Clinical Studies

CHD7 Expression Predicts Survival Outcomes in Patients with Resected Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with poor outcomes with current therapies. Gemcitabine is the primary adjuvant drug used clinically, but its effectiveness is limited. In this study, our objective was to use a rationale-driven approach to identify novel biomarkers for outcome in patients with early-stage resected PDAC treated with adjuvant gemcitabine. Using a synthetic lethal screen in human PDAC cells, we identified 93 genes, including 55 genes linked to DNA damage responses (DDR), that demonstrated gemcitabine sensitization when silenced, including CHD7, which functions in chromatin remodeling. CHD7 depletion sensitized PDAC cells to gemcitabine and delayed their growth in tumor xenografts. Moreover, CHD7 silencing impaired ATR-dependent phosphorylation of CHK1 and increased DNA damage induced by gemcitabine. CHD7 was dysregulated, ranking above the 90th percentile in differential expression in a panel of PDAC clinical specimens, highlighting its potential as a biomarker. Immunohistochemical analysis of specimens from 59 patients with resected PDAC receiving adjuvant gemcitabine revealed that low CHD7 expression was associated with increased recurrence-free survival (RFS) and overall survival (OS), in univariate and multivariate analyses. Notably, CHD7 expression was not associated with RFS or OS for patients not receiving gemcitabine. Thus, low CHD7 expression was correlated selectively with gemcitabine sensitivity in this patient population. These results supported our rationale-driven strategy to exploit dysregulated DDR pathways in PDAC to identify genetic determinants of gemcitabine sensitivity, identifying CHD7 as a novel biomarker candidate to evaluate further for individualizing PDAC treatment. Cancer Res; 74(10); 1–11. ©2014 AACR.

Introduction

Pancreatic adenocarcinoma has a poor prognosis with a 5-year overall survival (OS) rate around 5% (1). Patients with early-stage pancreatic adenocarcinoma who undergo resection demonstrate the best prognosis, particularly when resection is followed by adjuvant chemotherapy with or without radiotherapy (2, 3). Still, recurrence is common and OS remains poor even for patients who undergo complete resection and adjuvant therapy. Recent developments have suggested that pancreatic adenocarcinoma is a genetically heterogeneous disease (4) and, as such, patients may benefit from the identification of predictive biomarkers for responsiveness to adjuvant therapy. Gemcitabine is the primary chemotherapeutic agent used to treat patients with pancreatic adenocarcinoma in the adjuvant setting (2, 5). The cytotoxic effects of gemcitabine are mediated in part through incorporation into DNA as a terminal nucleoside analog and in part through inhibition of ribonucleotide reductase, which depletes nucleotides required for DNA synthesis. However, the efficacy of gemcitabine for pancreatic adenocarcinoma is limited. A better understanding of which patients are likely to respond to gemcitabine treatment would facilitate personalization of therapy and optimize the clinical benefit to toxicity ratio associated with adjuvant therapy.

The DNA damage response (DDR) pathway is critical for the maintenance of genome integrity and serves as a cancer barrier by mobilizing DNA repair, cell-cycle arrest, and/or apoptosis (6, 7). In human precancerous lesions, aberrant DNA replication induces DDR activation, which constrains tumor development. Thus, the DDR acts as a barrier against genomic instability and cancer development. Tumor cells may in turn develop mutations or epigenetic silencing of protective DDR genes, leading to the proliferation of genetically unstable cells and ultimately resulting in cancer. Indeed, a large number of DDR genes are somatically mutated in pancreatic adenocarcinoma.

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adenocarcinoma, including ATM, BRCA2, CDKN2A, FANCJ, HELB, and RAD9 (8). These genetic changes in the DDR pathway can lead to pancreatic adenocarcinoma and can also weaken the ability of cancer cells to respond to treatment by decreasing activity in DNA repair pathways. Often, the cancer cell will become reliant on backup pathways that can be targeted to cause cell death through the principal of synthetic lethality (inactivation of one gene or pathway is sublethal but inactivation of both causes cell death). As such, determining genetic alterations and cancer treatments that are synthetically lethal may lead to the identification of novel druggable targets as adjuncts to gemcitabine treatment or novel biomarkers to predict response to gemcitabine therapy. Using this rationale, we sought to exploit dysregulated DDR pathways in pancreatic adenocarcinoma by identifying genetic determinants that are synthetically lethal with gemcitabine treatment and evaluating their clinical relevance as biomarkers for outcome in patients with early-stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine.

