

## Glycogen Synthase Kinase 3 Beta Predicts Survival in Resected Adenocarcinoma of the Pancreas

Edgar Ben-Josef<sup>1</sup>, Asha George<sup>2</sup>, William F. Regine<sup>3</sup>, Ross Abrams<sup>4</sup>, Meredith Morgan<sup>5</sup>, Dafydd Thomas<sup>5</sup>, Paul L. Schaefer<sup>6</sup>, Thomas A. DiPetrillo<sup>7</sup>, Mitchel Fromm<sup>8</sup>, William Small Jr<sup>9</sup>, Samir Narayan<sup>10</sup>, Kathryn Winter<sup>2</sup>, Kent A. Griffith<sup>5</sup>, Chandan Guha<sup>11</sup>, and Terence M. Williams<sup>12</sup>

### Abstract

**Purpose:** GSK3 $\beta$  is a protein kinase that can suppress a number of key oncoproteins. We have previously shown in preclinical models of pancreatic ductal adenocarcinoma (PDAC) that inhibition of GSK3 $\beta$  causes stabilization and nuclear translocation of  $\beta$ -catenin, poor differentiation, proliferation, and resistance to radiation. The objective of this study was to determine its utility as a biomarker of clinical outcomes.

**Experimental Design:** Automated Quantitative Immunofluorescence Analysis (AQUA) of GSK3 $\beta$  was performed on a tissue microarray with samples from 163 patients treated on RTOG 9704. On the basis of findings in an exploratory cohort, GSK3 $\beta$  was analyzed as a categorical variable using its upper quartile (>Q3) as a cut point. Overall survival (OS) and disease-free survival (DFS) were estimated with the Kaplan–Meier method, and GSK3 $\beta$  groupings were compared using the log-rank test.

Univariable and multivariable Cox proportional hazards models were used to determine associations between GSK3 $\beta$  and OS/DFS.

**Results:** The 3-year OS rates for GSK3 $\beta$   $\leq$  Q3 versus GSK3 $\beta$  > Q3 were 16% (95% confidence intervals; CI, 10%–23%) and 30% (95% CI, 17%–44%), respectively,  $P = 0.0082$ . The 3-year DFS rates were 9% (95% CI, 5%–15%) and 20% (95% CI, 9%–33%) respectively,  $P$  value = 0.0081. On multivariable analysis, GSK3 $\beta$  was a significant predictor of OS. Patients with GSK3 $\beta$  > Q3 had a 46% reduced risk of dying of pancreatic cancer (HR, 0.54; 95% CI, 0.31–0.96,  $P$  value = 0.034). The HR for DFS was 0.65 (95% CI, 0.39–1.07;  $P$  value = 0.092).

**Conclusions:** GSK3 $\beta$  expression is a strong prognosticator in PDAC, independent of other known factors such as tumor (T) stage, nodal status, surgical margins and CA19-9. *Clin Cancer Res*; 21(24); 5612–8. ©2015 AACR.

### Introduction

GSK3 $\beta$  is a protein kinase involved in the regulation of cell cycle, transcription, proliferation, differentiation, and apoptosis. A number of key oncoproteins, including  $\beta$ -catenin, c-Myc, Cyclin D, Cyclin E, and c-Jun, are known substrates of GSK3 $\beta$ ; most are functionally inhibited by it (1–3).

Wnt signaling is essential for the embryonic development of the exocrine pancreas (4, 5), and deregulation of this pathway has been linked to pancreatic ductal adenocarcinoma (PDAC; ref. 6).

<sup>1</sup>University of Pennsylvania, Philadelphia, Pennsylvania. <sup>2</sup>Radiation Therapy Oncology Group-Statistical Center, Philadelphia, Pennsylvania. <sup>3</sup>University of Maryland Medical Systems, Baltimore, Maryland. <sup>4</sup>Rush University Medical Center, Chicago, Illinois. <sup>5</sup>University of Michigan Medical School, Ann Arbor, Michigan. <sup>6</sup>Toledo Community Hospital Oncology Program CCOP, Toledo, Ohio. <sup>7</sup>Providence, Rhode Island. <sup>8</sup>Akron General Medical Center, Akron, Ohio. <sup>9</sup>Northwestern Memorial Hospital, Chicago, Illinois. <sup>10</sup>Michigan Cancer Research Consortium CCOP, Ann Arbor, Michigan. <sup>11</sup>Montefiore Medical Center, Moses Campus, Bronx, New York. <sup>12</sup>Ohio State University Medical Center, Columbus, Ohio.

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

**Corresponding Author:** Edgar Ben-Josef, Department of Radiation Oncology, University of Pennsylvania, Perelman Center for Advanced Medicine, 2 West Room 306, 3400 Civic Center Boulevard, Philadelphia, PA 19104. Phone: 215-662-3932; Fax: 215-349-5445; E-mail: edgar.ben-josef@uphs.upenn.edu

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GSK3 $\beta$  is a well-characterized negative regulator of canonical Wnt signaling: it phosphorylates  $\beta$ -catenin, targeting it for degradation. Although mutations in pathway components such as  $\beta$ -catenin and APC are rare in PDAC (7, 8), abnormal accumulation of  $\beta$ -catenin in the nucleus and cytoplasm has been described in a large fraction of pancreatic intraepithelial neoplasm (PanIN) lesions and PDAC (9–11). In addition, genomic characterization revealed that 100% of patients with pancreatic cancer have aberrations of the Wnt or Notch pathways (12).  $\beta$ -Catenin aberrant localization is most pronounced in high-grade PanIN and invasive carcinoma (9) and its expression correlates with the degree of differentiation (13). Wnt pathway activity has been shown to be increased in the majority of human PDAC samples and cell lines tested and inhibition of the pathway resulted in a reduction in cell proliferation and an increase in apoptosis (10). Furthermore, ataxia telangiectasia group D–associated protein (ATDC) mediated accumulation of  $\beta$ -catenin and activation of its target genes was shown to promote PDAC growth and metastasis (14).

