β-catenin is independently associated with outcome in pancreatic ductal adenocarcinoma: An analysis of NRG Oncology/RTOG 9704

Short/Running Title β-catenin in pancreatic cancer

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Conflict of Interest Statement

Drs Ben-Josef, Bush, DiPetrillo, Fromm, Guha, Koenig, Leasure, Mowat, Narayan, Regine, Williams, and Mr Robb have nothing to declare. Dr Kim declares in the past 36 months grant to institution from Varian and Viewray, consulting fees from Novartis, speaker fees from Varian, Red Journal GI Editorial Board, unpaid. Ms Moughan declares since the initial planning of the work NRG Oncology SDMC Grant from NCI subaward to institution. Dr Timmers declares since the initial planning of the work grant R50 CA211529/CA/NCI NIH HHS/United States. Ms Winter declares since the initial planning of the work NRG Oncology SDMC Grant from NCI subaward to institution.

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Funding Statement

This project was supported by grants UG1CA189867 (NCORP), U10CA180868 (NRG Oncology Operations), U10CA180822 (NRG Oncology SDMC), and U24CA196067 (NRG Specimen Bank) from the National Cancer Institute (NCI).

Ethics approval and consent to participate:

The study was approved by Ohio State University IRB (IRB #2014C0077). The study was performed in accordance with the Declaration of Helsinki

Data sharing statement:

All data used in the publication will be de-identified and available for data sharing via NCI's NCTN/NCORP Data Archive at least 6 months from the publication date. Data dictionaries are provided with the data. Information about the archive and how to access the data can be found here: https://nctn-data-archive.nci.nih.gov/

Presentation Information: ASTRO 2021

Keywords: β-catenin, pancreatic ductal adenocarcinoma, Wnt signaling, NRG/RTOG 9704

www.ClinicalTrials.gov Registration: NRG/RTOG 9704-NCT00003216

Citation

Ben-Josef E, Winter K, Regine WF, Timmers C, Robb R, DiPetrillo TA, Leasure NC, Mowat R, Kim H, Koenig J, Narayan S, Bush DA, Fromm M, Moughan J, Guha C, Williams T. ß-catenin is independently associated with outcome in pancreatic ductal adenocarcinoma: An analysis of NRG Oncology/RTOG 9704. 2025 Apr 1.

NRG Oncology Unpublished Research Series. Uploaded 2025 Apr 14

https://www.nrgoncology.org/Portals/0/Publications/Unpublished%20Research%20Series/Ben-Josef%20(M%2017446)%20Manuscript%20w%20Citation.pdf?ver=9ktI1zMbcxWZTA96gm6JOQ%3d%3d

β-catenin in pancreatic cancer

Abstract

Introduction

The prognostic value of β-catenin signaling in pancreatic ductal adenocarcinoma (PDAC) is unknown.

Materials and Methods

Quantitative immunofluorescence for β-catenin and its active form (total, nuclear, and cytoplasmic) was performed on samples from NRG/RTOG 9704. Cox proportional hazards models were used to characterize the impact of expression on OS and DFS.

Results

Samples from 141 patients were analyzable. High levels of cytoplasmic β-catenin were associated with better OS [HR (95% CI): 0.65 (0.45, 0.93); log-rank p=0.02] and DFS [HR (95% CI): 0.70 (0.49, 0.99); log-rank p=0.04]. On multivariable analysis, high levels were associated with better OS [HR (95% CI): 0.63 (0.43, 0.92), p=0.02], after adjusting for CA19-9, age, and race, with a 37% risk reduction of death. There was a trend towards better DFS for patients with higher levels [HR (95% CI): 0.70 (0.48, 1.01), p=0.054], after adjusting for CA19-9. For cytoplasmic active β-catenin univariately, high levels were associated with better OS [HR (95% CI): 0.65 (0.45, 0.94); p=0.02], and this remained significant on multivariable analysis [HR (95% CI): 0.64 (0.44, 0.94); p=0.02].

Discussion(s)

Cytoplasmic and active β-catenin are significantly associated with OS, independently of other known prognostic factors. The effect size is clinically relevant, warranting further investigation.

β-catenin in pancreatic cancer

INTRODUCTION

Canonical Wnt signaling, which culminates in stabilization and nuclear translocation of β-catenin, is commonly activated in pancreatic ductal adenocarcinoma (PDAC) [1, 2]. In the nucleus, β-catenin binds to TCF/LEF1 transcription factors and induces transcription of Wnt target genes [3]. Many of the resulting proteins have critical roles in cell cycle regulation, proliferation, differentiation and apoptosis[4]. Accordingly, β-catenin has been implicated in promoting a variety of malignant biological features of cancer, most prominently in colorectal cancer[5].