Chromodomain helicase DNA binding protein 7 (CHD7), is a member of a family of chromodomain enzymes that belong to the ATP-dependent chromatin remodeling protein SNF2 superfamily. Mutations in CHD7 lead to congenital CHARGE syndrome, named for its characteristic traits: coloboma of the eye, heart defects, atresia of the nasal choanae, retardation of growth and/or development, genital and/or urinary abnormalities, and ear abnormalities and deafness (9), and Kallman Syndrome, a genetic disorder marked by hypogonadotropic hypogonadism and anosmia (10). CHD7 is also dysregulated in 13% to 35% of cases of pancreatic adenocarcinoma, with aberrant expression, copy-number variation, and somatic mutations (see Supplementary Table S3; refs. 11–13). CHD7 helps to regulate nuclear crest gene expression (14), regulates ribosomal RNA biogenesis (15), and interacts with SOX2 to regulate gene expression (16). CHD7 is also a putative substrate of the ATM/ATR checkpoint kinases, suggesting that it may play a role in the DDR (17, 18). The clinical significance of CHD7 expression in pancreatic adenocarcinoma has not previously been reported.

The purpose of this analysis was to use a rationale-driven approach to identify novel biomarkers for outcome in patients with early-stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine (Fig. 1A). We initially completed a synthetic lethal siRNA screen to identify genetic determinants of gemcitabine sensitivity in human pancreatic cancer cells and identified the top 15% of these genes for further analysis. Genes validated by a secondary screen and/or linked to the DDR were then analyzed for dysregulation and differential expression in pancreatic adenocarcinoma by mining published data sets to determine their potential as biomarkers. Finally, we correlated CHD7 gene expression characterized by immunohistochemistry (IHC) with clinical outcome in patients with early-stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine.

Materials and Methods

Cell culture, siRNA, and transfection

MIA PaCa-2 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS (Gibco) and 2.5% horse serum (Gibco). HPAC cells were grown in 1:1 DMEM:Hams F12 supplemented (Gibco) with 40 ng/mL hydrocortisone, 10 ng/mL EGF, and 5% FBS. BxPC-3 and AsPC-1 cells were grown in RPMI-1640 supplemented with 10% FBS, and CAPAN-1 cells were grown in Iscove’s Modified Dulbecco’s Medium supplemented with 20% FBS. Cell lines were grown in a humidified incubator at 37 °C with 5% carbon dioxide. Transfections were done using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s protocol. Primary and secondary screen siRNAs were purchased from Thermo Scientific. siRNA sequences are listed below.

NT: (ATGAAGCTGAATTGCTCAATT)
ATR: (CCUCUGCUGAGUGUCUGUA)
ATRIP: (GGCCACAGATTATAGA)
CHK1: (CTGAAGAAGCATGCGACGT)
CHD7-1: (UAACGUACCUAACCUAUUAA)
CHD7-2: (CGAACAGGCUAGUGUUGAAA)
CHD7-3: (GGGAAGCUAUUAUAUGCGA)
CHD7-4: (GUAGAUAACAGAGACUUAA)

TRC lentiviral short hairpin RNA (shRNA) was purchased from Thermo Scientific: shControl (RHS4080), shCHD7-1 (1RHS3979-201747986), shCHD7-2 (1RHS3979-201747990).

Gemcitabine sensitivity screen

MIA PaCa-2 cells were transfected in 96-well plates using HiPerFect Transfection Reagent (Qiagen) with 25 nmol/L siRNA from a custom siGENOME siRNA library (Thermo Scientific) of 4,024 siRNAs corresponding to 1,006 unique human nuclear enzyme genes (pools of four siRNAs targeting a unique sequence of each gene) using a one gene per well format. Twenty-four hours later, plates were split 1:4, and then treated following another 24 hours with or without 13 nmol/L gemcitabine (Hospira, Inc.) for 72 hours before examining for cell proliferation using WST-1 reagent (Roche Diagnostics). Each plate contained two positive controls (ATR and CHK1) and several negative controls (NT), and plate-to-plate variability was controlled by normalizing the values on each plate to the average of the negative control values on that plate. A ratio of gemcitabine treated/untreated viability was calculated and normalized to that of nontargeting siRNA. Principal components analysis (PCA) was used to account for possible variability between the cell viability of the three replicates for each gene. These genes were then sorted by increasing average cell viability via PCA, and the top 15% of genes were categorized as possible “hits.”

