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Publication Date

2020-11-01

DOI

10.1200/PO.19.00330

Peer reviewed

original reports

CES2 Expression in Pancreatic Adenocarcinoma Is Predictive of Response to Irinotecan and Is Associated With Type 2 Diabetes

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abstract

PURPOSE The combination chemotherapy of fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) has provided clinically meaningful improvement for pancreatic ductal adenocarcinoma (PDAC). We previously uncovered a role for the serine hydrolase carboxylesterase 2 (CES2) in mediating intratumoral activation of the prodrug irinotecan, a constituent of FOLFIRINOX. We aimed to further test the predictive value of CES2 for response to irinotecan using patient-derived xenograft (PDX) models and to elucidate the determinants of CES2 expression and response to FOLFIRINOX treatment among patients with PDAC.

METHODS PDXs were engrafted subcutaneously into nude mice and treated for 4 weeks with either saline control or irinotecan. CES2 and hepatocyte nuclear factor 4 alpha (HNF4A) expression in PDAC tissues was evaluated by immunohistochemical and Western blot analysis. Kaplan-Meier and Cox regression analyses were applied to assess the association between overall survival and hemoglobin A1C (HbA1C) levels in patients who underwent neoadjuvant FOLFIRINOX treatment.

RESULTS High CES2 activity in PDAC PDXs was associated with increased sensitivity to irinotecan. Integrated gene expression, proteomic analyses, and in vitro genetic experiments revealed that nuclear receptor HNF4A, which is upregulated in diabetes, is the upstream transcriptional regulator of CES2 expression. Elevated CES2 protein expression in PDAC tissues was positively associated with a history of type 2 diabetes (odds ratio, 4.84; $P = .02$). High HbA1C levels were associated with longer overall survival in patients who received neoadjuvant FOLFIRINOX treatment ($P = .04$).

CONCLUSION To our knowledge, we provide, for the first time, evidence that CES2 expression is associated with a history of type 2 diabetes in PDAC and that elevated HbA1C, by predicting tumor CES2 expression, may represent a novel marker for stratifying patients most likely to respond to FOLFIRINOX therapy.

JCO Precis Oncol 4:426-436. © 2020 by American Society of Clinical Oncology

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers, with a 5-year survival rate of 8%.[1] Patients typically present with locally advanced or metastatic disease and are ineligible for surgical intervention.[2] Recent combination chemotherapies—fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX)[3] and gemcitabine with nab-paclitaxel[4]—have nearly doubled the median survival for patients with advanced PDAC and gained acceptance as front-line therapies. However, response to these treatments is highly variable, with approximately one third of patients responding to a particular regimen.[3-5] Consequently, there is need to develop predictive markers to guide clinical management of PDAC for individual patients.

We previously uncovered a role for the serine hydrolase carboxylesterase 2 (CES2) in mediating activation of the prodrug irinotecan, a constituent of FOLFIRINOX, into its active form.[6,7] Importantly, high CES2 expression in PDAC tissue was associated with longer overall survival (OS) in patients who underwent neoadjuvant FOLFIRINOX treatment.[6] We further assessed the predictive value of CES2 using patient-derived xenograft (PDX) models in response to irinotecan and, using bioinformatics and in vitro genetic approaches, coupled with in-depth proteomic profiling of PDAC cell lines, identified determinants that mediate elevated CES2 expression in PDAC. We additionally explored the relationship between tumor CES2, type 2 diabetes (T2D) and response to FOLFIRINOX treatment.

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

Accepted on March 16, 2020 and published at ascopubs.org/journal/po on April 23, 2020: DOI https://doi.org/10.1200/PO.19.00330

CONTEXT

Key Objective

To elucidate the determinants of CES2 expression and response to fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) treatment amongst patients with pancreatic ductal adenocarcinoma (PDAC).

Knowledge Generated

We identify HNF4A as the upstream transcriptional regulator of CES2 expression. We further demonstrate that elevated CES2 protein expression in PDAC tissues was positively associated with a history of type 2 diabetes and that high HbA1c levels, through predicting tumor CES2 expression, were associated with longer overall survival in patients who underwent neoadjuvant FOLFIRINOX treatment.

Relevance

Elevated HbA1c, by predicting tumor CES2 expression, may represent a novel marker for stratifying patients most likely to respond to FOLFIRINOX therapy.

METHODS

Detailed information regarding methodologies and biospecimens are provided in the Data Supplement.

Patients and Clinical Specimens

PDAC specimens were obtained from patients who underwent resection with curative intent at MD Anderson Cancer Center (MDACC; Data Supplement). Written informed consent was obtained under an institutional review board–approved research protocol. Patient clinicopathologic characteristics for tissues used in xenograft models and tissue microarrays are described in the Data Supplement. Clinical features of 37 patients who underwent neoadjuvant FOLFIRINOX treatment are described in the Data Supplement. Our group has a well-documented bias toward the administration of preoperative chemotherapy and/or chemoradiation before intended surgical resection to most patients with resectable or borderline resectable pancreatic cancer[8]; the patients included in this study had localized cancers and a performance status and comorbidity profile appropriate for systemic FOLFIRINOX treatment and were treated either on or off protocol.[9]

Cell Culture, Transfection, and Viral Transduction

All pancreatic cancer cell lines used in this study (BxPC-3, SW1990, SU.86.86, PANC-1, Hs 766T, CFPAC-1, MIA PaCa-2, AsPC-1, Panc 03.27, HPAF-II, and Capan-2) were obtained from the American Type Culture Collection. Liquid chromatography tandem mass spectrometry analysis of cells was performed as previously described.[10] Knockdown of hepatocyte nuclear factor 4 alpha (*HNF4A*) was performed by transfecting the cells using the following siRNAs: siControl (Silencer Select Negative Control No. 1, ThermoFisher Scientific, Waltham, MA), and si*HNF4A* (SASI_Hs01_00124507 and SASI_Hs01_00124509; Sigma-Aldrich, St. Louis, MO).