It has been previously shown that inhibition of GSK3 $\beta$  in a preclinical PDAC model causes stabilization and nuclear translocation of  $\beta$ -catenin, and induces poor differentiation, proliferation, and resistance to radiation (15). To explore the potential utility of GSK3 $\beta$  as a prognostic biomarker of clinical outcomes, we examined its cytoplasmic expression in a tissue microarray (TMA) generated from patients enrolled in Radiation Therapy Oncology Group (RTOG) 9704, a prospective intergroup multicenter phase III trial of adjuvant chemotherapy and chemoradiation for resected PDAC (16).

### Translational Relevance

There is a great need for good biomarkers in pancreatic ductal adenocarcinoma (PDAC). GSK3 $\beta$  is a protein kinase that suppresses a number of oncoproteins, including Wnt signaling. In preclinical models, GSK3 $\beta$  inhibition causes nuclear translocation of  $\beta$ -catenin, increased proliferation and resistance to radiation. Herein, we show that GSK3 $\beta$  is an independent prognosticator in patients with PDAC. On the basis of exploratory analysis in an independent cohort, we assayed GSK3 $\beta$  expression in a tissue microarray from RTOG-9704. We show that high expression of GSK3 $\beta$  is associated with a clinically meaningful significant improvement in overall survival (HR, 0.54) and that this effect is independent of other known prognostic factors such as T- and N-stage, resection margins, and CA19-9. This represents an important step forward in personalized therapy as low GSK3 $\beta$  defines a group of patients with particularly poor outcomes. This novel biomarker can also be used for stratification in future clinical trials.

## Patients and Methods

### Patient population

The exploratory cohort consisted of a 38-sample TMA from patients who underwent pancreaticoduodenectomy at the University of Michigan (Ann Arbor, MI). The samples were linked to a clinical database with complete details on patient population, tumor characteristics, treatment, and clinical outcomes. A full description of this cohort has been previously published (17). IHC peroxidase staining with a GSK3 $\beta$  antibody (Abcam) was performed. Stained slides were scored by a gastrointestinal pathologist using a three-tier system (none, low, and high).

The test cohort consisted of TMA slides of patients treated on RTOG 9704. The eligibility criteria for RTOG 9704 included histologically confirmed PDAC, pathologic stages T1–4, N0–1, M0, gross total tumor resection, Karnofsky performance status of  $\geq 60$ , and adequate hematologic, renal, and hepatic function. After resection, patients were randomly assigned to either continuous infusion 5-fluorouracil (5-FU), 250 mg/m<sup>2</sup>/day (arm 1) or gemcitabine, 1,000 mg/m<sup>2</sup>, 30-minute infusion once weekly (arm 2) for 3 weeks before and 12 weeks after chemoradiotherapy (CRT). CRT was identical in both arms. It consisted of 50.4 Gy in 28 fractions to the tumor bed and regional nodes delivered concurrently with 5-FU, 250 mg/m<sup>2</sup>/day. Post-CRT chemotherapy consisted of 3 months of 5-FU or gemcitabine in arm 1 and 2, respectively. The study accrued 451 eligible patients and showed no statistically significant difference in OS between the arms.

### GSK3 $\beta$ assay

Automated Quantitative Immunofluorescence Analysis (AQUA) was conducted as described by Camp and colleagues (18). AQUA is a method of determining protein levels based on automated quantification of fluorescence intensity in targets of interest. Briefly, slides were stained for cytokeratin 8 (Novus Biologicals, NBP1-04926, 1:1000) and GSK3 $\beta$  (AbCam, AB31826, clone M131, 1:600). The optimization of antibody concentrations and other conditions was performed as described by Bordeaux (19) and Dolled-Filhart (20). The antibodies were

extensively validated using IHC on several different University of Michigan TMAs (a multitumor TMA, a breast cancer TMA, a pancreatic TMA, a urological TMA, and a TMA of normal tissues). A pathologist (D.G. Thomas) verified that the fluorescent stain was done properly, and that the cytokeratin stain was correctly staining the carcinoma cells.

Images of each core were captured with a microscope at three different extinction/emission wavelengths. Within each tumor core, areas of tumor were distinguished from stroma and necrotic areas by the cytokeratin stain (an epithelial marker). The pixel intensity of the GSK3 $\beta$  protein/antibody complex was then machine-read and reported. GSK3 $\beta$  was read only within the tumor-specific mask. An example of the GSK3 $\beta$  stain in one patient is depicted in Fig. 1. Cores that did not pass the quality-assurance checks in the software were excluded from scoring. Each patient's tumor in the TMA was represented by two cores, and results were averaged, providing a better assessment of the degree of GSK3 $\beta$  staining within the tumor of each patient.

### Statistical analysis

Overall survival (OS) was calculated from date of randomization to date of death due to any cause or last follow-up for censored patients. Disease-free survival (DFS) events were defined as local, regional, or distant relapse, appearance of a second primary lesion or death due to any cause. DFS was calculated from date of randomization to date of first documented failure or last follow-up for censored patients. OS and DFS were estimated univariately with the Kaplan–Meier method (21).