Secondary validation screen

MIA PaCa-2, BxPC3, or HPAC cells were transfected in 96-well plates with 25 nmol/L siRNA, split 1:4 24 hours later, and then treated following another 24 hours with or without gemcitabine at ICS, ICS25, or ICS50 for 72 hours before assaying for cell proliferation using WST-1 reagent. A ratio of gemcitabine treated/untreated viability was calculated and normalized to that of nontargeting siRNA. MIA PaCa-2, HPAC, CAPAN-1, BxPC3, and AsPC-1 cells were treated
with the indicated concentrations of gemcitabine for 72 hours before assaying for cell proliferation using the WST-1 reagent.

**Colony formation assay**

Cells were transfected with 25 nmol/L siRNA. Following a 24-hour knockdown, 500 cells were seeded into 6-well plates in triplicate. Cells were allowed to culture overnight and were then treated for 24 hours with increasing concentrations of gemcitabine. Following the gemcitabine incubation, the plates were washed with PBS and fresh media were added for 8 to 12 days before staining colonies with a 0.5% crystal violet (Ampresco) solution.

**Western blot analysis**

MIA PaCa-2 cells transfected with siRNA for 48 hours or MIA PaCa-2, HPAC, CAPAN-1, BxPC3, and AsPC-1 cells were harvested with NP40 buffer containing 200 mmol/L NaCl, 1% NP40, 50 mmol/L Tris-HCl (pH 8.0), and supplemented with fresh protease inhibitors. Samples were loaded into a SDS-PAGE gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and subsequently probed with an anti-CHD7 antibody.
antibody (NBP1-77393; Novus Biologicals) and anti-GAPDH antibody (GeneTex; GTX627408) followed by LI-COR IRDye secondary antibodies. Detection was performed using the Odyssey system (LI-COR Biosciences).

To analyze phosphorylation of CHK1, MIA PaCa-2 cells were transfected with 25 nmol/L siRNA for 48 hours and treated with 1 mmol/L gemcitabine for 6 hours. Cells then were harvested, washed with PBS, and lysed in cold RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% SDS) with protease and phosphatase inhibitors for 30 minutes. Lysates were clarified by centrifugation at 16,000 × g for 10 minutes and 150 μg of protein for each sample were used for Western blot analysis. Primary antibody pCHK1 S317 (Cell Signaling Technology; #2344) and CHK1 (Santa Cruz Biotechnology; sc-8408) were used for detection of phosphorylated and total CHK1, respectively.

**In vivo tumor growth inhibition assay**

Male nude mice were maintained in a pathogen-free environment, and all *in vivo* procedures were approved by the Emory University, Institutional Animal Care and Use Committee. Stable MIA PaCa-2 shCHD7-2 and MIA PaCa-2 shControl cells (1.5 × 10<sup>6</sup>/0.1 mL of 20% Matrigel gel in serum-free media) were injected subcutaneously into the flank of 5-week-old mice. Mice bearing established tumors (100 to 125 mm<sup>3</sup>) were randomized into treatment groups of four. Animals were treated on days 0, 7, and 14 via the tail vein with either vehicle or 100 mg/kg gemcitabine. Tumor growth inhibition was determined as described previously (19).

**γH2AX DNA damage assay**

MIA PaCa-2 cells were treated with or without 13 mmol/L gemcitabine for 20 hours, washed, released for the indicated time points, and processed for γH2AX staining by indirect immunofluorescence. Cells were washed with 1× PBS, fixed with 2% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.5% Triton X-100 (Fisher Scientific), and blocked in a 5% bovine serum albumin solution (Sigma). Cells were then immunostained with anti-phospho-histone H2AX (Ser139) antibody (Millipore; 05-636) and anti-mouse secondary antibody with AlexaFluor-488 (Invitrogen; A21206). After incubation, cells were mounted onto slides with a mounting media containing 4′, 6-diamidino-2-phenylindole (DAPI) and dried. Analysis was performed using a Zeiss Observer Z1 microscope with Axiosvision Rel 4.8 software using the 63× oil objective. Foci quantitation was conducted by counting 250 healthy cells and scoring cells with 10 or more foci as positive. Experiments were done in triplicate.

**Cell-cycle analysis**

MIA PaCa-2 cells were transfected with 25 nmol/L siRNA for 48 hours and treated with or without gemcitabine (13 mmol/L) for 24 hours. After fixing with ice-cold 70% ethanol, cells were washed in PBS, and propidium iodide (PI; 25 μg/mL; Sigma) and RNase A (10 μg/mL; Qiagen) were added to determine DNA content. Cells were analyzed on a FACSCanto II (BD Biosciences) and FlowJo software.