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

TaqMan polymerase chain reaction (PCR) assay was performed with a 7500 Fast Real-Time PCR System, TaqMan PCR master mix, commercially available primers, FAM-labeled probes for the *CES2* gene (Hs01077945_m1), *HNF4A* gene (Hs00230853_m1) and VIC-labeled probes for *18S* (Hs99999901_S1), according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). Values are reported as $\Delta\Delta$Ct values.

Cell Line RNA Sequencing

RNA sequencing (RNA-seq) libraries were prepared and sequenced using standard Illumina (San Diego, CA) reagents and protocols. Paired-end sequencing with a read length of 50 bases was performed on Illumina HiSEquation 2000 platform. CASAVA 1.8.2 was used to demultiplex and generate FASTQ files. Sequencing data were analyzed using Tophat (Johns Hopkins University Center for Computational Biology, Baltimore, MD)[11] mapping against hg19 and Cufflinks.[11a,12] The number of reads was normalized and expressed as fragments per kilobase of exon per million fragments mapped.[12]

Western Blot Analysis

The following antibodies were used: CES2 (HPA018897; Sigma-Aldrich, St Louis, MO), HNF4α (#3113; Cell Signaling, Salt Lake City, UT), and β-actin (Sigma-Aldrich).

CES2 Activity Assay

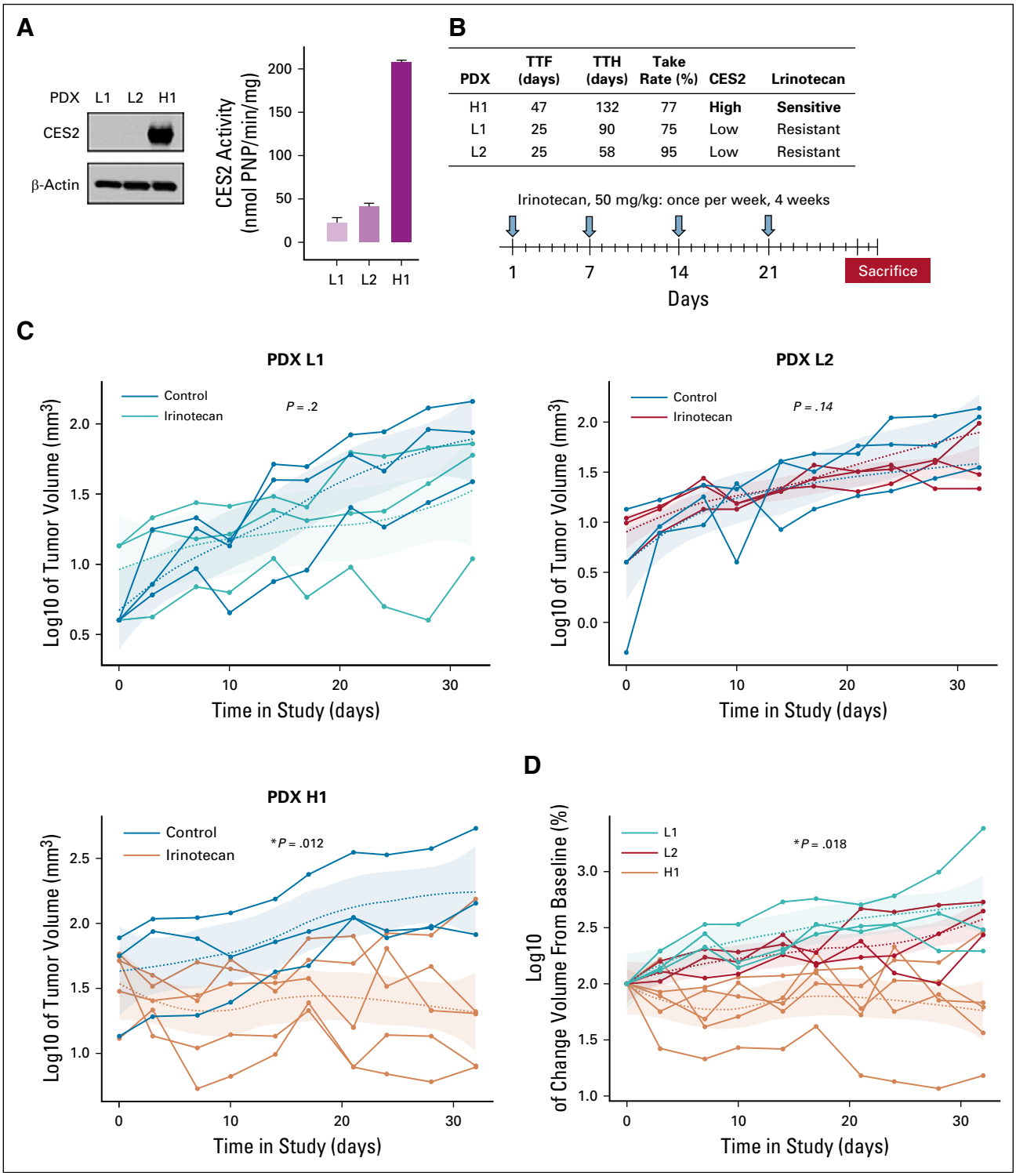
CES2 activity was measured as previously described.[13,14] Detailed information is provided in the Data Supplement

Gene Expression Datasets

Gene expression and methylation data datasets were downloaded from The Cancer Genome Atlas (TCGA; level-3 data)[15] for pancreatic samples (n = 112) and the International Cancer Genome Consortium (ICGC) Australian Pancreatic Cancer Genome Initiative (AU; 68 PDAC samples)[16] for data portals. Gene expression data of the pancreatic progenitor subtype gene signature from the TCGA (n = 180) and ICGC (n = 96) datasets were downloaded from cBioportal[17] and Bailey et al,[18] respectively.

