions in gemcitabine IC50 and increases in gemcitabine-induced apoptosis and caspase-3 activation. In contrast, forced HMGA1 overexpression in MiaPaCa2 cells promoted chemoresistance to gemcitabine, with increases in gemcitabine IC50 and reductions in gemcitabine-induced apoptosis and caspase-3 activation. Dominant negative Akt abrogated HMGA1 overexpression–induced increases in chemoresistance to gemcitabine. Finally, HMGA1 silencing promoted chemosensitivity to gemcitabine in vivo in a nude mouse xenograft model of pancreatic adenocarcinoma.

Conclusion: Our findings suggest that HMGA1 promotes chemoresistance to gemcitabine through an Akt-dependent mechanism. Targeted therapies directed at HMGA1 represent a potential strategy for ameliorating chemoresistance in pancreatic adenocarcinoma.

Overall prognosis for patients diagnosed with pancreatic adenocarcinoma remains dismal, with 5-year survival rates averaging <5% (1). At the time of diagnosis, most patients have locally advanced or metastatic disease precluding surgical resection (2). First line therapy for most patients with advanced pancreatic cancer is based on the nucleoside analogue gemcitabine. However, the clinical response rate to gemcitabine remains modest, in part due to the profound chemoresistance inherent in pancreatic cancer cells. Therefore, characterization of mechanisms mediating chemoresistance in pancreatic adenocarcinoma is an important priority. The human HMGA1 gene, located on chromosomal locus 6p21, encodes two HMGA1 splice variants (HMGA1a and HMGA1b; ref. 3). These HMGA1 proteins are architectural transcription factors that form stereospecific, multiprotein complexes termed “enhanceosomes” on the promoter/enhancer regions of genes they regulate (4–6). Each HMGA1 protein has three AT-hook domains that can bind to the minor groove of AT-rich DNA sequences (4, 7). HMGA1 proteins are overexpressed in a wide range of human cancers, including pancreatic adenocarcinoma (8–15). Further, tumoral HMGA1 overexpression has been reported to be associated with poor prognosis in cancer patients (9, 10, 16).

Our group has previously reported the important roles played by HMGA1 in mediating cellular invasiveness and metastatic potential of pancreatic adenocarcinoma cells (17). Overexpression of HMGA1 promotes cellular invasion in vitro whereas posttranscriptional silencing of HMGA1 inhibits the ability of pancreatic adenocarcinoma cells to form metastases in vivo. Furthermore, we showed that overexpression of HMGA1 is associated with increased resistance to apoptosis under anchorage-independent culture (“anoikis resistance”; ref. 18). In these studies, we have reported that Akt is a key downstream effector of HMGA1-dependent signaling in pancreatic cancer (17). Given the central role of Akt in mediating chemoresistance to gemcitabine (19–21), we hypothesized that HMGA1 would promote chemoresistance to this agent through an Akt-dependent mechanism. In this study, we have confirmed this hypothesis to be correct, and in addition, we have shown that targeted posttranscriptional silencing of HMGA1 promotes chemosensitivity in vivo. In conclusion, our findings are of particular clinical importance as they suggest that targeted therapies directed against HMGA1 may ameliorate chemoresistance to gemcitabine.

Materials and Methods

Cells and cell culture. MiaPaCa2 and PANC1 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection. Cells were maintained in DMEM containing 10% fetal bovine serum (Life Technologies) and incubated in a humidified
(37°C, 5% CO2) incubator, grown in 75-cm2 culture flasks, and passaged on reaching 80% confluence.

Reagents and dominant negative Akt constructs. Anti-HMGA1, anti–lamin B1, anti–phospho-Akt (Ser473), anti-Akt1, and anti-hemagglutinin antibodies were obtained from Santa Cruz Biotechnology. Hemagglutinin-tagged dominant negative Akt adenovirus (Ad-DN-Akt) and its control adenovirus (Ad-CMV-Null), both titrated at 1 x 10^10 plaque-forming units per milliliter, were purchased from Vector Bioslams.

