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Glycogen Synthase Kinase 3 beta predicts survival in resected adenocarcinoma of the pancreas

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Abstract

Purpose—GSK3β 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 GSK3β causes stabilization, nuclear translocation of β-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 GSK3β was performed on a tissue microarray with samples from 163 patients treated on RTOG 9704. Based on findings in an exploratory cohort, GSK3β was analyzed as a categorical variable using its upper quartile (>Q3) as a cut point. Overall Survival (OS) and Disease-Free Survival

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(DFS) were estimated with the Kaplan-Meier method and GSK3β groupings were compared using the log-rank test. Univariable and multivariable Cox proportional hazards models were used to determine associations between GSK3β and OS/DFS.

Results—The 3-yr. OS rates for GSK3β≤Q3 vs. GSK3β >Q3 were 16% (95% CI: 10%–23%) and 30% (95% CI: 17%–44%), respectively, p=0.0082. The 3-yr. DFS rates were 9% (95% CI: 5%–15%) and 20% (95% CI: 9%–33%) respectively, p-value =0.0081. On multivariable analysis, GSK3β was a significant predictor of OS. Patients with GSK3β >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—GSK3β expression is a strong prognosticator in PDAC, independent of other known factors such as tumor (T) stage, nodal status, surgical margins and CA-19-9.

Keywords

Wnt signaling; Glycogen Synthase Kinase 3 beta; pancreatic cancer; RTOG 9704; molecular marker

INTRODUCTION

GSK3β is a protein kinase involved in the regulation of cell cycle, transcription, proliferation, differentiation and apoptosis. A number of key oncoproteins, including β-catenin, c-Myc, Cyclin D, Cyclin E, and c-Jun, are known substrates of GSK3β; 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) (6). GSK3β is a well-characterized negative regulator of canonical Wnt signaling: it phosphorylates β-catenin, targeting it for degradation. Although mutations in pathway components such as β-catenin and APC are rare in PDAC (7, 8), abnormal accumulation of β-catenin in the nucleus and cytoplasm have 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). β-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). Further, ataxia telangiectasia group D-associated protein (ATDC) mediated accumulation of β-catenin and activation of its target genes was shown to promote PDAC growth and metastasis (14).

It has been previously shown that inhibition of GSK3β in a preclinical PDAC model causes stabilization and nuclear translocation of β-catenin, and induces poor differentiation, proliferation, and resistance to radiation (15). To explore the potential utility of GSK3β 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).

PATIENITS AND METHODS

Patient population

The exploratory cohort consisted of a 38-sample TMA from patients who underwent pancreaticoduodenectomy at the University of Michigan. 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). Immunohistochemical peroxidase staining with a GSK3β antibody (Abcam, Cambridge, MA) was performed. Stained slides were scored by a gastrointestinal pathologist using a three-tier system (none, low and high).

The test cohort consisted of tissue microarray slides of patients treated on RTOG 9704. The eligibility criteria for RTOG 9704 included histologically confirmed PDAC, pathological stages T1–4, N0–1, M0, gross total tumor resection, Karnofsky performance status of ≥ 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^2/day, for 3 weeks (arm 1) or gemcitabine, 1000 mg/m^2, 30 minute infusion once weekly for 3 weeks (arm 2) before and 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^2/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.

GSK3β assay

Automated Quantitative Immunofluorescence Analysis (AQUA) was conducted as described by Camp et al. (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, Littleton, CO, NBP1-04926, 1:1000) and GSK3β (AbCam, Cambridge, MA, 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 multi-tumor TMA, a breast cancer TMA, a pancreatic TMA, a urological TMA and a TMA of normal tissues). A pathologist (DT) 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 3 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 GSK3β protein/antibody complex was then machine-read and reported. GSK3β was read only within the tumor-specific mask. An example of the GSK3β stain in one patient is depicted in Figure 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 GSK3β 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).

Based on the findings in the exploratory cohort, GSK3β was categorized using its upper quartile as a cut point. Although the exploratory cohort had three categories of GSK3β 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 to patients in the lower three quartiles (≤Q3) in the test cohort.

GSK3β groupings were compared using the log-rank test. Potential associations between baseline characteristics and GSK3β groupings were carried out using the chi-square or Fisher's exact test.

Univariate and multivariate Cox proportional hazards models (22) were used to determine if there are any associations of GSK3β with OS and DFS. For the multivariable analysis, only GSK3β was forced into the models and a backwards 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 GSK3β: 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: pathological 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)) vs. log of survival time for the GSK3β groupings, which should result in parallel curves.