On the basis of the findings in the exploratory cohort, GSK3 $\beta$  was categorized using its upper quartile as a cut point. Although the exploratory cohort had three categories of GSK3 $\beta$  expression, only approximately one quarter of patients were in the highest expression category. For this reason, to more closely approximate how the analysis was done in the exploratory cohort, patients in the upper quartile ( $>Q3$ ) were compared with patients in the lower three quartiles ( $\leq Q3$ ) in the test cohort.

GSK3 $\beta$  groupings were compared using the log-rank test. Potential associations between baseline characteristics and GSK3 $\beta$  groupings were carried out using the  $\chi^2$  or Fisher exact test.

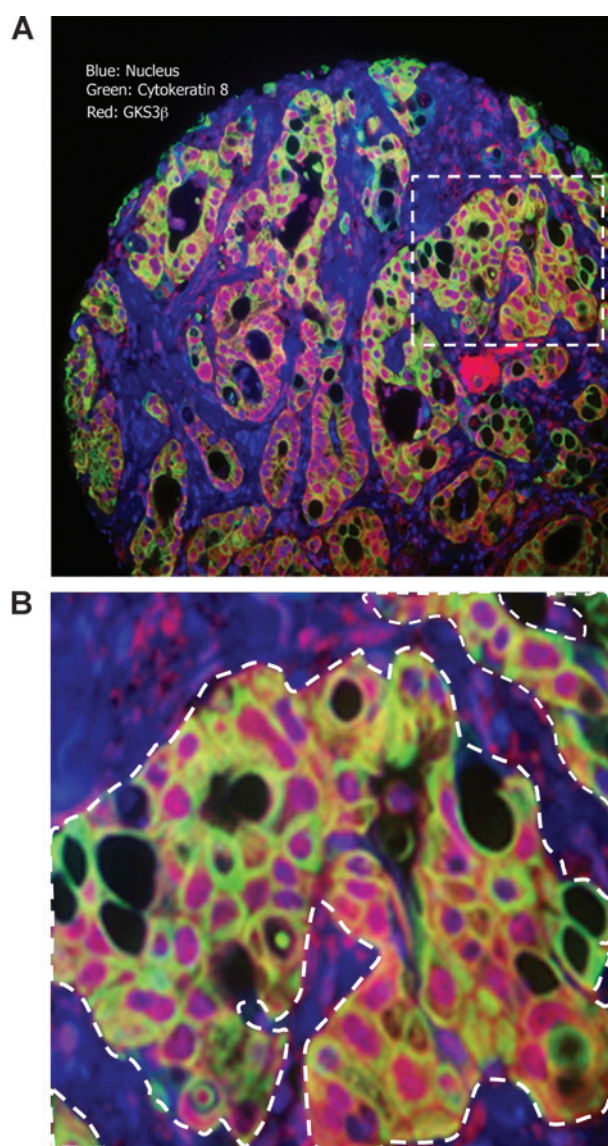
Univariate and multivariate Cox proportional hazards models (22) were used to determine whether there are any associations of GSK3 $\beta$  with OS and DFS. For the multivariable analysis, only GSK3 $\beta$  was forced into the models and a backward selection procedure was used to choose other variables using  $\alpha \geq 0.05$  level as the exit criteria for the model building. The following variables were assessed in the models along with GSK3 $\beta$ : treatment arm, age, gender, race, primary tumor location, nodal status (stratification variable), largest tumor dimension (stratification variable), and surgical margin status (stratification variable). The following baseline characteristics were dichotomized: pathologic T-stage (T1, T2 vs. T3, T4) and AJCC stage (I, II vs. III, IV). Race was categorized as White vs. African American/other. The proportional hazards assumption was evaluated by graphing the log(-log (survival)) versus log of survival time for the GSK3 $\beta$  groupings, which should result in parallel curves.

## Results

### Exploratory cohort

On the basis of preclinical data generated at the University of Michigan, GSK3 $\beta$  was first tested for its prognostic value in an

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**Figure 1.**

Automated quantitative immunofluorescence analysis (AQUA). Example of a tumor with GSK3 $\beta$  expression (AQUA score 3819) at low magnification (A) and high magnification (B). Dashed box in A, represents area of high magnification. Dashed areas in B represent the portions of tumor that were scored for GSK3 $\beta$  expression. The cells in this area exhibited positive cytochrome staining and thereby formed the "tumor mask."

exploratory dataset of a group of patients treated at the University of Michigan with chemotherapy and chemoradiation after resection of their pancreatic cancer (17). It was hypothesized that, as a negative regulator of Wnt, substantial expression would be required for it to exert an effect. The analysis revealed a trend toward improved progression-free survival (PFS) when all three groups were compared with each other (Supplementary Fig. S1A). Because the outcome of patients with no- and low GSK3 $\beta$  expression was not statistically different, these two groups were combined into one. Comparing this new low expression group to the original group of high expression revealed a statistically signifi-

cant difference in PFS in favor of high GSK3 $\beta$  expression (Supplementary Fig. S1B).

The estimated 3-year PFS rates were 16.7% and 66.7% in the high and low GSK3 $\beta$  groups, respectively. To validate these findings, in order to detect a difference between 20% and 60% 3-year PFS, using a two-sided test with  $\alpha < 0.05$  and 90% power, a minimum of 30 patients per expression group would be required.

#### Test cohort

GSK3 $\beta$  was then assayed in a TMA from 199 eligible patients treated on RTOG 9704. Of these, 36 patients failed the AQUA quality test and were excluded from analysis. The remaining 163 eligible and analyzable patients form the test cohort. This number exceeded the minimal sample size calculated based on the exploratory analysis. The distribution of GSK3 $\beta$  in this cohort by treatment arm is shown in Supplementary Table S1.