Tissue Microarray

Detailed information is provided in the Data Supplement. The tissue microarray was constructed using standard



PDX	TTF (days)	TTH (days)	Take Rate (%)	CES2	Lrinotecan
H1	47	132	77	**High**	**Sensitive**
L1	25	90	75	Low	Resistant
L2	25	58	95	Low	Resistant

FIG 1. Tissue carboxylesterase 2 (CES2) predicts response to irinotecan in pancreatic ductal adenocarcinoma (PDAC) patient-derived xenograft (PDX) models. (A) Western blot analysis of CES2 expression (left panel) and of CES2 activity (right panel) in PDX tumors. β-actin served as a loading control. Activity was assessed by monitoring transformation of *para*-nitrophenolic acetate into its hydrolyzed analog, *para*-nitrophenol (*p*-NP), in the presence or absence of CES2-selective inhibitor, loperamide. Activity is expressed in unit of nmoles of *p*-NP formed per minute per 1 mg of protein lysate. Results are the mean of 3 independent experiments ± the standard deviation. L1 and L2 PDXs: low CES2 activity and expression; ^{1}H PDX: high CES2 activity and expression. (B) Summary of tumor graft characteristics and in vivo irinotecan drug administration schedule. Average time of tumor formation (TTF), time of tumor harvesting (TTH), and overall tumor take rate. L1 and L2 PDXs: low CES2 activity and expression; ^{1}H PDX: high CES2 activity and expression. PDXs were engrafted subcutaneously into immunodeficient mice. One month after implantation, mice (n = 3-5 per group) were treated for 4 weeks, via intraperitoneal injection, with either saline control or 50 mg/kg irinotecan, and euthanized 32 days after the (continued on following page)

instrumentation (Beecher Instruments Sun Prairie, Wisconsin) as described previously.[19]

Immunohistochemical Analysis

Immunohistochemical staining for CES2 (Sigma-Aldrich, HPA018897) and HNF4α (#3113; Cell Signaling) was performed on 5 μm of unstained sections from tissue microarray blocks constructed using formalin-fixed paraffin-embedded archival tissue blocks from pancreatectomy specimens of 188 patients with PDAC, as well as normal adjacent tissue that was available from 125 of the 188 patients (Data Supplement).

Animal Studies

Animal experiment protocols were approved by the MDACC Institutional Animal Care and Use Committee in accordance with the published National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. PDXs with different ranges of CES2 expression and activity were reimplanted in nude mice (tumor size, approximately 10 mm^3). After 1 month, mice were randomized into 2 groups (3-5 per group) and treated weekly via intraperitoneal injection with irinotecan (50 mg/kg) or saline control for 4 weeks. Tumor volume was monitored and calculated using the following formula: length × $width^2$ × 0.5. Mice were euthanized after 32 days of treatment or immediately on becoming moribund, as required in the animal care guidelines. Tumors were snap frozen or formalin fixed for downstream analyses.[20]

Data Analysis and Statistics

Detailed information is provided in the Data Supplement. Statistical analysis was performed using R (version 3.2.3), Statistical Package for Social Sciences (IBM SPSS Statistics 22, Armonk, NY), MedCalc (New York, NY; Version 14.10.2), and GraphPad Prism (La Jolla, CA; Version 6.0). Fisher's exact, Student's *t* test, and Wilcoxon rank sum tests were used to assess the differences in continuous variables, as appropriate. Pearson's correlation was applied to analyze normalized datasets, and Spearman's correlation was used for non-normally distributed data. Mixed linear effect analysis was performed to quantify growth curves of PDX tumor volume for groups treated with irinotecan versus saline control. Ingenuity Pathway Analysis (IPA; Qiagen, Hildon, Germany) was used to identify upstream regulators, top biologic functions, detoxification pathways, and networks linked to gene and protein expression changes associated with *CES2* expression in the analyzed datasets.

Odds ratio (OR) was used to measure the relationship between CES2 expression and patient clinicopathologic features. Receiver operating characteristic curve analysis was performed to assess the performance of hemoglobin A1C (HbA1C) in distinguishing PDAC tissues according to CES2 expression levels. A Cox model was applied to construct survival curves. OS curves were constructed using the Kaplan-Meier method; the log-rank test was used to evaluate the statistical significance.

RESULTS

High CES2 Activity Is Associated With Increased Sensitivity to Irinotecan Therapy in PDX Models of PDAC

We previously reported CES2 as a determinant of response to irinotecan and neoadjuvant FOLFIRINOX therapy in PDAC.[6] In this study, we evaluated CES2 activity and response to irinotecan in PDX models. To this end, tumors from 3 PDXs with either high (^{1}H) or low (L1 and L2) CES2 activity and expression were subcutaneously engrafted into nude mice (Fig 1A; Data Supplement). PDXs were selected based on similar latency to tumor formation and harvesting as well as comparable tumor take rates (Fig 1B). One month after implantation, PDX-bearing mice were treated for 4 weeks with either saline control or irinotecan (50 mg/kg; Fig 1B). ^{1}H mice exhibited a significant reduction in tumor volume over time after irinotecan compared with saline control treatment (2-sided $P = .012$ using linear mixed effect model [LMEM]; see Methods; Fig 1C). No significant effect on tumor volume over time was observed after irinotecan treatment in any L1 and L2 mice (2-sided $P > .05$ using LMEM; Fig 1C). Four of 5 ^{1}H mice exhibited regression in tumor volume and a durable response to irinotecan over time; significant differences were observed in tumor volume over time between low and high CES2 expression groups (2-sided $P = .018$ using LMEM; Fig 1D). These results reaffirm our previous findings and provide additional evidence showing that CES2 predicts response to irinotecan.[6]

CES2 Expression Is Regulated via HNF4A in PDAC

Next, we aimed to identify determinant(s) of CES2 expression in PDAC. Using TCGA and ICGC-AU RNA-seq datasets, we first performed Pearson correlation analyses to identify genes with *CES2*-concordant mRNA expression. A total of 439 shared genes were identified that were significantly positively correlated with *CES2* mRNA expression in both TCGA and ICGC-AU RNA-seq datasets ($r > 0.3$; $P < .005$). IPA of these 439 genes identified the transcription factor HNF4A as the top predicted upstream regulator (Fig 2A; Data Supplement). Top biologic functions were related to metabolic pathways, particularly lipid metabolism (Data Supplement).