RESULTS

Exploratory cohort

Based on preclinical data generated at the University of Michigan, GSK3β was first tested for its prognostic value in an 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 towards improved progression-free survival (PFS) when all three groups were compared to each other (Figure S1A). Since the outcome of patients with no- and low GSK3β 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 significant difference in PFS in favor of high GSK3β expression (Figure S1B).

The estimated 3-year PFS rates were 16.7% and 66.7% in the high and low GSK3β 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 α <0.05 and 90% power, a minimum of 30 patients per expression group would be required.

Test cohort

GSK3β 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 GSK3β in this cohort by treatment arm is shown in 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 GSK3β-analyzable cases and all other eligible cases on the trial. There were no statistically significant differences. Similarly, baseline characteristics were not 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 hazard ratios (HR) still appropriate.

The 3-yr. OS rates for GSK3β ≤ Q3 vs. GSK3β >Q3 were 16% (95% CI: 10%–23%) and 30% (95% CI: 17%–44%), respectively [log rank p-value=0.0082 (Figure 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).

The 3-yr. 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-value =0.0081 (Figure 2b)]. Table 2 shows the Cox proportional hazards model for this grouping. Patients with GSK3β >Q3 had a 39% decrease in the risk of disease recurrence as compared to patients with GSK3β ≤ Q3 (HR=0.61, 95% CI: [0.42, 0.88], p-value=0.0087).

Potential correlations between GSK3β expression and CA19-9 and tumor grade were tested. There were no statistically significant correlations.

Since CA-19-9, 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 CA-19-9 were conducted. Table 3 shows the multivariable Cox proportional hazards model of OS for the 95 patients who had a pre-treatment CA-19-9. In the final model, GSK3β was a significant predictor of OS (as were surgical margins, age and CA-19-9). Patients with GSK3β >Q3 have 46% reduced risk of dying of pancreatic cancer than patients with GSK3β ≤ 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.

Table S2 shows the multivariable Cox proportional hazards model of OS for all 163 patients, including those who did not have pre-treatment CA19-9. GSK3β 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. GSK3β 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 CA-19-9 were statistically significant. Table S3 shows the multivariable Cox model of DFS in all patients. In this model, GSK3β was the only factor that was statistically significant.

To determine if GSK3β 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 GSK3β is not a predictive biomarker for either 5-FU or gemcitabine based therapies.

DISCUSSION

The main finding in this study is that GSK3β is an important independent prognostic factor in PDAC. We noted markedly superior survival and disease-free survival (8.8 and 6.8 months improvement in median, respectively) in patients with high expression of GSK3β. 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. By comparison, the addition of erlotinib to gemcitabine in patients with metastatic pancreatic cancer in the NCIC trial resulted in a hazard ratio 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, GSK3β is a negative regulator of β-catenin; it is part of a complex that ubiquitinates β-catenin, thereby tagging it for proteasomal degradation. β-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 regarding 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 (CA-19-9 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 CA-19-9 (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 CA-19-9 have been previously shown to have a statistically significant independent effect on survival. We now show that GSK3β 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 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 GSK3β exceeds that of nodal status, but is only additive to that of CA-19-9. Also, although the proportional hazards assumption was not fully met, it is robust. In this regard, our data is 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 pathological factors discussed above, a large number of molecular biomarkers have been examined with inconsistent findings. In a recent meta-analysis, vascular endothelial growth factor (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 et al. (28) reported nuclear accumulation of GSK3β 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 β-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 β-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 GSK3β is associated with a better prognosis in pancreatic cancer. However, it is not clear whether GSK3β is driving a tumor-suppressive state or is merely a biomarker for a more favorable disease. If the former, the mechanistic underpinnings of this observation are not yet known. Certainly, the results are consistent with previous observations that Wnt activation, with consequent β-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 GSK3β as a suppressor of Wnt activity. Furthermore, other oncoproteins, such as c-Myc, Cyclin D, Cyclin E, and c-Jun, are functionally inhibited by GSK3β (1–3) and it is possible that GSK3β influences outcome through regulation of multiple pathways. It is also possible that the effects of GSK3β in the cytoplasm are different than they are in the nucleus, as suggested by

Ougolkov's report (28). In future work, cytoplasmic and nuclear GSK3β expression and their associations with outcomes should be assessed. It is also interesting to note that the other GSK3 isoform, GSK3α, 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-β-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 GSK3β 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 pre-clinical pancreatic cancer models and potential development of clinically useful inhibitors of this pathway if pre-clinical results are promising.