To ensure that the test cohort is a representative sample of patients treated on RTOG 9704, we tested for differences in baseline characteristics of the 163 eligible and GSK3 $\beta$ -analyzable cases and all other eligible cases on the trial. There were no statistically significant differences. Similarly, baseline characteristics were not

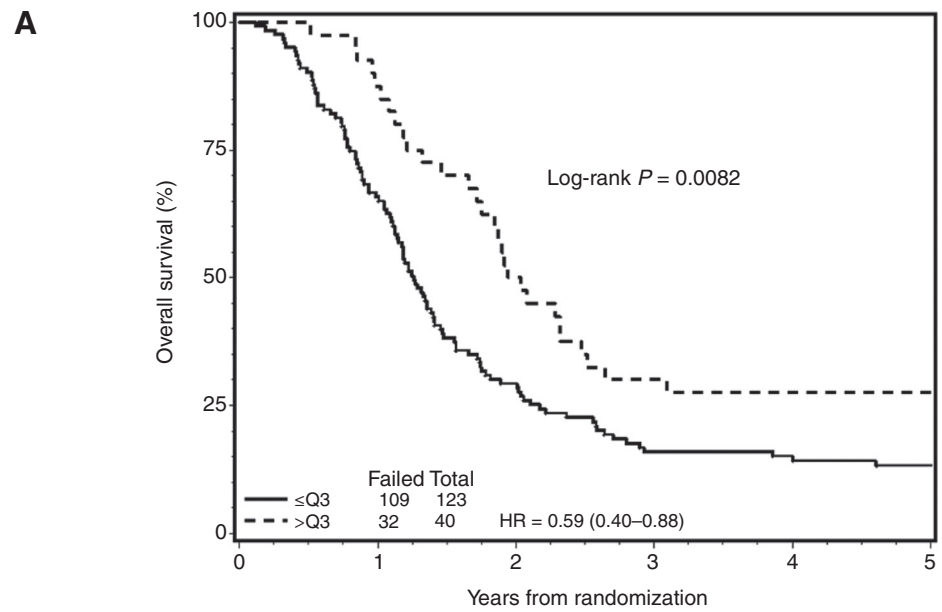
**Table 1.** Characteristics of patients entered on RTOG 9704 with GSK3 $\beta$  expression ( $n = 163$ )

	$\leq$ Q3 ( $n = 123$ )	$>$ Q3 ( $n = 40$ )	$P^a$
Age, y			
Median	60	63	0.21 <sup>b</sup>
Min-max	35-80	42-80	
Gender			0.76
Male	71 (57.7%)	22 (55.0%)	
Female	52 (42.3%)	18 (45.0%)	
Race			0.75
White	113 (91.9%)	36 (90.0%)	
African-American/Other	10 (8.1%)	4 (10.0%)	
Primary tumor location			0.25
Head	105 (85.4%)	31 (77.5%)	
Neck/body/tail	18 (14.6%)	9 (22.5%)	
KPS			0.30
60,70,80	48 (39.0%)	12 (30.0%)	
90,100	75 (61.0%)	28 (70.0%)	
T stage			0.44
T1,T2	32 (26.0%)	8 (20.0%)	
T3,T4	91 (74.0%)	32 (80.0%)	
N stage			0.85
N0	41 (33.3%)	14 (35.0%)	
N1	82 (66.7%)	26 (65.0%)	
AJCC stage			0.93
I and II	39 (31.7%)	13 (32.5%)	
III and IV	84 (68.3%)	27 (67.5%)	
Largest dimension of primary			0.39
$<$ 3 cm	43 (35.0%)	17 (42.5%)	
$\geq$ 3 cm	80 (65.0%)	23 (57.5%)	
Primary tumor status			0.93
Complete resection/negative margins	47 (38.2%)	16 (40.0%)	
Complete resection/positive margins	44 (35.8%)	13 (32.5%)	
Complete resection/unknown margins	32 (26.0%)	11 (27.5%)	
RX			0.49
RT + 5-FU	60 (48.8%)	22 (55.0%)	
RT + Gemcitabine	63 (51.2%)	18 (45.0%)	

<sup>a</sup> $P$  value from the  $\chi^2$ /Fisher exact test.

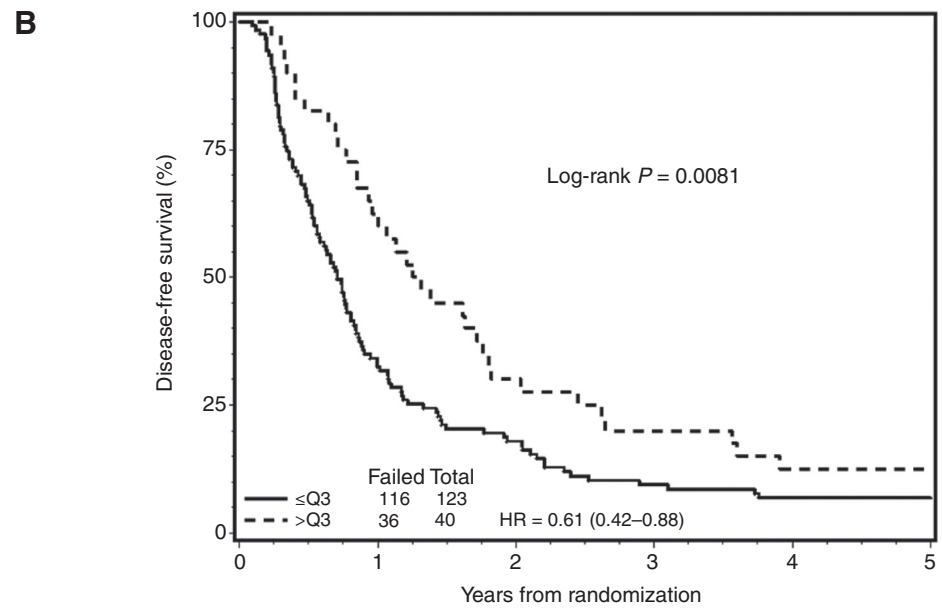
<sup>b</sup>Kruskal-Wallis test.