**Biomarker selection**

Gemcitabine sensitivity genes validated on secondary screen or known DDR genes were analyzed for evidence of dysregulation by identifying genes overexpressed in The Compendium of Potential Biomarkers of Pancreatic Cancer (20) or somatically mutated in the pancreatic Catalogue for Somatic Mutations in Cancer (COSMIC) database (11). For determination of differential expression, we extracted expression data from the two Gene Expression Omnibus (GEO) submissions based on the Affymetrix U133 Plus 2.0 platform: (i) GSE12654, a 22 pancreatic cancer cell line study (19); and (ii) GSE16515, 20 pancreatic patient tumors (21, 22). Within each study, after processing and normalization, we performed a genomewide filter to identify genes with "large" expression differences among tumors, and separately, among cell lines, using a variance approach. We define "differential expression" as genes whose expression variability is "large" relative to all other genes on the array, in which "large" is defined according to whether expression variability associated with a gene was greater than the 90th percentile from all genes. We then compared this list of genes with the lists in Supplementary Tables S2 and S4. CHD7 was chosen as a potential biomarker based on evidence of both dysregulation and differential expression.

**IHC patient selection**

Patients were selected for this analysis from a prospectively maintained database of patients who underwent resection for early-stage pancreatic adenocarcinoma between January 2000 and October 2008; data for these patients have been included in other cohorts previously reported (23–26). These 59 patients received adjuvant chemotherapy with or without adjuvant radiation. The gemcitabine patient population was composed of 42 of these patients who received gemcitabine as a component of the adjuvant chemotherapy regimen. An additional 17 patients received adjuvant chemotherapy with agents other than gemcitabine. OS was calculated from date of surgery to patient death. Recurrence-free survival (RFS) was measured on the basis of surveillance imaging obtained at regular intervals after resection. Patient demographics, pathologic characteristics, and treatment characteristics were originally collected from pathologic record and chart review. Permission was obtained from the Emory Institutional Review Board 00048816, and patient confidentiality was maintained according to the Health Insurance and Patient Accessibility Act of 1996.

**Immunohistochemical analysis**

An experienced pathologist identified representative sections of tumor and normal tissue from formalin-fixed paraffin-embedded slides. The tissue was stained using an anti-CHD7 mouse monoclonal antibody (NBP1-77393; Novus Biologicals) at a concentration of 1:200. Specificity of the anti-CHD7 antibody was validated by Western blot analysis following siRNA silencing (Fig. 2B). An expression score was calculated using a previously defined scoring system (23, 27). Overall score was dichotomized into low (<3.1) and high (≥3.1) expression groups for this analysis (Supplementary Fig. S1).
Statistical analysis

Descriptive statistics were generated for patient characteristics, tumor characteristics, and treatment characteristics. Similar statistical analyses were performed for patients receiving adjuvant therapy, patients receiving gemcitabine-based therapy, and patients receiving non-gemcitabine-based therapy. Kaplan–Meier log-rank survival analysis was performed to determine prognostic factors for RFS and OS. Univariate and multivariate Cox regression analyses were performed for all patients to examine the correlation of CHD7 expression level on both RFS and OS. Factors examined on univariate analysis included age, sex, ethnicity, receipt of adjuvant and neoadjuvant therapy, tumor size, margin status, grade, nodal status, perineural invasion, lymphovascular invasion, receipt of radiotherapy, CA19-9 levels, and type of adjuvant chemotherapy. Clinically relevant covariates significant to a level of \( P < 0.2 \) on univariate analysis for either RFS or OS were included in the multivariate model; these included tumor size, margin status, nodal status, perineural invasion, lymphovascular invasion, and tumor grade. Data were analyzed using the Statistical Package for the Social Sciences 19.0 software for Windows (IBM).