Cytoplasmic levels of β-catenin are tightly regulated and in the absence of a Wnt signal are low[3]. β-catenin is immobilized at adherens junctions on the plasma membrane by binding to E-cadherin. It can be released from the adherens junctions by a number of kinases or by downregulation of E-cadherin. Free, non-bound β-catenin is rapidly phosphorylated by a destruction complex (the assembly of APC, GSK3□, Axin and CK1), thereby tagged for degradation. The degradation complex, in turn, is inhibited by Wnt signaling.

It has been previously shown that overexpression of GSK3□ in pancreatic cancer patients treated with surgery and chemoradiotherapy was strongly and statistically significantly associated with better survival[6]. It was proposed that this was likely mediated through suppression of β-catenin signaling. However, only few studies evaluated the role of β-catenin in pancreatic cancer and the results have been conflicting. Herein, this question was investigated using samples from a large prospective phase III clinical trial, the NRG Oncology/RTOG 9704.

MATERIALS AND METHODS

Patient population

NRG/RTOG 9704 was a phase III trial which compared adjuvant 5-FU- to gemcitabine-based chemotherapy in PDAC patients after pancreatectomy[7]. The eligibility criteria 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. All patients received 5-

FU-based chemoradiotherapy. The study showed no statistically significant difference in OS between the arms. The assays were conducted on a tissue microarray from participating patients with available tissue.

β-catenin assay

Quantitative immunofluorescence analysis for β-catenin and its active form (total, nuclear, and cytoplasmic) were assayed in pan-cytokeratin positive tumor cells using the Akoya OPAL reagents (Akoya Biosciences, Marlborough, MA) on the Ventana Discovery Ultra system (Roche Diagnostics, Indianapolis, IN).

Tissue microarray slides were de-paraffinized on the system and tissue underwent antigen retrieval using a Tris/EDTA buffer pH 9 (Agilent/Dako Cat. # S236784-2) for 24 minutes at 100°C. A list of the antibodies used, their dilutions and the order in which they were stained, are listed in Supplemental Table S1. A peroxidase inhibitor (Roche Tissue Diagnostics, Cat. # 760-4840) was applied for 12 minutes after the first primary antibody. Appropriate species specific secondary antibodies (Roche Tissue Diagnostics, OmniMap anti-Rb HRP, Discovery Cat. # 760-4311 and Omni-Map anti-Ms HRP Discovery Cat. # 760-4310) were used directly. For detection we used different OPAL reagents (520, 570, and 620) and incubated for 16 minutes. After each round of primary, secondary and OPAL detection, slides were heated to 90°C for 8 minutes to strip the primary/secondary complex off before starting the next round. Slides were counterstained with Spectral DAPI (Akoya Biosciences Cat. # FP1490) at a 1:100 dilution for 8 minutes. Slides were coverslipped using Prolong Diamond Antifade mounting media (Thermo Fisher Scientific, Cat. # P36970). Slides were scanned on a Vectra Polaris microscope and analyzed with inForm® image analysis software (Perkin-Elmer).

Assay performers were blinded to all clinical outcomes.

Statistical Analysis

Scores were dichotomized at their median (< vs. ≥). Overall survival (OS) and disease-free survival (DFS) were estimated univariately with the Kaplan-Meier method [8] and groups compared using the log-rank test [9]. Cox proportional hazards models [10] were used to further identify the impact of expression on OS and DFS. The following variables were included in the models: β-catenin (<median vs. ≥ median),

treatment, age (continuous), sex, race (white vs. other), CA19-9 (<90 [includes Lewis antigen negative] vs. ≥ 90), tumor location (head vs. everything else), nodal involvement (N0 vs. N+), tumor diameter (<3 cm vs. ≥ 3 cm), and surgical margin status (negative vs. positive vs. unknown). Only β-catenin was forced into the models and a stepwise selection procedure at α=0.05 was use for model building.

RESULTS

Of 451 eligible patients, β-catenin/active β-catenin was assayed on 141 patients (Supplemental Figure S1: CONSORT). To assess potential differences between patients for whom samples were available and analyzable vs. those with no such samples, patient and tumor characteristic were compared between groups. There were no significant differences in baseline characteristics for patients with and without analyzable tissue for β-catenin or active β-catenin (Supplemental Tables S2 and S3). Similarly, there were no significant differences in OS or DFS between the groups (Supplemental Table S4). Median follow-up for all patients is 1.41 years, and 6.76 years for surviving patients.