**Figure 2.** OS (A) and DFS (B), by GSK3β expression. The 3-year OS rates for GSK3β ≤Q3 versus GSK3β >Q3 were 16% (95% CI, 10%–23%) and 30% (95% CI: 17%–44%), respectively (log rank *P* = 0.0082). The 3-year DFS rates for those with GSK3β ≤Q3 and GSK3β >Q3 were 9% (95% CI, 5%–15%) and 20% (95% CI, 9%–33%), respectively (log-rank *P* = 0.0081).

Patients at risk	0	1	2	3	4	5
≤Q3	123	81	36	19	16	14
>Q3	40	35	20	12	10	10



Patients at risk	0	1	2	3	4	5
≤Q3	123	40	22	11	8	8
>Q3	40	25	12	8	5	5

significantly different among patients in the upper quartile (>Q3) and lower three quartiles (≤Q3) for GSK3β expression (Table 1). We also tested the proportional hazards assumption. While it was not fully met (as is usually the case), the curves for assessing this assumption were roughly parallel, making reporting the HRs still appropriate.

The 3-year OS rates for GSK3β ≤Q3 versus GSK3β >Q3 were 16% (95% CI, 10%–23%) and 30% (95% CI, 17%–44%), respectively (log-rank *P* value = 0.0082; Fig. 2A). Table 2 shows the Cox proportional hazards model for this grouping. Patients with GSK3β >Q3 have a 41% decrease in the risk of dying than those with GSK3β ≤Q3 (HR, 0.59; 95% CI, 0.40–0.88, *P* value = 0.009).

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**Table 2.** Univariate Cox proportional hazard model for GSK3 $\beta$  expression ( $n = 163$ )

Endpoint	GSK3 $\beta$ expression	HR <sup>a</sup>	P <sup>b</sup>
Overall survival	$\leq Q3$	1.00	—
	$>Q3$	0.59 (0.40–0.88)	0.0090
DFS	$\leq Q3$	1.00	—
	$>Q3$	0.61 (0.42–0.88)	0.0087

<sup>a</sup>HR < 1 indicates a decreased risk of death (in the OS model) or disease recurrence (in the DFS model) for the second level of the variables listed.

<sup>b</sup>P value from the Wald  $\chi^2$  test using the Cox proportional hazards model.

The 3-year DFS rates for those with GSK3 $\beta$   $\leq Q3$  and GSK3 $\beta$   $>Q3$ , were 9% (95% CI, 5%–15%) and 20% (95% CI, 9%–33%), respectively (log-rank  $P$  value = 0.0081; Fig. 2B). Table 2 shows the Cox proportional hazards model for this grouping. Patients with GSK3 $\beta$   $>Q3$  had a 39% decrease in the risk of disease recurrence as compared with patients with GSK3 $\beta$   $\leq Q3$  (HR, 0.61; 95% CI, 0.42–0.88,  $P$  value = 0.0087).

Potential correlations between GSK3 $\beta$  expression and CA19-9 and tumor grade were tested. There were no statistically significant correlations.

Because CA19-9, a known prognostic factor in PDAC, was not available for all patients, separate multivariable analyses for OS and DFS in all patients and in patients with CA19-9 were conducted. Table 3 shows the multivariable Cox proportional hazards model of OS for the 95 patients who had a pretreatment CA19-9. In the final model, GSK3 $\beta$  was a significant predictor of OS (as were surgical margins, age and CA19-9). Patients with GSK3 $\beta$   $>Q3$  have 46% reduced risk of dying of pancreatic cancer than patients with GSK3 $\beta$   $\leq Q3$  (HR, 0.54; 95% CI, 0.31–0.96,  $P$  value = 0.034). No other variables (including treatment arm, nodal status, and tumor diameter) were significantly associated with OS. Supplementary Table S2 shows the multivariable Cox proportional hazards model of OS for all 163 patients, including those who did not have pretreatment CA19-9. GSK3 $\beta$  was a significant predictor of OS in the final model as well.

Table 4 shows the multivariable Cox model of DFS in patients with CA19-9. GSK3 $\beta$  expression had a borderline-significant association with DFS, with a HR of 0.65 (95% CI, 0.98–1.07;  $P$  value = 0.092) while surgical margins and CA19-9 were statistically significant. Supplementary Table S3 shows the multivariable Cox model of DFS in all patients. In this model, GSK3 $\beta$  was the only factor that was statistically significant.

To determine whether GSK3 $\beta$  is a prognostic factor or predictive of chemotherapy benefit in PDAC, the analyses above were also conducted within each treatment arm separately (i.e., 5-FU or gemcitabine-based treatment arms). There were no significant differences in the observed effects by treatment arm, indicating that GSK3 $\beta$  is not a predictive biomarker for either 5-FU or gemcitabine-based therapies.

**Table 3.** Multivariable Cox proportional hazards model of overall survival ( $n = 95^a$ )

Adjustment variables	Comparison	HR <sup>b</sup>	95% CI LL	95% CI UL	P <sup>c</sup>
GSK3 $\beta$	$\leq Q3$ vs. $>Q3$	0.54	0.31	0.96	0.034
Surgical margin status	Negative vs. positive	0.87	0.52	1.48	0.62
	Negative vs. unknown	0.48	0.27	0.87	0.016
Age	Continuous	0.97	0.95	0.99	0.0032
CA19-9	Continuous (unit increase = 30)	1.05	1.02	1.08	0.0031

<sup>a</sup>This multivariable model excludes 68 patients with no pretreatment CA19-9 value. The Lewis antigen-negative patients were analyzed as CA19-9 = 0.