Results

Gemcitabine sensitivity screen

To identify genetic determinants of gemcitabine sensitivity, we completed a siRNA screen to identify genes that when silenced cause either sensitization or resistance to a low dose of gemcitabine.
gemcitabine in human pancreatic cancer cells. Because gemcitabine induces DNA damage and replication stress, we reasoned that gemcitabine sensitivity genes would likely be involved in the DDR. We, therefore, optimized a high-throughput assay using ATR and CHK1 siRNA as positive controls and a nontargeting siRNA as a negative control with cell proliferation as a read-out (Fig. 1B). The primary screen was completed in MIA PaCa-2 cells, which consistently gave the highest signal-to-noise ratio among several tested cell types (Supplementary Fig. S3A and data not shown). Briefly, cells were transfected with pools of four siRNAs targeting a unique sequence of each gene arrayed in a one gene per one well format in 96-well plates. Forty-eight hours after transfection, cells were treated with or without 13 nmol/L gemcitabine (equivalent to IC25 under these conditions, see Fig. 2E) for 72 hours before assaying for cell proliferation using WST-1 reagent. Each plate contained two positive controls (ATR and CHK1) and several negative controls (NT), and plate-to-plate variability was controlled by normalizing the values on each plate to the average of the negative control values on that plate. We completed three replicas of the primary screen using a library of 4,024 siRNAs, corresponding to four unique siRNA duplexes, targeting each of 1,006 unique human genes (Fig. 1C). The library consisted predominantly of nuclear enzymes, which we reasoned were more likely to function directly in the DDR and be targetable. Results of the primary screen were ranked by PCA score (Supplementary Table S1). The top 15% of these genes (156 genes) included 55 genes linked to the DDR (Fig. 1D; Supplementary Fig. S2, and Supplementary Table S2) including well-characterized ATR signaling pathway genes ATR, CHK1, RAD9, RAD1, and Hus1 and nucleotide metabolism genes RRM1 and RRM2, known to regulate gemcitabine sensitivity (28), demonstrating that our screen can yield DDR genes that determine gemcitabine sensitivity.

CHD7 knockdown causes gemcitabine sensitization

Sixty-eight of our hits were identified in previously published DNA damage sensitivity screens (17, 20, 29–36) and 27 are putative ATM/ATR substrates (17; Supplementary Table S3). We used these criteria to validate 47 of the 99 hits not characterized in the DDR in a secondary screen using deconvoluted siRNA to confirm their gemcitabine sensitivity and eliminate false positives due to off-target effects, and 38 of these genes induced gemcitabine sensitivity in at least two out of four siRNAs tested, including CHD7 (Supplementary Table S4). Four of 4 siRNAs targeting CHD7 caused gemcitabine sensitization (Fig. 2A). Western blot analysis confirmed decreased levels of CHD7 following siRNA knockdown as well as specificity of the anti-CHD7 antibody used for IHC analysis (Fig. 2B). A similar gemcitabine sensitization after CHD7 silencing was observed using a range of gemcitabine concentrations and in BxPC-3 and HPAC pancreatic cancer cells, suggesting that the phenotype is not cell-type specific (Supplementary Fig. S3A–C). CHD7 silencing in the absence of gemcitabine treatment reduced cell viability (Supplementary Fig. S3D). We also determined the gemcitabine sensitivity of CHD7-depleted cells using a colony formation assay. MIA PaCa-2 cells silenced for CHD7 demonstrated a significantly reduced percentage of surviving colonies following a 24-hour pulse of gemcitabine in a dose-dependent manner compared with a NT control (Fig. 2C), confirming the gemcitabine sensitization of CHD7-depleted cells observed with WST-1 reagent. Consistent with these findings, MIA PaCa-2 and BxPC3 pancreatic cancer cells, which express lower levels of CHD7 than HPAC, CAPAN-1, and AsPC-1 pancreatic cancer cells, demonstrated increased gemcitabine sensitivity (Fig. 2D and E), suggesting that CHD7 expression may predict response to gemcitabine in pancreatic adenocarcinoma cells. To determine if CHD7 silencing causes gemcitabine sensitization of pancreatic cancer tumors in vivo, we generated a xenograft model using MIA PaCa-2 cells stably expressing shCHD7 or shControl (Fig. 2F). CHD7 silencing significantly delayed tumor growth in mice treated with gemcitabine compared with a control treated with gemcitabine (Fig. 2G), suggesting that CHD7 silencing also causes gemcitabine sensitization in vivo. No significant difference in body weight was observed in mice bearing tumors with shCHD7 compared with shControl and treated with or without gemcitabine (Supplementary Fig. S4).