To complement these findings, we performed RNA-seq analyses and proteomic profiling of 11 PDAC cell lines and conducted analyses to identify mRNAs or proteins positively correlated (Spearman $r > 0.6$; $P < .05$) with CES2 expression. IPA analyses of positively correlated genes from

FIG 1. (Continued). start of the treatment. (C) PDX growth curves. The graph represents the mean tumor volume ± SEM. Day 0 indicates the start of treatment. Curves were compared by linear mixed effects analysis (see Methods). (D) Spaghetti plot[43] providing over time tumor size percent change from baseline of irinotecan-treated PDX mice. H1, high CES2 activity and expression.

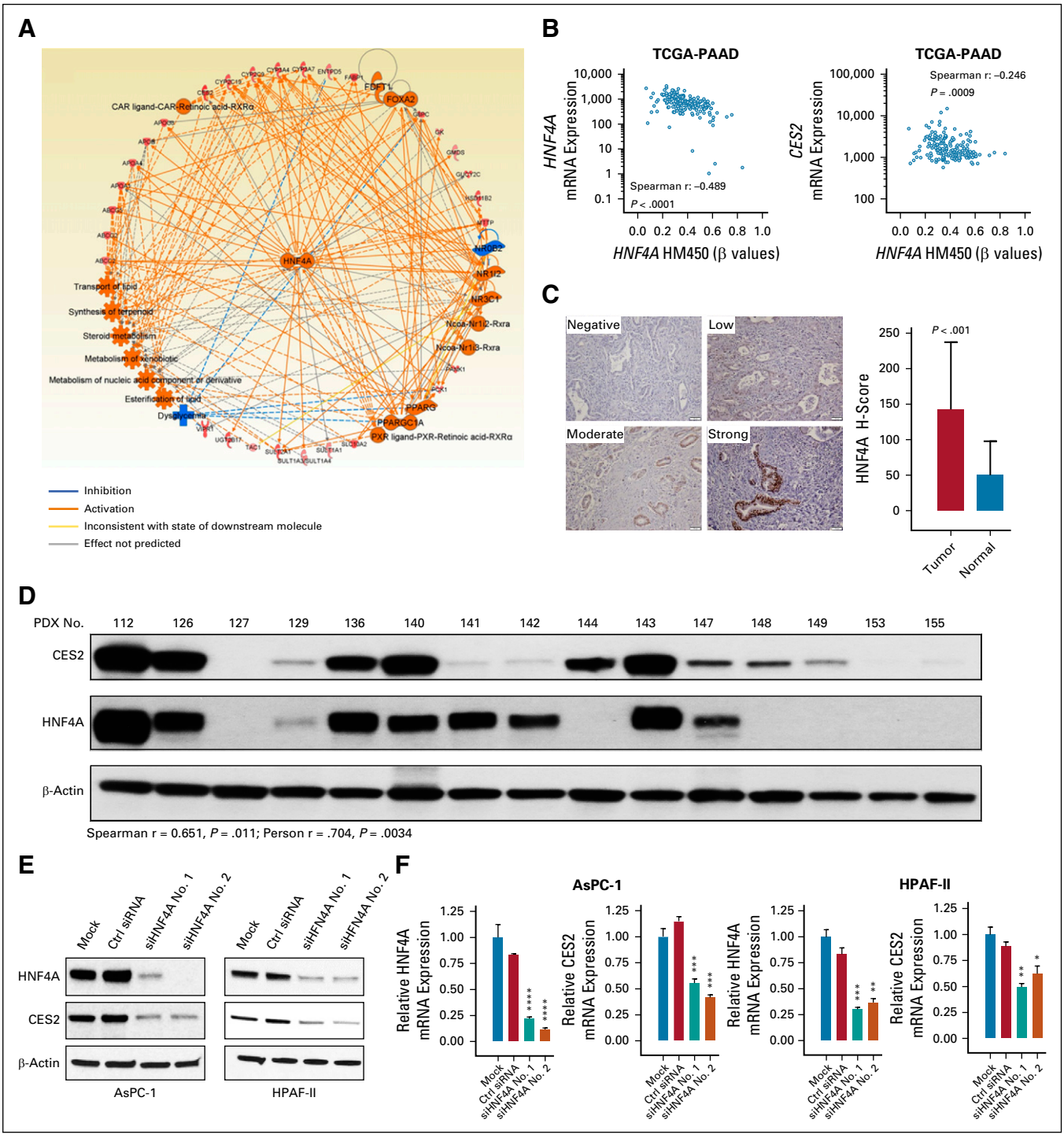


FIG 2. Hepatocyte nuclear factor 4 alpha (HNF4A) is the transcriptional regulator of carboxylesterase 2 (CES2) expression. (A) HNF4A-centered network showing transcription regulators and biologic functions predicted to be activated (orange) or inhibited (blue) by the Ingenuity Pathway Analysis of genes positively correlated with CES2 expression (red) in both the International Cancer Genome Consortium Australian Pancreatic Cancer Genome Initiative (ICGC-AU) and The Cancer Genome Atlas (TCGA) pancreatic cancer gene expression datasets. Solid lines indicate direct interaction; dashed lines indicate indirect interaction. (B) Correlation analyses between methylation beta-values for *HNF4A* and mRNA expression of *HFN4A* and *CES2* in pancreatic ductal adenocarcinoma (PDAC) TCGA gene expression dataset. (C) Immunohistochemical analysis, using tissue microarray, of HNF4A expression in PDAC. (Left panel) Representative micrographs showing different HNF4A immunostaining intensities. Scale bars represent 50 μm. (Right panel) Graph showing HNF4A H-score in PDAC and normal adjacent tissues. The graph represents mean ± standard deviation (SD). *P* value was calculated by 2-sided unpaired *t* test. (D) Western blot and correlation analysis of CES2 and HNF4A expression in patient-derived xenograft (PDX) tumors. β-actin served as a loading control. (E) Western blot analysis and (F) qRT–polymerase chain reaction of HNF4A and CES2 mRNA and protein expression levels, respectively, in AsPC-1 and HPAF-II PDAC cell lines untreated or treated with negative control (ctrl) and HNF4A siRNAs. β-actin was used as loading control. Graphs illustrate the mean result of 3 independent experiments ± SD. *P* value was calculated by 2-sided unpaired *t* test. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

TABLE 1. Association Between Clinicopathologic Variables and CES2 Expression

		Crude OR			Adjusted OR[a]		
Variable	**Value**	**OR**	**95% CI**	***P*** [b]	**OR**	**95% CI**	***P***
HNF4A	≥ Median *v* < median	4.11	1.31 to 12.89	**.021**	4.11	1.31 to 12.89	**.015**
Tumor size	≤ 2 cm *v* > 2 cm	0.70	0.24 to 2.06	.583	—	—	.455
Tumor margin	Negative *v* positive	0.89	0.33 to 2.43	1.000	—	—	.863
Lymph node metastasis	Negative *v* positive	1.12	0.45 to 2.78	1.000	—	—	.692
Tumor differentiation	Well/moderately *v* poor	2.45	1.10 to 5.44	**.039**	2.89	1.27 to 6.59	**.012**
Type 2 diabetes	Positive *v* negative	4.82	1.34 to 17.34	**.014**	4.84	1.34 to 17.54	**.016**
BMI	< 25 *v* ≥ 25	0.50	0.22 to 1.2	.106	—	—	.112