<sup>b</sup>HR of 1 indicates no difference between the two subgroups. The variables were coded such that a HR < 1 indicates a decreased risk of death for the second level of the variables listed.

<sup>c</sup>P value from the Wald  $\chi^2$  test using the Cox proportional hazards model.

## Discussion

The main finding in this study is that GSK3 $\beta$  is an important independent prognostic factor in PDAC. We noted markedly superior survival and DFS (8.8 and 6.8 months improvement in median, respectively) in patients with high expression of GSK3 $\beta$ . This novel biomarker performed remarkably well in separating two distinct subgroups of patients with widely varying prognosis and clinical outcomes. These differences were clinically meaningful, essentially doubling of overall survival and DFS in high expressors. In comparison, the addition of erlotinib to gemcitabine in patients with metastatic pancreatic cancer in the NCIC trial resulted in HR of 0.82, a statistically significant difference prompting FDA approval, but clinically not meaningful – an increase of only two weeks in median survival (23).

Biologically, GSK3 $\beta$  is a negative regulator of  $\beta$ -catenin; it is part of a complex that ubiquitinates  $\beta$ -catenin, thereby tagging it for proteasomal degradation.  $\beta$ -catenin is an oncogenic transcription factor that has been linked to numerous processes that drive carcinogenesis, differentiation, tumor growth, and metastasis. However, despite the established role of Wnt signaling in cancer pathogenesis, little is known about the expression of proteins of this pathway in PDAC or of any relation of this expression to clinical outcomes. This study is the first to demonstrate the clinical significance of a protein of the Wnt pathway in patients with PDAC and the first to validate a molecular biomarker in this disease.

Prognostic factors are important for optimizing care and in clinical trial design. They allow selection of therapy appropriate for the individual patient and provide potential stratification variables to minimize bias in the evaluation of new treatments. This is particularly important in PDAC where the TNM staging system provides little prognostic fidelity and treatment paradigms have been based on gross (and often controversial) categorization based on resectability. Many patient- and disease-related factors have been examined for their prognostic utility: age, sex, performance status, socioeconomic status, ethnicity, tumor markers (CA19-9 and CEA), location within the pancreas, tumor size, extent, grade, differentiation, perineural and blood/lymph vessel invasion, and lymph node status. Tumor diameter, lymph node status, differentiation, negative resection margins (24, 25), and CA19-9 (26) seem to be the most important factors, although there is substantial disagreement between studies. In RTOG 9704, the study from which our samples were obtained, only nodal involvement and CA19-9 have been previously shown to have a statistically significant independent effect on survival. We now show that GSK3 $\beta$  is an additional independent factor in that dataset.

It is worth noting that the backwards selection multivariable modeling did not include nodal status in the final model, as the

**Table 4.** Multivariable cox proportional hazards model of disease-free survival ( $n = 95^a$ )

Adjustment variables	Comparison	HR <sup>b</sup>	95% CI LL	95% CI UL	P e <sup>c</sup>
GSK3 $\beta$	$\leq$ Q3 vs. $>$ Q3	0.65	0.39	1.07	0.092
Surgical margin status	Negative vs. positive	1.22	0.74	1.99	0.44
	Negative vs. unknown	0.57	0.33	0.97	0.04
CA19-9	Continuous (unit increase = 30)	1.04	1.01	1.07	0.0091

<sup>a</sup>This multivariable model excludes 68 patients with no pretreatment CA19-9 value. The Lewis antigen-negative patients were analyzed as CA19-9 = 0.

<sup>b</sup>HR of 1 indicates no difference between the two subgroups. The variables were coded such that a HR < 1 indicates a decreased risk of death for the second level of the variables listed.

<sup>c</sup>P value from the Wald  $\chi^2$  test using the Cox proportional hazards model.

modeling algorithm frequently selected nodal status for exit. However, the final models did include CA-19-9 (Tables 3 and 4) suggesting that the predictive power of GSK3 $\beta$  exceeds that of nodal status, but is only additive to that of CA19-9. Also, although the proportional hazards assumption was not fully met, it is robust. In this regard, our data are not different from data reported from most clinical trials. It is important to keep in mind that each reported HR represents an average effect over the range of times observed.

In addition to the clinical and pathologic factors discussed above, a large number of molecular biomarkers have been examined with inconsistent findings. In a recent meta-analysis, VEGF, Bcl-2, bax, and p16 were found to be significant prognostic factors; p53, smad4, and EGFR were not (27). Importantly, none of these biomarkers have been validated in a prospective clinical trial.

Wnt activity has been studied in human samples of PDAC very rarely. Ougolkov and colleagues (28) reported nuclear accumulation of GSK3 $\beta$  in 62 of 122 human samples and found that this accumulation correlated with poor differentiation. However, the authors did not link this finding to clinical outcomes. The relationship between Wnt signaling and clinical outcomes in other cancers is also not well understood. Dickkopf-1 (a Wnt antagonist) and  $\beta$ -catenin may be of prognostic value in breast cancer (29). Epigenetic silencing of Dickkopf-3 was found to be common in gastric cancer and associated with poor outcome (30). A number of investigators examined the correlation of  $\beta$ -catenin expression with outcomes in colorectal cancer and reported inconsistent results. Some found shorter survival with cytoplasmic/nuclear expression (31, 32) while others have not (33).