CHD7 is a DDR protein

The gemcitabine hypersensitivity of CHD7-depleted cells suggests that CHD7 may function in the DDR. CHD7 silencing significantly increased the percentage of cells staining with γH2AX, a marker for DNA damage, following treatment with gemcitabine (Fig. 3A), suggesting that CHD7 silencing potentiates gemcitabine-induced DNA damage. However, no significant difference in repair kinetics was observed between cells silenced with CHD7 compared with a nontargeting siRNA (Fig. 3A). CHD7-silenced cells showed a decreased percentage of cells in S phase and an increased percentage of cells in G2–M in the absence of gemcitabine treatment (Supplementary Fig. S5A); however, no significant difference in cell-cycle profile was observed between CHD7-depleted compared with NT control cells following gemcitabine treatment (Supplementary Fig. S5B). There was also no significant difference in protein levels of CHD7 in response to gemcitabine treatment (Supplementary Fig. S6). To determine whether CHD7 functions in ATR-dependent signaling in response to gemcitabine treatment, we examined cells for the phosphorylation of CHK1 Ser317. CHD7 silencing significantly reduced CHK1 Ser317 phosphorylation but not total CHK1 protein levels in response to gemcitabine treatment (Fig. 3B), suggesting that CHD7 functions in controlling ATR-dependent phosphorylation of CHK1 in response to gemcitabine treatment.

CHD7 is dysregulated and differentially expressed in pancreatic adenocarcinoma

Genes validated by our secondary screen or linked to the DDR were then analyzed for dysregulation and differential expression in pancreatic adenocarcinoma by mining of published data sets to determine their potential as biomarkers. Of these, eight genes demonstrate aberrant expression or somatic mutations in pancreatic adenocarcinoma (Fig. 3C and Supplementary Table S3) as reported in The Compendium of Potential Biomarkers (20) and the COSMIC database (11). Twelve of the genes are above the 90th percentile in differential
expression among a panel of 22 pancreatic adenocarcinoma cell lines or 20 pancreatic adenocarcinoma tissue samples (Fig. 3C and Supplementary Table S3; refs. 21, 22). Four of the genes exhibit both dysregulation and differential expression, including CHD7, which was selected for further analysis as a biomarker.

Survival analyses

Patient demographics, pathologic and treatment characteristics can be seen in Table 1. CHD7 expression was low in 84.7% of patients. Median tumor size was 3.4 cm (range, 1–6 cm), and 60% of patients were node positive. In addition to CHD7 expression, significant covariates on univariate analysis included tumor size, margin status, lymph node status, perineural invasion (PNI), lymphovascular invasion (LVI), and grade (P < 0.2). On Kaplan–Meier analysis for patients receiving gemcitabine as a component of adjuvant therapy (n = 42), low CHD7 expression was associated with increased RFS (15 vs. 7 months; P = 0.025; Fig. 4A) and increased OS (18 vs. 10 months; P = 0.015; Fig. 4B). On multivariate analysis (Table 2), low CHD7 expression was associated with increased RFS (15 months vs. 7 months; P = 0.015; Fig. 4C) and increased OS (19.5 months vs. 9 months; P = 0.001; Fig. 4D). These results remained significant on multivariate analysis (Table 3). To ensure stability of the multivariate model given the small number of events, the three least significant covariates on univariate analysis were removed from the model (grade, PNI, and LVI) and the significance of CHD7 expression remained unchanged.

Discussion

In this study, we demonstrate a rationale-driven approach for identifying novel biomarkers for outcome in patients with early-stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine. Using a synthetic lethal screen to identify genetic determinants of gemcitabine sensitivity in human pancreatic cancer cells, we identified 93 genes that, when

| Table 1. Patient demographics, tumor characteristics, and treatment characteristics for all patients (N = 59) |
|---------------------------------|---------|---------------------------------|
| **Patient demographics** | **Median (range)** | **N** |
| Male sex | 31 | 52.5 |
| Ethnicity | | |
| Asian | 2 | 3.4 |
| Black | 12 | 20.3 |
| White | 42 | 71.2 |
| Age (y) | 60.0 (37–84) |
| OS (mo) | 17.3 (4.8–114.6) |
| RFS (mo) | 14.5 (0.6–109.8) |
| **Tumor characteristics** | | |
| Positive margins | | |
| Well differentiated | 5 | 6.3 |
| Moderately differentiated | 46 | 57.5 |
| Poorly differentiated | 28 | 35.0 |
| Positive nodes | 48 | 60.0 |
| PNI | 70 | 87.5 |
| LVI | 38 | 47.5 |
| Low CHD7 expression | 50 | 84.7 |
| **Tumor size (cm)** | 3.4 (1–6) |
| **Treatment characteristics** | | |
| Neoadjuvant therapy | 2 | 3.4 |
| Radiation therapy | 39 | 66.1 |
| Received gemcitabine | 42 | 69.5 |
silenced, demonstrate gemcitabine sensitization, including CHD7. CHD7 deficiency caused gemcitabine sensitization in pancreatic adenocarcinoma cells and delayed pancreatic tumor xenograft growth in mice treated with gemcitabine. We further found that CHD7 knockdown impaired ATR-dependent phosphorylation of CHK1 and increased DNA damage induced by gemcitabine, revealing a novel function for CHD7 as a DDR protein, which maintains genome integrity in response to gemcitabine. We examined CHD7 as a potential biomarker based on its dysregulation and differential expression in a panel of pancreatic adenocarcinoma cell lines and tissues. Finally, we found that low CHD7 expression is associated with improved RFS and OS in patients with early-stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine. These findings support our rationale-driven approach in exploiting dysregulated DDR pathways in pancreatic adenocarcinoma to identify genetic determinants of gemcitabine sensitivity that can be translated to novel biomarkers or drug targets.