Lentivirus-mediated HMGA1 RNA interference. Hairpin RNA interference plasmids (pLKO.1-HMGA1, TRCN00000018949) were obtained from the RNAi Consortium (Sigma Aldrich). The sequences of short hairpin RNA targeting the human HMGA1 gene (Genbank accession no. NM_002131) was 5′-CAACCCTCGCAGGAAAGCAA-3′ (shHMGA1 targets coding region positions 446-466 of HMGA1 mRNA transcript variant 2). The control plKO.1 plasmid, which has a scrambled nontargeting short-hairpin RNA sequence, was obtained from Addgene (22). High-titer lentivirus expressing shHMGA1 and a scrambled nontargeting short-hairpin RNA sequence, was obtained from The RNAi Consortium (Sigma Aldrich). The sequences forming units per milliliter, were purchased from Vector Biolabs. Antibodies were obtained from Santa Cruz Biotechnology. Hemagglutinin. Cell viability was determined after 48 to 72 hours in appropriate restriction sites at their 5′ end. The primers used were as follows: forward, 5′-TTTTGAATTCTCACTGCTCCTCCTCCGAGGA-3′ and backward, 5′-TTTTGATATCATGAGTGAGTCGAGCTCGAAG-3′. Purified PCR products were digested with EcoRV and EcoRI, before ligation into a EcoRV/EcoRI–digested pIREs-puro3 vector (Clontech). The expression plasmids were named pIREs-HMGA1. MiaPaCa2 cells were transfected with pIREs-HMGA1 or empty pIREs-puro3 vector using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s protocol. Stable clones were selected by exposure to increased concentrations of puromycin (Invivogen), isolated using cloning cylinders, and maintained in medium containing 3 μg/ml puromycin (Invivogen). Clones pIREs-HMGA1.1 and pIREs-HMGA1.2, which expressed the highest levels of HMGA1, were used for further studies.

Western blotting. Cells were harvested and rinsed twice with PBS. Total cell extracts were prepared with Phosphosafe lysis buffer (Pierce). Protein concentration was measured using the BCA assay (Pierce) according to the manufacturer’s instructions. Plates were washed and resuspended in 100 μL of PBS s.c. After blocking with PBS containing 3% bovine serum albumin for 1 hour at room temperature, membranes were incubated with 3 to 5 mg/ml antibody in PBS containing 0.1% Tween 20 overnight at 4°C. Chemiluminescence detection (Amersham Biosciences) according to the manufacturer’s protocol (Chemicon). The number of apoptotic cells in at least five random fields from each section was counted. Apoptotic staining was done using the following formula: volume = 1/2 a x b^2, where a and b represent the larger and smaller tumor diameters, respectively. After 6 weeks of gemcitabine administration and 4 days after final gemcitabine injection, necropsy was done, and the primary tumor was excised, formalin-fixed, and paraffin embedded.

Immunohistochemistry. Tumor sections (5 μm) were deparaffinized, rehydrated through graded alcohol, and processed using a streptavidin-biotin-peroxidase complex method. Sections were then incubated with StrepABComplex/horseradish peroxidase (1:50 dilution). These second antibodies were biotinylated rabbit anti-goat antibodies (DAKO) used at a dilution of 1:200 for 30 minutes at 37°C. Sections were then incubated with StrepABCComplex/horseredish peroxidase (1:100) for 30 minutes at 37°C. Immunolocalization was done by exposure to 0.05% 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. Normal serum was used in the place of primary antibody as a negative control.

Apoptosis staining. Following preparation of 5-μm tumor sections, apoptosis was quantified using a commercially available terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) kit, in accordance with the manufacturer’s protocol (Chemicon). The number of apoptotic cells in at least five random fields from each section was counted. The apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of nuclei counted in each field. Statistical analysis. Differences between groups were analyzed using Student’s t test, multifactorial ANOVA of initial measurements, and Mann-Whitney U test, for nonparametric data, as appropriate, using Statistica 5.5 software (StatSoft, Inc.). In cases where averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final p value when p < 0.05 was considered statistically significant.
Results

HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma cells. Lentivirus-mediated HMGA1 silencing resulted in a marked increase in chemosensitivity to gemcitabine in BxPC3 cells (in which we achieved almost 90% silencing of HMGA1 protein expression; Fig. 1A), with ~4-fold reductions in IC_{50} to gemcitabine (mean IC_{50}, control shRNA versus shHMGA1: 50 versus 12 nmol/L, *P* = 0.001; Fig. 1C and D). Interestingly, BxPC3 cells in which HMGA1 had been silenced developed spiculated morphology on exposure to 1 μmol/L gemcitabine for 48 hours, whereas control cells did not (Fig. 1B). In our previous study, we achieved up to 90% silencing of HMGA1 expression in MiaPaCa2 cells using lentivirus-expressing shRNA with the same target sequence (23). In the current study, we achieved a similar degree of HMGA1 knockdown in MiaPaCa2 cells (data not shown). Lentivirus-mediated shHMGA1 silencing had similar effects on MiaPaCa2 cells, with shifting of the gemcitabine IC_{50} curve to the left (Fig. 2B) and ~2-fold reductions on IC_{50} (mean IC_{50}, control shRNA versus shHMGA1: 60 versus 30 nmol/L, *P* = 0.001; Fig. 2D).

We then tested the effect of forced overexpression of HMGA1 on cellular chemoresistance to gemcitabine. MiaPaCa2 cells (which have low inherent expression of HMGA1) were stably transfected with the pRES-HMGA1 vector, as described in Materials and Methods. We selected two transfectant clones with highest expression levels of HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2). In our previous studies, we have characterized the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones and verified their overexpression of HMGA1 by Western analysis (17, 18). pIRES-HMGA1.1 and pIRES-HMGA1.2 clones overexpress HMGA1 by 4-fold and 3.5-fold, respectively, when compared with empty pIRES-puro3 transfectants (data not shown). Overexpression of HMGA1 resulted in significant increases in chemoresistance to gemcitabine, with increases in IC_{50} to gemcitabine (Fig. 2D) and shifting of the IC_{50} curves to the right for both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Fig. 2C). The mean IC_{50} for pIRES-HMGA1.1 and pIRES-HMGA1.2 were 130 and 100 nmol/L, respectively (*P* = 0.003 and *P* = 0.006, respectively, versus empty pIRES-puro3 controls). Notably, pIRES-HMGA1.1 consistently overexpressed HMGA1 to a greater degree than pIRES-HMGA1.2; this greater HMGA1 overexpression was associated with a higher mean IC_{50} value. Forced HMGA1 overexpression enhanced the viability of cells exposed to 1 μmol/L of gemcitabine for 72 hours, whereas HGMA1 silencing was associated with the opposite effect (Fig. 2A).

**HMGA1 expression status modulates gemcitabine-induced apoptosis and caspase-3 activation.** Following exposure to 1 μmol/L gemcitabine for 24 hours, cells were subjected to flow cytometric quantitation of apoptosis and fluorometric caspase-3 profiling. Lentivirus-mediated HMGA1 silencing was associated with increases in gemcitabine-induced apoptosis (Fig. 3A) and caspase-3 activation in both BxPC3 and MiaPaCa2 cells (Fig. 3C). In contrast, HMGA1 overexpression was
associated with reductions in gemcitabine-induced apoptosis and in caspase-3 activation (Fig. 3B and D).

**HMGA1-induced chemoresistance to gemcitabine is dependent on Akt signaling.** We have previously reported that Akt is a downstream effector of HMGA1 (18, 23). We have found that HMGA1 silencing is associated with reductions in Akt phosphorylation (a marker of Akt activation), whereas forced HMGA1 overexpression is associated with increases in Akt kinase activity and in Akt phosphorylation (17, 18). Given the importance of the phosphatidylinositol 3-kinase/Akt pathway in antiapoptotic signaling, particularly in the context of chemoresistance, we sought to determine if chemoresistance to gemcitabine-induced HMGA1 overexpression is Akt dependent. Each of pIRE5-HMGA1.1 and pIRE5-HMGA1.2 clones were transduced with dominant negative Akt adenovirus at multiplicity of infection of 10. The efficiency of transduction and expression of dominant negative Akt were confirmed by immunoblotting for the hemagglutinin tag of the dominant negative Akt construct (Fig. 4). We next assessed the effects of dominant negative Akt on chemosensitivity to gemcitabine in each HMGA1-overexpressing clone. Dominant negative Akt was found to reverse the chemoresistance induced by HMGA1 overexpression, with reductions of IC50 to gemcitabine in both pIRE5-HMGA1.1 and pIRE5-HMGA1.2 clones. As such, HMGA1 overexpression–induced chemoresistance is dependent on Akt signaling.