This study shows that GSK3 $\beta$  is associated with a better prognosis in pancreatic cancer. However, it is not clear whether GSK3 $\beta$  is driving a tumor-suppressive state or is merely a biomarker for a more favorable disease. Certainly, the results are consistent with previous observations that Wnt activation, with consequent  $\beta$ -catenin cytoplasmic accumulation and nuclear translocation, promotes PDAC growth, metastasis, and resistance to therapy (15). The observations are also in line with the well-established role of GSK3 $\beta$  as a suppressor of Wnt activity. Furthermore, other oncoproteins, such as c-Myc, Cyclin D, Cyclin E, and c-Jun, are functionally inhibited by GSK3 $\beta$  (1–3) and it is possible that GSK3 $\beta$  influences outcome through regulation of multiple pathways. It is also possible that the effects of GSK3 $\beta$  in the cytoplasm are different than they are in the nucleus, as suggested by Ougolkov's report (28). In future work, cytoplasmic and nuclear GSK3 $\beta$  expression and their associations with outcomes should be assessed. It is also interesting to note that the other GSK3 isoform, GSK3 $\alpha$ , may have an opposite effect in pancreatic cancer (34, 35). This may have significant implications in the development of specific inhibitors targeting GSK3 or the Wnt- $\beta$ -catenin pathways for therapy. Taken together with preclinical data, our

findings also raise the question of whether inhibition of Wnt signaling would be a worthwhile therapeutic endeavor in this disease. If so, it is possible that lower levels of GSK3 $\beta$  in tumor cells may define a subgroup of tumors that might be particularly suitable for such an intervention. Future efforts should also be directed at testing of Wnt- $\beta$  catenin pathway targeting in preclinical pancreatic cancer models and potential development of clinically useful inhibitors of this pathway if preclinical results are promising.

The major strengths of this study are: (i) it is hypothesis-driven and based on results derived from preclinical models; (ii) the prospective nature of the clinical trial from which patient samples was derived. This increases the homogeneity of the patient population and treatment and reduces bias and confounding factors. This also ensures unbiased collection of high-quality clinical outcome data; (iii) the analysis in the test cohort was informed and guided by previous findings in an independent exploratory dataset; (iv) AQUA, the method used to determine GSK3 $\beta$  expression, is an objective automated and quantitative method that eliminates human inconsistencies and bias in the scoring of a biomarker expression levels; (v) the investigators involved in generation of the GSK3 $\beta$  expression data were blinded to the clinical outcomes of the patients from which the assayed samples were obtained.

In summary, we hereby show that GSK3 $\beta$  is a strong and clinically meaningful prognostic biomarker in PDAC, independent of other known factors such as T stage, nodal status, surgical margins, and CA19-9. The finding that GSK3 $\beta$  can serve as a prognostic biomarker is important in the setting of personalized therapy for pancreatic cancer, and GSK3 $\beta$  expression should be considered for stratification in future clinical trials.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** E. Ben-Josef, W. Regine, M.A. Morgan, D.G. Thomas, K. Winter, C. Guha, T.M. Williams

**Development of methodology:** E. Ben-Josef, D.G. Thomas, K. Winter, T.M. Williams

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** E. Ben-Josef, W. Regine, R.A. Abrams, D.G. Thomas, P. Schaefer, T. DiPetrillo, M. Fromm, W. Small, S. Narayan, K. Winter

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** E. Ben-Josef, A. George, R.A. Abrams, D.G. Thomas, T. DiPetrillo, W. Small, S. Narayan, K. Winter, K.A. Griffith, T.M. Williams

**Writing, review, and/or revision of the manuscript:** E. Ben-Josef, A. George, W. Regine, M.A. Morgan, D.G. Thomas, T. DiPetrillo, M. Fromm, W. Small, S. Narayan, K. Winter, K.A. Griffith, C. Guha, T.M. Williams

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** E. Ben-Josef, T.M. Williams