NOTE. Bold type indicates statistically significant.

Abbreviations: BMI, body mass index; CES2, carboxylesterase 2; HNF4A, hepatocyte nuclear factor 4 alpha; OR, odds ratio.

[a]From binary logistic regression model adjusted for age (< median *v* ≥ median), sex (female *v* male), and tumor stage (IIA *v* IIB), using a forward stepwise method (likelihood ratio). HNF4A, tumor differentiation, and type 2 diabetes were not included in the same model but assessed separately after adjusting for age (< median vs ≥ median), sex (female *v* male), and tumor stage (IIA *v* IIB)

[b]Fisher exact test.

these analyses similarly identified HNF4A as a major network node (Data Supplement).

HNF4A was overexpressed and its promoter hypomethylated in PDAC compared with adjacent nontumor tissue.[21-23] Consistently, analysis of the PDAC TCGA dataset indicated a significant inverse association between methylation beta-values for *HNF4A* and mRNA expression of *HFN4A* and *CES2* in PDAC, further implicating a positive relationship between *HNF4A* and *CES2* (Fig 2B). Immunohistochemical analysis of 188 PDACs by tissue microarray (TMA; Data Supplement) indicated significantly higher HNF4A protein levels in PDAC compared with normal adjacent tissue (H-score–based *t* test $P < .001$; Fig 2C) and a significant positive association between HNF4A and CES2 (adjusted OR, 4.11; 95% CI, 1.31 to 12.89; $P = .015$; Table 1). Western blot analyses for HNF4A and CES2 in 15 PDXs further established a positive correlation between HNF4A and CES2 expression (Spearman R, 0.651; $P = .011$; Fig 2D). To confirm that HNF4A is the upstream regulator of CES2, we performed *HNF4A* knockdown by siRNA in PDAC cell lines AsPC-1 and HPAF-II that express high mRNA levels of both *HNF4A* and *CES2*. Knockdown of *HNF4A* in AsPC-1 and HPAF-II resulted in significantly reduced mRNA and protein levels of CES2 (Figs 2E and 2F). Based on the Match algorithm of TRANSFAC database (Qiagen) 1 predicted and 3 reported ChIP assay–based HNF4A binding sites are known to be in the human *CES2* distal promoter. Our findings demonstrate that HNF4A is the upstream transcriptional regulator of CES2 expression in pancreatic cancer.

CES2 Expression Is Associated With the Pancreatic Progenitor Subtype of PDAC

The ICGC-AU genomic analysis delineates 4 PDAC molecular subtypes with distinct transcriptional and epigenetic profiles.[18] The pancreatic progenitor subtype (PPS) is characterized by hypomethylation and overexpression of *HNF4A,* together with other master regulators of GI differentiation (Data Supplement).[18,22-25] Given that our data implicates HNF4A as the upstream transcriptional regulator of CES2 (Figs 2E and 2F), we evaluated whether increased CES2 expression in pancreatic tumors would associate with the PPS. We used ICGC-AU and TCGA RNA-seq datasets and developed an estimated aggregate signature score for the PDAC PPS, based on mRNA expression of its distinctive transcription factors.[18] Stratification of the PPS mRNA expression score into tertiles and Spearman's correlation analyses using continuous values indicated significant positive association between *CES2* mRNA expression and PPS signature in both the ICGC-AU and TCGA datasets (Fig 3A; Data Supplement).