The major strengths of this study are: (1) It is hypothesis-driven and based on results derived from preclinical models. (2) The prospective nature of the clinical trial from which patient samples were 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. (3) The analysis in the test cohort was informed and guided by previous findings in an independent exploratory dataset. (4) AQUA, the method used to determine GSK3β expression, is an objective automated and quantitative method that eliminates human inconsistencies and bias in the scoring of a biomarker expression levels. (5) The investigators involved in generation of the GSK3β expression data were blinded to the clinical outcomes of the patients from which the assayed samples were obtained.

In summary, we hereby show that GSK3β is a strong and clinically meaningful prognostic biomarker in PDAC, independent of other known factors such as T stage, nodal status, surgical margins and CA-19-9. The finding that GSK3β can serve as a prognostic biomarker is important in the setting of personalized therapy for pancreatic cancer, and GSK3β expression should be considered for stratification in future clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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STATEMENT OF TRANSLATIONAL RELEVANCE

There is a great need for good biomarkers in pancreatic ductal adenocarcinoma (PDAC). GSK3β is a protein kinase that suppresses a number of oncoproteins, including Wnt signaling. In preclinical models, GSK3β inhibition causes nuclear translocation of β-catenin, increased proliferation and resistance to radiation. Herein we show that GSK3β is an independent prognosticator in patients with PDAC. Based on exploratory analysis in an independent cohort, we assayed GSK3β expression in a tissue microarray from RTOG-9704. We show that high expression of GSK3β 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 CA-19-9. This represents an important step forward in personalized therapy as low GSK3β defines a group of patients with particularly poor outcomes. This novel biomarker can also be used for stratification in future clinical trials.

Fig 1A

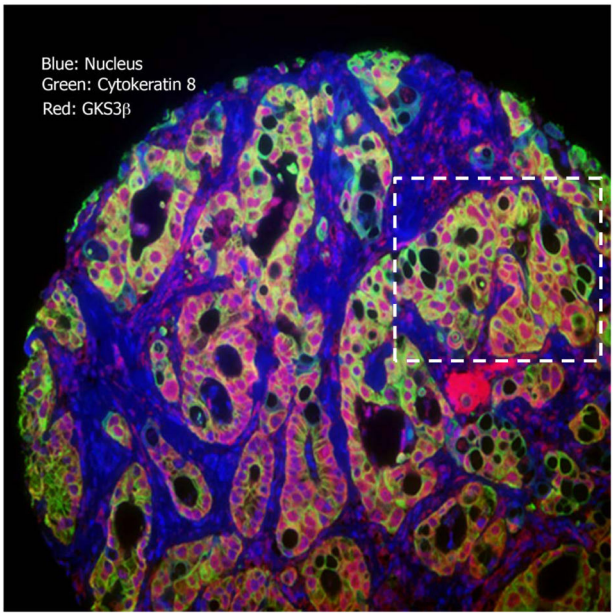

Fig 1B

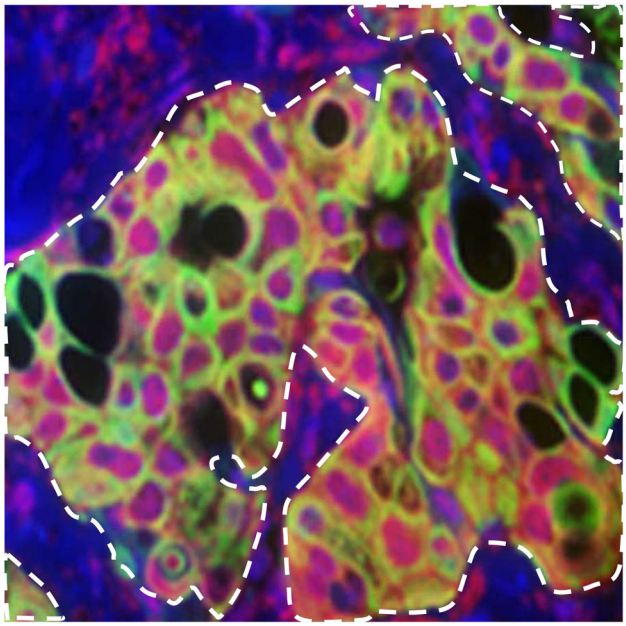

Figure 1.

Automated Quantitative Immunofluorescence Analysis (AQUA). Example of a tumor with GSK3β 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 which were scored for GSK3β expression. The cells in this area exhibited positive cytokeratin staining and thereby formed the “tumor mask”.