A third of the genes identified in our primary gemcitabine sensitivity screen are linked to the DDR, including ATR signaling pathway genes ATR, CHK1, RAD9, RAD1, HUS1, and CDK9 (37) and nucleotide metabolism genes RRM1 and RRM2. CHD7 was previously identified as a putative ATM/ATR substrate (17). Our finding that CHD7 silencing in human pancreatic cancer cells potentiates gemcitabine-induced DNA damage and impairs CHK1 Ser317 phosphorylation in response to gemcitabine treatment suggests that CHD7 also functions in the ATR signaling pathway and helps to explain at least in part why CHD7 knockdown causes gemcitabine sensitization in cells and in vivo. Still, the cell-cycle effects of CHD7 expression require further understanding through future studies, which remain ongoing. For example, CHK1 inhibition has been shown to potentiate gemcitabine-induced cytotoxicity by inducing premature mitosis (38). A number of genes identified in our screen, including RRM1, RRM2, and CHK1 have previously been shown to determine gemcitabine sensitivity in pancreatic adenocarcinoma cells and xenografts.
Low CHD7 Expression Predicts Improved Outcome in PAC

Table 2. Multivariate\(^{\text{a}}\) Cox regression analyses for patients receiving gemcitabine therapy (\(N = 42\))

<table>
<thead>
<tr>
<th>Outcome</th>
<th>RFS</th>
<th></th>
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<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>(P)</td>
<td>HR</td>
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<td>Tumor size</td>
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<td>Higher grade</td>
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<td>0.566–2.242</td>
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<td>PNI</td>
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<td>0.210–1.567</td>
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<td>LVI</td>
<td>1.004</td>
<td>0.462–2.180</td>
<td>0.992</td>
<td>1.429</td>
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<td>Low CHD7 expression</td>
<td>0.122</td>
<td>0.035–0.420</td>
<td>0.001</td>
<td>0.086</td>
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NOTE: Bold denotes statistical significance.
\(^{\text{a}}\)Multivariate analysis includes all clinically relevant covariates with \(P < 0.2\) on univariate analysis.

Table 3. Multivariate\(^{\text{a}}\) Cox regression analyses for patients receiving adjuvant therapy (\(N = 59\))

<table>
<thead>
<tr>
<th>Outcome</th>
<th>RFS</th>
<th></th>
<th>OS</th>
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<td></td>
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<tr>
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</tr>
<tr>
<td>Higher grade</td>
<td>1.051</td>
<td>0.579–1.907</td>
<td>0.870</td>
<td>1.057</td>
</tr>
<tr>
<td>Positive nodes</td>
<td>1.638</td>
<td>0.777–3.452</td>
<td>0.194</td>
<td>1.729</td>
</tr>
<tr>
<td>PNI</td>
<td>0.410</td>
<td>0.161–1.044</td>
<td>0.062</td>
<td>2.028</td>
</tr>
<tr>
<td>LVI</td>
<td>1.064</td>
<td>0.529–2.139</td>
<td>0.862</td>
<td>1.082</td>
</tr>
<tr>
<td>Low CHD7 expression</td>
<td>0.271</td>
<td>0.107–0.687</td>
<td>0.006</td>
<td>0.203</td>
</tr>
</tbody>
</table>

NOTE: Bold denotes statistical significance.
\(^{\text{a}}\)Multivariate analysis includes all clinically relevant covariates with \(P < 0.2\) on univariate analysis.

human pancreatic cancer cells (39), and low RRM2 expression has been shown to be associated with improved outcome in patients with pancreatic adenocarcinoma (24) and specifically those treated with adjuvant gemcitabine (28), providing validation for our screen in identifying gemcitabine sensitivity genes that may function as potential biomarkers. Several of the gemcitabine sensitivity genes, including PLK1 and AURKB, are involved in mitotic progression that is in part targeted by nanoparticle albumin bound (nab)-paclitaxel (Abraxane; Celgene), which potentiates gemcitabine sensitivity and improves survival in patients with metastatic pancreatic adenocarcinoma treated with gemcitabine (40, 41). It is thus possible that the gemcitabine sensitivity genes reported in this study may also be novel druggable targets to be used in combination with gemcitabine. Indeed, PARP2, a target of PARP inhibitors that sensitizes pancreatic cancer cells to gemcitabine (42, 43), was also identified in our screen.