HbA1C Is a Predictive Marker for CES2 Expression and Response to FOLFIRINOX Therapy

These findings provide supportive evidence that elevated CES2 mRNA expression is highly associated with the PPS. HNF4A and genes that are characteristic of the PPS have been directly linked to maturity onset of diabetes.[18,26-29] Moreover, long-standing T2D has been linked to increased risk for PDAC.[30] Given these observations, we evaluated whether tumor CES2 protein expression would similarly associate with a history of T2D. CES2 expression in PDAC TMAs (Data Supplement) exhibited a significant positive association with tumor differentiation (adjusted OR, 2.89; 95% CI, 1.27 to 6.59; $P = .012$) and history of T2D among patients with tumors, most of whom had long-standing T2D (diagnosed more than 3 years before PDAC; adjusted OR, 4.84; 95% CI, 1.34 to 17.54; $P = .016$; Table 1). Notably, patients with high tumor CES2 protein expression exhibited significant increased plasma HbA1C levels compared with patients with low tumor CES2 expression (Wilcoxon rank

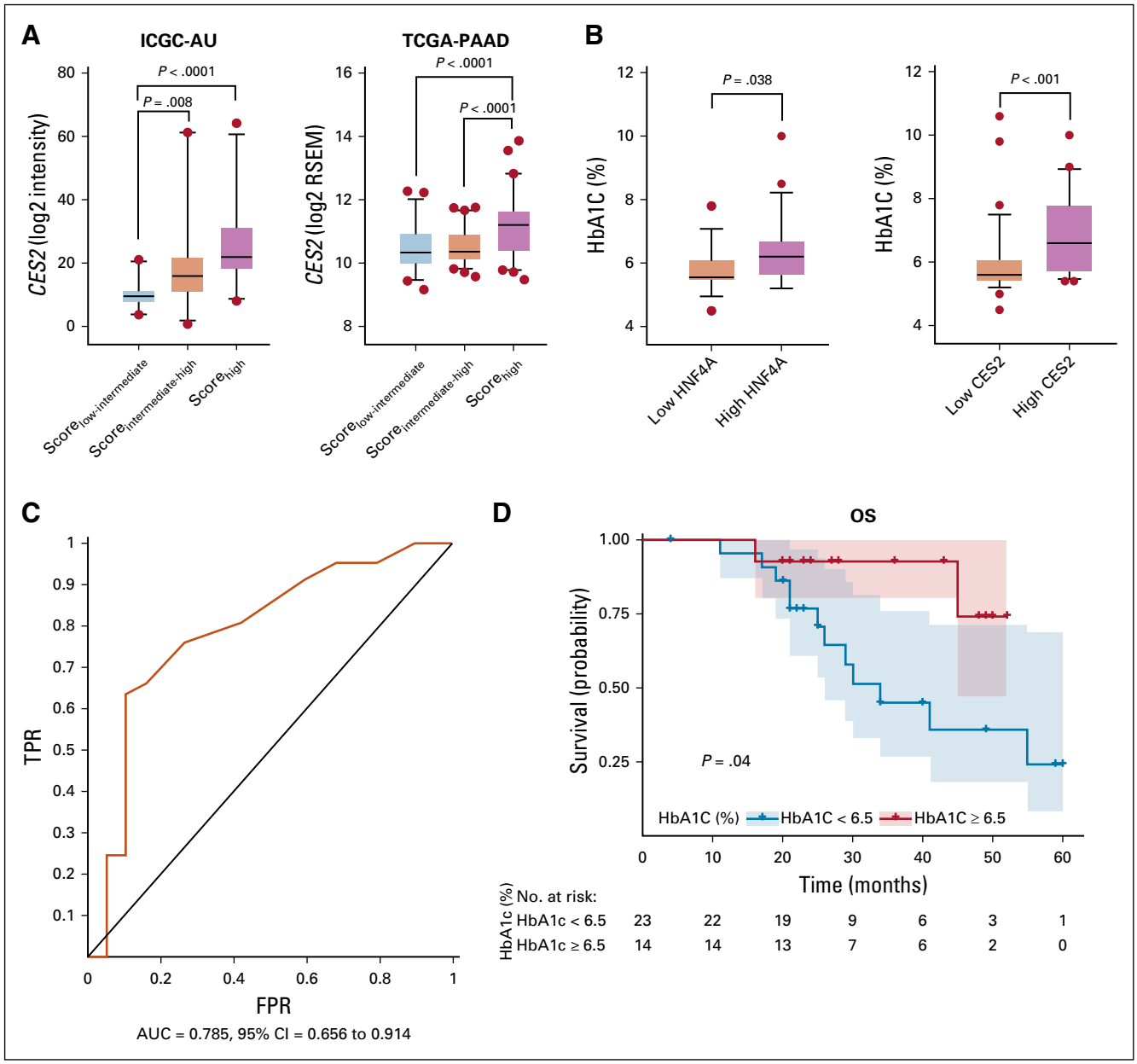


FIG 3. High hemoglobin A1C (HbA1C) is associated with carboxylesterase 2 (CES2) tumor expression and a significant increase in patient overall survival (OS) after neoadjuvant fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) therapy. (A) Boxplot showing CES2 mRNA expression in pancreatic tumors stratified into tertiles by aggregate signature score for the pancreatic progenitor subtype (PPS) in International Cancer Genome Consortium Australian Pancreatic Cancer Genome Initiative (ICGC-AU) and The Cancer Genome Atlas (TCGA) gene expression datasets. The PPS was defined by gene expression of the transcription factors *PDX1*, *MNX1*, *HNF4G*, *HNF4A*, *HNF1B*, *HNF1A*, *FOXA2*, *FOXA3*, and *HES1*[18,36,39] (Data Supplement). Significance was determined using the Kruskal Wallis test; pairwise comparisons were determined using Dunnett's post hoc test. (B) HbA1C (%) levels in patients stratified by tumor hepatocyte nuclear factor 4 alpha (HNF4A; left) or CES2 (right) protein expression. Significance was determined by Wilcoxon rank sum test. (C) Receiver operating characteristic curve showing HbA1C blood level distributions in patients with pancreatic ductal adenocarcinoma (PDAC) with CES2-positive and CES2-negative tumors. Treatment-naïve patients with resected PDAC were stratified based on CES2 expression. CES2 expression was assessed with trough immunohistochemical analysis. Positive CES2: strong or intermediate staining in 10% or more cells. Negative CES2: absent or weak staining. (D) Kaplan-Meier survival plot of patients who underwent first-line neoadjuvant FOLFIRINOX therapy stratified according to HbA1C blood levels ≥ 6.5%. AUC, area under the curve; PAAD, pancreatic adenocarcinoma; RSEM, RNA-seq by Expectation Maximization; TPR, true-positive rate.

sum $P < .001$; Fig 3B), yielding an area under the curve of 0.785 (95% CI, 0.656 to 0.914; 1-sided Wilcoxon rank sum test < 0.001) in discriminating the 2 groups (Fig 3C). HbA1C levels, but not cholesterol, also exhibited a significant positive correlation with HNF4A (Pearson's r, 0.36; $P = 0.022$; Data Supplement) and tended to be elevated in patients with high tumor HNF4A protein expression (1-sided Wilcoxon rank sum test $P = .038$; Fig 3B).

TABLE 2. Univariate and Multivariate Analyses of Overall Survival of Patients With PDAC Who Underwent First-Line Neoadjuvant FOLFIRINOX Therapy

		Univariate Analysis[a]			Multivariate Analysis[b]		
Characteristic	**Variable**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Sex	Male *v* female	1.04	0.37 to 2.98	.935	—	—	—