**Study supervision:** E. Ben-Josef, W. Regine, K. Winter, T.M. Williams

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## References

- Gregory MA, Qi Y, Hann SR. Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *J Biol Chem* 2003;278:51606–12.
- Wei W, Jin J, Schlisio S, Harper JW, Kaelin WG Jr. The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. *Cancer Cell* 2005;8:25–33.
- Welcker M, Singer J, Loeb KR, Grim J, Bloecher A, Gurien-West M, et al. Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. *Mol Cell* 2003;12:381–92.
- Murtaugh LC, Law AC, Dor Y, Melton DA. Beta-catenin is essential for pancreatic acinar but not islet development. *Development* 2005;132:4663–74.
- Wells JM, Esni F, Boivin GP, Aronow BJ, Stuart W, Combs C, et al. Wnt/beta-catenin signaling is required for development of the exocrine pancreas. *BMC Dev Biol* 2007;7:4.
- Morris JPt, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer* 2010;10:683–95.
- Abraham SC, Klimstra DS, Wilentz RE, Yeo CJ, Conlon K, Brennan M, et al. Solid-pseudopapillary tumors of the pancreas are genetically distinct from pancreatic ductal adenocarcinomas and almost always harbor beta-catenin mutations. *Am J Pathol* 2002;160:1361–9.
- Gerdes B, Ramaswamy A, Simon B, Pietsch T, Bastian D, Kersting M, et al. Analysis of beta-catenin gene mutations in pancreatic tumors. *Digestion* 1999;60:544–8.
- Al-Aynati MM, Radulovich N, Riddell RH, Tsao MS. Epithelial-cadherin and beta-catenin expression changes in pancreatic intraepithelial neoplasia. *Clin Cancer Res* 2004;10:1235–40.
- Paasca di Magliano M, Biankin AV, Heiser PW, Cano DA, Gutierrez PJ, Deramaudt T, et al. Common activation of canonical Wnt signaling in pancreatic adenocarcinoma. *PLoS ONE* 2007;2:e1155.
- Zeng G, Germinaro M, Micsenyi A, Monga NK, Bell A, Sood A, et al. Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma. *Neoplasia* 2006;8:279–89.
- Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008;321:1801–6.
- Lowy AM, Fenoglio-Preiser C, Kim OJ, Kordich J, Gomez A, Knight J, et al. Dysregulation of beta-catenin expression correlates with tumor differentiation in pancreatic duct adenocarcinoma. *Ann Surg Oncol* 2003;10:284–90.
- Wang L, Heidt DG, Lee CJ, Yang H, Logsdon CD, Zhang L, et al. Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. *Cancer Cell* 2009;15:207–19.
- Watson RL, Spalding AC, Zielske SP, Morgan M, Kim AC, Bommer GT, et al. GSK3beta and beta-catenin modulate radiation cytotoxicity in pancreatic cancer. *Neoplasia* 2010;12:357–65.
- Regine WF, Winter KA, Abrams R, Safran H, Hoffman JP, Konski A, et al. Fluorouracil-based chemoradiation with either gemcitabine or fluorouracil chemotherapy after resection of pancreatic adenocarcinoma: 5-year analysis of the U.S. Intergroup/RTOG 9704 phase III trial. *Ann Surg Oncol* 2011;18:1319–26.
- Desai S, Ben-Josef E, Griffith KA, Simeone D, Greenson JK, Francis IR, et al. Gemcitabine-based combination chemotherapy followed by radiation with capecitabine as adjuvant therapy for resected pancreas cancer. *Int J Radiat Oncol Biol Phys* 2009;75:1450–5.
- Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* 2002;8:1323–7.
- Bordeaux J, Welsh A, Agarwal S, Killiam E, Baquero M, Hanna J, et al. Antibody validation. *BioTechniques*. 2010;48:197–209.
- Dolled-Filhart M, Gustavson M, Camp RL, Rimm DL, Tonkinson JL, Christiansen J. Automated analysis of tissue microarrays. *Methods Mol Biol* 2010;664:151–62.
- Kaplan EL, Meier P. Nonparametric Estimation from Incomplete Observations. *J Am Stat Assoc* 1958;53:457–81.
- Cox DR. Regression Models and Life-Tables. *J Royal Stat Soc Ser B (Methodological)*. 1972;34:187–220.
- Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007;25:1960–6.
- Garcea G, Dennison AR, Pattenden CJ, Neal CP, Sutton CD, Berry DP. Survival following curative resection for pancreatic ductal adenocarcinoma. A systematic review of the literature. *JOP* 2008;9:99–132.
- Neuzillet C, Sauvanet A, Hammel P. Prognostic factors for resectable pancreatic adenocarcinoma. *J Visc Surg* 2011;148:e232–43.
- Berger AC, Garcia M Jr, Hoffman JP, Regine WF, Abrams RA, Safran H, et al. Postresection CA 19–9 predicts overall survival in patients with pancreatic cancer treated with adjuvant chemoradiation: a prospective validation by RTOG 9704. *J Clin Oncol* 2008;26:5918–22.
- Smith RA, Tang J, Tudur-Smith C, Neoptolemos JP, Ghaneh P. Meta-analysis of immunohistochemical prognostic markers in resected pancreatic cancer. *Br J Cancer* 2011;104:1440–51.
- Ougolkov AV, Fernandez-Zapico ME, Bilim VN, Smyrk TC, Chari ST, Billadeau DD. Aberrant nuclear accumulation of glycogen synthase kinase-3beta in human pancreatic cancer: association with kinase activity and tumor dedifferentiation. *Clin Cancer Res* 2006;12:5074–81.
- Xu WH, Liu ZB, Yang C, Qin W, Shao ZM. Expression of dickkopf-1 and beta-catenin related to the prognosis of breast cancer patients with triple negative phenotype. *PLoS ONE* 2012;7:e37624.
- Yu J, Tao Q, Cheng YY, Lee KY, Ng SSM, Cheung KF, et al. Promoter methylation of the Wnt/beta-catenin signaling antagonist Dkk-3 is associated with poor survival in gastric cancer. *Cancer* 2009;115:49–60.
- Chen S, Liu J, Li G, Mo F, Xu X, Zhang T, et al. Altered distribution of beta-catenin and prognostic roles in colorectal carcinogenesis. *Scand J Gastroenterol* 2008;43:456–64.
- Lugli A, Zlobec I, Minoo P, Baker K, Tornillo L, Terracciano L, et al. Prognostic significance of the wnt signalling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: a tissue microarray-based analysis. *Histopathology* 2007;50:453–64.
- Chung GG, Provost E, Kielhorn EP, Charette LA, Smith BL, Rimm DL. Tissue microarray analysis of beta-catenin in colorectal cancer shows nuclear phospho-beta-catenin is associated with a better prognosis. *Clin Cancer Res* 2001;7:4013–20.
- Bang D, Wilson W, Ryan M, Yeh JJ, Baldwin AS. GSK-3alpha promotes oncogenic KRAS function in pancreatic cancer via TAK1-TAB stabilization and regulation of noncanonical NF-kappaB. *Cancer Discov* 2013;3:690–703.
- Wilson WIII, Baldwin AS. Maintenance of constitutive IkkappaB kinase activity by glycogen synthase kinase-3alpha/beta in pancreatic cancer. *Cancer Res* 2008;68:8156–63.

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Edgar Ben-Josef, Asha George, William F. Regine, et al.

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