In our clinical data, low CHD7 expression was associated with increased OS and RFS in all patients receiving adjuvant therapy, although this was likely driven by the inclusion of patients receiving gemcitabine. The association of low CHD7 expression with increased survival was magnified in patients receiving gemcitabine as a component of their adjuvant therapy despite smaller patient numbers, indicating that low CHD7 expression may indeed be associated with gemcitabine sensitivity in these patients. In contrast, CHD7 expression in patients not receiving gemcitabine was not statistically significant. This analysis is underpowered with limitation of small sample size and selection bias, but our findings provide valuable hypothesis-generating data suggesting that CHD7 may have predictive value in these patients.

Given the evidence that patients with low CHD7 expression demonstrate improved outcomes, it is possible that adjuvant therapy regimens could be tailored to individualize patient treatment based on CHD7 expression. This should be examined in future prospective trials and in larger secondary analyses of completed prospective studies. Although adjuvant chemotherapy for patients with pancreatic adenocarcinoma is advantageous, the ideal drug regimen remains unclear. The benefit of adjuvant gemcitabine compared with adjuvant 5-FU in patients with early-stage resected pancreatic adenocarcinoma has not been demonstrated in any large trials. Both the ESPAC-3 trial, which randomized patients with resected pancreatic adenocarcinoma to adjuvant gemcitabine versus 5-FU, and the RTOG 97-04 trial, which randomized patients with resected pancreatic adenocarcinoma to adjuvant pre- and postchemoradiotherapy gemcitabine versus 5-FU, reported no significant difference in disease-free survival (DFS) or OS.
between the two arms (3, 44). Our finding that low CHD7 expression is associated with improved outcome in patients with early-stage pancreatic adenocarcinoma treated with adjuvant gemcitabine suggest that, once validated, CHD7 expression could potentially be used as a predictive biomarker to individualize adjuvant therapy for these patients. In addition, the optimal radiation dose and fractionation for patients with resected pancreatic adenocarcinoma remains unknown, and molecular biomarkers to guide adjuvant therapy decisions are essential (45). The potential utility of CHD7 expression as a prognostic and potentially predictive biomarker still remains a hypothesis-generating observation and requires validation in a prospective clinical trial, in which regimen dosing and duration are more homogenous.

Interest in genetic sequencing data, such as with The Cancer Genome Atlas and other similar projects, continues to increase (46, 47), leading to rapidly increasing knowledge of genes expressed and mutated in specific cancer types including pancreatic adenocarcinoma. As this knowledge becomes available, it is crucial that an approach be developed to help identify those genes that may serve as clinically relevant prognostic or predictive biomarkers or potential drug targets for novel therapeutics. The successful identification and validation of CHD7 as a novel gemcitabine sensitivity gene that is associated with outcome in patients with early-stage pancreatic adenocarcinoma treated with adjuvant gemcitabine is evidence that our approach may be successful in identifying other clinically relevant biomarkers or drug targets.

It is worth noting that recent advances in chemotherapy have increased the use of FOLFIRINOX therapy in the metastatic setting, impacting the potential utility of this study. Still, National Comprehensive Cancer Network guidelines in the metastatic setting equivalently recommend FOLFIRINOX or two gemcitabine-based regimens (gemcitabine with the addition of erlotinib or nab-paclitaxel), both with category one evidence (24). In addition, gemcitabine or fluoropyrimidine therapies are still recommended in the adjuvant setting, which is where this study’s clinical focus remains. Future studies should evaluate the predictive role of CHD7 in a larger, randomized prospective trial to validate potential gemcitabine sensitivity genes in a similar fashion to other identified predictive biomarkers (48). The current study suggests that CHD7 may be a useful biomarker for determining which patients will derive greater benefit from gemcitabine therapy, providing clinicians a way to better select patients for specific adjuvant therapy regimens in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Authors’ Contributions
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References
Low CHD7 Expression Predicts Improved Outcome in PAC


CHD7 Expression Predicts Survival Outcomes in Patients with Resected Pancreatic Cancer


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