**HMGA1 silencing promotes chemosensitivity to gemcitabine in vivo.** BxPC3 cells in which HMGA1 had been silenced through stable lentiviral shRNA-mediated RNAi or control BxPC3 cells transduced with lentivirus carrying nontargeting shRNA were s.c. implanted into nude mice. Once the resulting xenograft tumors had grown to reach ~ 50 mm3 in diameter, a 6-week course of gemcitabine administration was initiated. Tumors derived from BxPC3 cells in which HMGA1 had been silenced (n = 8 animals) regressed during the treatment period, whereas tumors derived from control cells (n = 8 animals) continued to...
Fig. 3. A, lentivirus-mediated silencing of HMGA1 promoted gemcitabine-induced apoptosis as assessed by flow cytometric analyses of YO-PRO-1/propidium iodide–stained cells. Silencing of HMGA1 resulted in ~2-fold increases in the relative apoptotic rates in both BxPC3 and MiaPaCa2 cells following exposure to 1 µmol/L gemcitabine for 24 h. *, P = 0.001 versus control shRNA. Representative flow cytometric images of three experiments are shown, with the apoptotic fractions being highlighted in triangles drawn. B, forced overexpression of HMGA1 in pIRES-HMGA1.1 and HMGA1.2 clones protected the cells from gemcitabine-induced apoptosis with ~70% to 80% reductions in relative apoptotic rates, as assessed by flow cytometry [P = 0.001 (pIRES-HMGA1.1) and P = 0.002 (pIRES-HMGA1.2) versus empty pIRES-puro3 control]. *, P < 0.05 versus empty pIRES-puro3 control. Representative flow cytometric images of three independent experiments are shown, with the apoptotic fractions being highlighted in triangles drawn. C, relative caspase-3 activities were determined using a fluorometric caspase-3 substrate assay following exposure of cells to 1 µmol/L gemcitabine for 24 h. Lentivirus-mediated silencing of HMGA1 promoted gemcitabine-induced caspase-3 activities. *, P = 0.008 (BxPC3) and P = 0.005 (MiaPaCa2) versus control shRNA transfectants. D, as expected, overexpression of HMGA1 in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones resulted in reductions in caspase-3 activation following exposure to 1 µmol/L gemcitabine for 24 h, indicating protection from gemcitabine-induced caspase-mediated apoptosis. *, P = 0.001 versus empty pIRES-puro3 transfectants.

grow during the treatment period (Fig. 5A–C). Stable suppression of HMGA1 expression in tumors derived from shHMGA1 transfectants was confirmed on Western blotting of nuclear extracts of tumor homogenates (Fig. 6A) and on immunohistochemical analysis of xenografts harvested at the end of the study period (Fig. 6B). TUNEL staining revealed significantly higher apoptotic index in shHMGA1 transfectant–derived tumors than in control cell–derived tumors (Fig. 6B and C).
Discussion

HMGA1 proteins are overexpressed in a wide range of human cancers, including pancreatic adenocarcinoma (8). Experimental data implicating biologically important roles for HMGA1 in cancer pathogenesis are rapidly accumulating (24, 25). Our study provides the first data suggesting that HMGA1 may mediate a critical feature of malignant phenotype: chemoresistance. First, our findings show that forced HMGA1 overexpression promotes chemoresistance to gemcitabine in pancreatic cancer cells in vitro, whereas HMGA1 silencing promotes gemcitabine-induced cytotoxicity and therefore abrogates chemoresistance to gemcitabine. Second, we have confirmed that HMGA1 silencing promotes gemcitabine-induced cytotoxicity and reduces tumor growth in vivo in a nude mouse xenograft model of pancreatic cancer. Finally, our findings suggest a plausible mechanism by which HMGA1 promotes chemoresistance to gemcitabine: activation of Akt signaling. Phosphatidylinositol 3-kinase/Akt signaling is well described as a mediator of chemoresistance to gemcitabine in the context of pancreatic cancer (19, 26, 27). These findings imply that targeted suppression or inactivation of HMGA1 could be a potential therapeutic strategy for increasing chemosensitivity to gemcitabine in this highly chemoresistant cancer.