Fig 2A

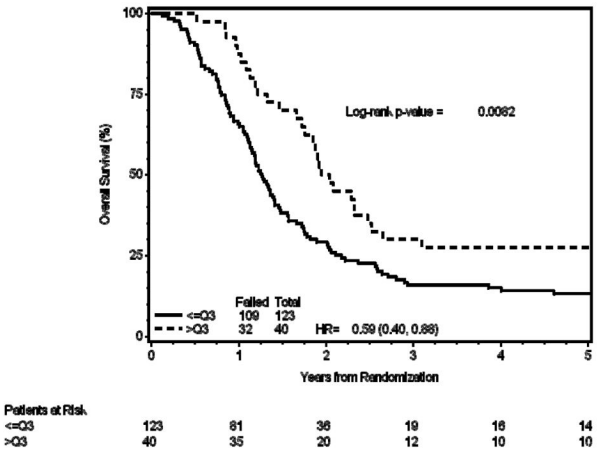

Fig 2B

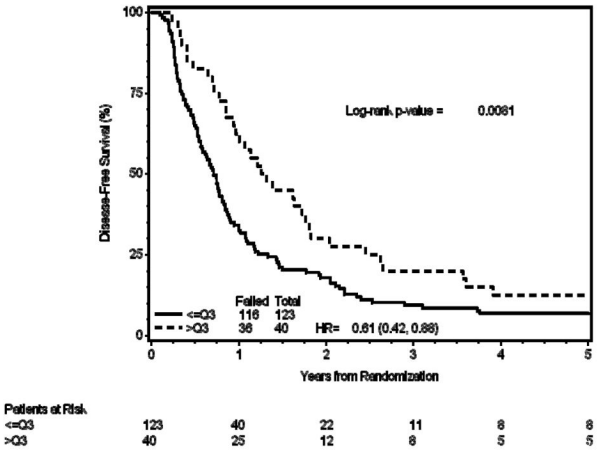

Figure 2.

Overall survival (A) and Disease-free survival (B) by GSK3β expression. The 3-yr. OS rates for GSK3β ≤ Q3 vs. GSK3β > Q3 were 16% (95% CI: 10%–23%) and 30% (95% CI: 17%–44%), respectively [log rank p-value=0.0082]. The 3-yr. 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-value =0.0081].

Table 1

Characteristics of Patients Entered on RTOG 97-04 with GSK3β Expression (n=163)

	≤Q3 (n=123)	> Q3 (n=40)	p-value*
Age (years)			
Median	60	63	0.21†
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%)	
Everything else	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 (surgical)			0.85
N0	41 (33.3%)	14 (35.0%)	
N1	82 (66.7%)	26 (65.0%)	
AJCC Stage			0.93
I,II	39 (31.7%)	13 (32.5%)	
III,IV	84 (68.3%)	27 (67.5%)	
Largest tumor dimension of primary			0.39
<3 cm	43 (35.0%)	17 (42.5%)	
≥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%)	

*p-value from Chi-square/Fisher's Exact Test

†Kruskal Wallis test

Table 2

Univariate Cox Proportional Hazard Model for GSK3β Expression (n=163)

Endpoint	GSK3β expression	HR*	p-value†
Overall Survival	≤Q3	1.00	--
	>Q3	0.59 (0.40, 0.88)	0.0090
Disease-Free Survival	≤Q3	1.00	--
	>Q3	0.61 (0.42, 0.88)	0.0087

*Hazard ratio; a 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.

†p-value from the Wald Chi-square test using the Cox proportional hazards model

Table 3

Multivariable Cox Proportional Hazards Models of Overall Survival (n=95*)

Adjustment Variables	Comparison	Hazard Ratio**	95% C.I. LL	95% C.I. UL	p-value†
GSK3β	≤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

*This multivariablee model excludes 68 patients with no pretreatment CA19-9 value. The Lewis antigen-negative patients were analyzed as CA19-9=0

**A hazard ratio 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.

†p-value from the Wald Chi-square test using the Cox proportional hazards model

Table 4

Multivariable Cox Proportional Hazards Models of Disease-free Survival Including CA19-9 (n=95*)

Adjustment Variables	Comparison	Hazard Ratio**	95% C.I. LL	95% C.I. UL	p-value†
GSK3β	≤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

* This multivariable model excludes 68 patients with no pretreatment CA19-9 value. The Lewis antigen-negative patients were analyzed as CA19-9=0

** A hazard ratio 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.

† p-value from the Wald Chi-square test using the Cox proportional hazards model