Ethnicity	White *v* other	1.21	0.76 to 11.35	.271	—	—	—
Age	≥ Median *v* < median	0.82	0.28 to 2.42	.709	—	—	—
Clinical stage	BR/LA *v* PR	4.19	1.24 to 14.19	.116	0.18	0.22 to 1.44	.105
No. FOLFIRINOX cycles	5-6 *v* 4	1.43	0.50 to 4.11	.515	—	—	—
BMI	≥ 25 *v* < 25	0.78	0.27 to 2.22	.635	—	—	—
HbA1C	> 6.5 *v* ≤ 6.5	0.25	0.08 to 0.72	**.040**	0.20	0.04 to 0.93	**.040**
T2D medication[c]	Yes *v* no	0.44	0.14 to 1.42	.260	—	—	—

NOTE. Bold type indicates statistically significant.

Abbreviations: BR, borderline resectable; FOLFIRINOX, fluorouracil, leucovorin, irinotecan, and oxaliplatin; HbA1C, hemoglobin A1C; HR, hazard ratio; LA, locally advanced; PDAC, pancreatic ductal adenocarcinoma; PR, potentially resectable; T2D, type 2 diabetes.

[a]Calculated using Kaplan-Meier analysis with comparisons performed with the log-rank test.

[b]Variables included in the equation after selection using a backward stepwise method (likelihood ratio).

[c]Insulin or oral agent.

Remarkably, patients with high HbA1C levels (> 6.5%; cutoff applied for T2D diagnosis) exhibited a significant increase in OS compared with patients with low HbA1C (≤ 6.5%; log-rank $P = .04$; hazard ratio [HR], 0.25; 95% CI, 0.08 to 0.72; Fig 3D; Table 2) in an independent set of patients with PDAC (n = 37), who underwent first-line neoadjuvant FOLFIRINOX treatment (Data Supplement). Multivariate Cox regression analyses indicated that HbA1C was the only independent predictor of OS (log-rank $P = .04$; HR, 0.20; 95% CI, 0.04 to 0.93; Table 2) after stepwise selection of clinicopathologic variables. To avoid dichotomization approaches, we used a Cox model (Data Supplement). Survival curves for elevated HbA1C values (7% and 8%) yielded significantly higher survival probabilities compared with the curves corresponding to lower HbA1C levels (4%, 5%, and 6%; Data Supplement). CES2 expression, HbA1C levels, and presence of T2D were not associated with OS in patients with stage II PDAC (Data Supplement) who did not receive any type of therapy (Data Supplement).

DISCUSSION

We expanded on our previous findings[6] and provided complementary in vivo evidence that high CES2 activity in PDAC PDXs is associated with increased sensitivity to irinotecan. We further identified HNF4A as the upstream transcriptional regulator of CES2 expression and provided a mechanistic framework, wherein CES2-mediated catabolism of choline-containing phospholipids promotes the biogenesis of endogenous lipid ligands that stimulate activation of HNF4A, thereby sustaining elevated CES2 expression. We additionally provided compelling evidence that elevated *CES2* mRNA expression is highly associated with the PPS and uncover a novel relationship that exists between CES2 and history of T2D. Specifically, our data suggest a positive association between tumor CES2, a history of T2D, and blood HbA1C levels. Remarkably, our analyses indicated that blood HbA1C, which is significantly positively correlated with tumor CES2, is a positive predictor for response to neoadjuvant FOLFIRINOX therapy.

HNF4A is a member of the steroid hormone receptor superfamily, expressed mainly in the liver.[31,32] HNF4A has been shown to be upregulated during neoplastic transformation.[22-25] Consistently, we observed overexpression of HNF4A in PDAC compared with normal adjacent tissue. HNF4A has been shown to regulate *CES2* expression in mouse liver[33,34] and to bind to the *CES2* promoter in human hepatocytes.[35] Concordantly, we observed a significant positive correlation between CES2 and HNF4A protein expression in PDAC primary and PDX tissues and demonstrated that *CES2* is a downstream target of HNF4A in PDAC cells.