Proposed mechanisms by which HMGA1 regulates gene expression include derepression of gene promoters by displacement of histone H1 nucleoproteins, which are strong repressors of gene transcription, from scaffold attachment regions, thus
allowing for a more open chromatin structure that facilitates transcriptional activation (28). Further, HMGA1 is able to bind to AT-rich promoter regions, where it modifies DNA conformation to facilitate binding of other transcriptional factors that promote gene transcription (29). By binding to promoter regions, HMGA1 is also able to form multiprotein complexes, the enhanceosomes, that serve as transcription-activating complexes (29). Given these putative functions, it is not surprising that HMGA1 is involved in the regulation of a large number of target genes. Previous studies have shown that HMGA1 overexpression is associated with increased expression of growth factors/cytokines (e.g., fibroblast growth factors, IFNs α and β, and interleukins 10-14 and 17), growth factor receptors (e.g., fibroblast growth factor receptor, epidermal growth factor receptor, ERBB3, and ERBB4), and multiple integrins (α1, α2, αv, αv, β1, β3, β6; ref. 24). Clearly, it is not surprising that through induction of these growth factor–related signaling pathways, HMGA1 could have an effect on prosurvival phosphatidylinositol 3-kinase/Akt pathways, as shown in this study. It is also plausible that by increasing integrin expression, HMGA1 may stimulate integrin-linked kinase, which is known to directly interact with integrins and phosphorylate Akt in a phosphatidylinositol 3-kinase–dependent manner (30).

In the current study, we have examined the effects of silencing HMGA1 on chemoresistance in BxPC3 and MiaPaCa2 pancreatic adenocarcinoma cells. BxPC3 cells inherently express relatively high levels of HMGA1 whereas MiaPaCa2 cells inherently express relatively low levels of HMGA1 under baseline conditions. Because these cell lines differ in ways other than in HMGA1 expression levels alone, we examined the effects of modulating HMGA1 expression in a single cell line system. This approach allowed us to control for potential confounders, such as variable K-ras mutation status (31), inherent in comparisons involving multiple cell lines. We did both loss-of-function (HMGA1 silencing) and gain-of-function (HMGA1 overexpression) experiments on MiaPaCa2 cells. As such, by using the same cell line and theoretically controlling for variations in genetic background, we were able to show that HMGA1 silencing reduces chemoresistance whereas HMGA1 overexpression results in the reverse effects on chemoresistance.

Although gemcitabine-based regimens are currently standard of care for the treatment of advanced pancreatic cancer, their efficacy is limited by profound chemoresistance. Recently, the combination of gemcitabine and the human epidermal growth factor receptor tyrosine kinase inhibitor erlotinib has been reported, for the first time, to be associated with improved

![Fig. 6. A, in vivo HMGA1 silencing was confirmed by Western blot analysis of nuclear extracts from explanted xenograft tumors. Columns, mean; bars, SD. *, P = 0.001 versus control shRNA xenografts. B and C, immunohistochemistry of xenograft sections showed little or absent staining for HMGA1 in the shHMGA1 xenografts, when compared with the control shRNA xenografts that showed intense staining for HMGA1. Photomicrograph of HMGA1 staining was obtained at ×40 magnification. In the same sections, TUNEL staining was done. In each tumor slide stained with TUNEL, the number of TUNEL-positive cells was counted in at least five randomly selected fields at ×40 magnification. *, P < 0.001 versus control shRNA xenografts. Representative tumor sections stained for TUNEL photographed at ×20 magnification. Columns, mean; bars, SD.](Fig. 6)
survival over single-agent gemcitabine in patients with advanced pancreatic cancer in a phase III clinical trial (32). Although these results are encouraging, the benefits provided by the addition of erlotinib are only incremental. Truly transformative increases in efficacy are likely to arise only through incorporation of targeted therapies selected on the basis of rational understanding of mechanisms mediating chemoresistance in pancreatic cancer. In this context, our findings suggest that HMGA1 warrants further investigation as a novel therapeutic target in this deadly cancer.

References

HMGA1 Is a Molecular Determinant of Chemoresistance to Gemcitabine in Pancreatic Adenocarcinoma

Siong-Seng Liau and Edward Whang


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/5/1470

Cited articles  This article cites 32 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/5/1470.full#ref-list-1

Citing articles  This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/5/1470.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.

Permissions  To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/14/5/1470.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC)
Rightslink site.