The PPS is defined by gene programs regulating fatty acid oxidation, steroid hormone biosynthesis, and drug metabolism,[18] and by the hypomethylation and expression of the transcription factors including *HNF4A*.[18,36,37] Using 2 independent gene expression datasets, we provide evidence that elevated *CES2* mRNA expression is additionally a feature associated with the PPS. Recent studies have provided evidence that the classic/progenitor PDAC subtype responds significantly better to FOLFIRINOX therapy compared with gemcitabine-based treatment and to other PDAC subtypes.[38,39] Our findings point to a role of CES2 in this favorable response.

Given that our current and previous studies implicate tumor CES2 expression as a predictor of response to irinotecan-containing therapies, such as FOLFIRINOX,[6] and that tumor CES2 protein expression is significantly associated with elevated HbA1C levels, we assessed whether HbA1C can

serve as a proxy marker for CES2 expression that could be applied to define a subset of patients with PDAC likely to respond to neoadjuvant FOLFIRINOX therapy. Indeed, high HbA1C levels were associated with a significant increase in OS compared with low HbA1C in patients who underwent neoadjuvant FOLFIRINOX therapy. A previous study reported association between T2D and worse OS in patients with PDAC who received neoadjuvant FOLFIRINOX or gemcitabine-abraxane therapy.[40] This inconsistency with our results could be related to several differences in their study design: (1) the association was limited to patients with insulin-dependent but not noninsulin-dependent diabetes mellitus; (2) patients with both resected and unresected PDAC were included in the analysis; and (3) enrollment in the study was not restricted to patients being treated with FOLFIRINOX but also included patients being treated with gemcitabine-abraxane.[40]

We did not observe any association among CES2 expression, HbA1C levels, or presence of T2D in patients with resectable PDAC without FOLFIRINOX therapy. It has previously been reported that patients with T2D exhibit decreased OS compared with nondiabetic patients with PDAC.[41,42] The discrepancy with our study may reflect differences in the type of cohorts, because those patients were enrolled in prospective studies, the majority of them were diagnosed with advanced-stage PDAC, and, most importantly, patient treatment information was limited or absent.[41,42]

In summary, we demonstrated that the nuclear receptor HNF4A is the upstream transcriptional regulator of CES2 expression. We further provided persuasive evidence that CES2 expression is significantly elevated in the PPS and, to our knowledge, demonstrated for the first time that a history of T2D in PDAC is associated with improved response to FOLFIRINOX. HbA1C may represent a novel marker that, by predicting CES2 expression, can improve the stratification of patients with PDAC for FOLFIRINOX therapy.

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SUPPORT

Supported by the National Pancreas Foundation (M.C.), MD Anderson's Moonshot Program, 2 faculty fellowships from The University of Texas MD Anderson Cancer Center Duncan Family Institute for Cancer Prevention and Risk Assessment (J.F.F. and M.C.), 2 National Institutes of Health T32 fellowships (T32 CA009599 and P30 CA016672; M.V.R.P. and D.R.), and the Viragh Family Foundation.

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Financial support: Oliver Fiehn
Administrative support: Alia Fleury, Jennifer B. Dennison, Oliver Fiehn
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Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The following represents disclosure information provided by authors of this manuscript. All relationships are considered compensated unless otherwise noted. Relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information about ASCO's conflict of interest policy, please refer to www.asco.org/rwc or ascopubs.org/po/author-center.
Open Payments is a public database containing information reported by companies about payments made to US-licensed physicians (Open Payments).

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Employment: Janssen Research & Development
Patents, Royalties, Other Intellectual Property: There is an intellectual property related to biomarkers for early detection of pancreatic cancer

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Patents, Royalties, Other Intellectual Property: There is an intellectual property related to biomarkers for early detection of pancreatic cancer

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Patents, Royalties, Other Intellectual Property: Royalty distribution related to the license and collaboration agreement with Hangzhou Cosmos Wisdom Biotechnology. These refer to 2 blood-based biomarker panels for the detection of lung and pancreatic cancer

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Patents, Royalties, Other Intellectual Property: An intellectual property related to biomarkers for early detection of pancreatic cancer

Jennifer B. Dennison
Research Funding: Cosmos Wisdom (Inst), Dynex (Inst)
Patents, Royalties, Other Intellectual Property: Intellectual property on pancreas cancer early detection biomarkers

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Patents, Royalties, Other Intellectual Property: Royalties from Hangzhou Guangkeande (Cosmos) Biotechnology Company for blood-based biomarkers of early pancreatic cancer. I do not own stocks in the company nor do I have any research or grant funding from them. Johns Hopkins University has licensed a patent related to pancreatic cancer to Thrive Earlier Detection. I have 1.8% contribution on that patent. No royalties have been received by me

Ziding Feng
Patents, Royalties, Other Intellectual Property: I am one of the co-inventors for a biomarker panel for pancreatic cancer. The patent was filed by The University of Texas MD Anderson Cancer Center and was licensed to a company by the UT MD Anderson Cancer Center

Matthew H. Katz
Consulting or Advisory Role: Alcresta Therapeutics, AbbVie

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Leadership: Biopath Holdings
Consulting or Advisory Role: Johnson and Johnson, Glycobio, Moleculin Biotech, Perthera
Patents, Royalties, Other Intellectual Property: US Application No. 15/780,799, based on International Application No. PCT/US2016/065763, entitled "Polymeric Drug Delivery Systems for Treatment of Disease," by Chun Li et al; In the Name of Board of Regents, The University of Texas System

Samir M. Hanash
Patents, Royalties, Other Intellectual Property: Patents submitted for lung and pancreatic cancer diagnostic markers

No other potential conflicts of interest were reported.

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