Cytoplasmic β-catenin

Table 1 shows patient, tumor, and treatment characteristics for patients with cytoplasmic β-catenin (levels below and ≥ median). The only statistically significant difference between the groups was a lower proportion of patients with negative-margin resection in the group with β-catenin levels ≥ median. OS and DFS by cytoplasmic β-catenin are shown in Figure 1. On univariate analysis, levels ≥ median were associated with better OS [HR (95% CI): 0.65 (0.45, 0.93); log-rank p=0.02] and DFS [HR (95% CI): 0.70 (0.49, 0.99); log-rank p=0.04]. On multivariable analysis, levels ≥ median were associated with better OS [HR (95% CI): 0.63 (0.43, 0.92), p=0.02], after adjusting for CA19-9, age, and race, with a 37% risk reduction of death. There was a trend towards better DFS for patients with levels ≥ median [HR (95% CI): 0.70 (0.48, 1.01), p=0.054], after adjusting for CA19-9. The results are summarized in Table 2.

Cytoplasmic active β-catenin

Table 3 shows patient, tumor, and treatment characteristics for patients with cytoplasmic active β-catenin (levels below and ≥median). As seen for β-catenin, there was a statistically significant lower proportion of patients with negative-margin resection in the group with active β-catenin levels ≥ median. OS and DFS

by cytoplasmic active β-catenin are shown in Figure 2. On univariate analysis, levels ≥ median were associated with better OS [HR (95% CI): 0.65 (0.45, 0.94); log-rank p=0.02]. On multivariable analysis (Table 4), active β-catenin remained significant for OS [HR (95% CI): 0.64 (0.44, 0.94); log-rank p=0.02] after adjusting for CA19-9, age, and race, but not for DFS.

Nuclear and total β-catenin

On univariate analysis, higher levels of total and nuclear β-catenin were both associated with improved OS [HR (95% CI): 0.68 (0.48, 0.98)] and [HR (95% CI): 0.68 (0.47, 0.97)], respectively. This, however, did not hold on multivariable analysis. Total active β-catenin was associated with OS in univariate [HR (95% CI): 0.65 (0.45, 0.93)] and multivariable analysis [HR (95% CI): 0.59 (0.40, 0.86)] after adjusting for CA19-9, sex, and nodal involvement.

CONCLUSSION

The main finding in this study is that cytoplasmic β-catenin and its active form are important independent prognostic factors in PDAC. In samples from NRG/RTOG 9704, on multivariable analysis, high levels of cytoplasmic β-catenin were associated with a 37% reduction in the risk of death and a similar trend was noticed for its active (non-phosphorylated) form.

β-catenin is a crucial intermediary in canonical Wnt signaling, a pathway which has been implicated in the pathogenesis and progression of many malignancies[5]. Most notably, mutations in APC, a key protein in the β-catenin destruction complex, have been shown to be a strong oncogenic driver of colorectal cancer. Various other mutations causing Wnt overactivation have been shown to be important in gastric, lung, prostate, ovarian, endometrial, and breast cancer. Mutations in pathway proteins are rare in PDAC[11-13] but abnormal accumulation of β-catenin in the nucleus and cytoplasm have been described in a large fraction of both pancreatic intraepithelial neoplasm (PanIN) and PDAC[1, 12, 14]. Consistently, investigators noted reduced expression of membranous β-catenin in PDAC, when compared to the normal pancreas. The significance of these alterations, either association with or contribution to clinical outcomes, in PDAC remain unclear.

β-catenin in pancreatic cancer

Lowy et al.[15] reported that reduced membranous expression of β-catenin correlated with loss of tumor differentiation, but they did not attempt to correlate this with prognosis. Li et al.[16] reported abnormal expression of beta-catenin in 47 PDAC patients, which was significantly correlated to metastasis and one-year survival. However, there was no relation with size, differentiation or cell proliferation. Karayiannakis et al.[17] studied the E-cadherin-catenin cell adhesion complex in 43 patients with PDAC. They found that all three catenins, as well as E-cadherin, were associated with a poor prognosis, but only E-cadherin and alpha-catenin were independent prognostic factors for cancer-specific survival. Wang et al. found no prognostic value in cytoplasmic β-catenin expression in a study that included 48 patients [18], whereas Qiao et al.[19] showed that reduced membranous and positive cytoplasmic expression of β-catenin was associated with poor survival. Their study involved 43 patients. More recently, Saukkonen et al.[20] investigated this in 156 patients with PDAC. They noted that in PDAC, there was both membranous and cytoplasmic expression, but little or no nuclear expression. Further, low total expression was significantly associated with higher tumor grade and high expression was significantly associated with lower risk of death in multivariate analysis (HR = 0.54; 95 % CI 0.35-0.82).

Taken together, the literature suggests that high expression of β-catenin is associated with favorable prognosis, but the studies in general are limited by small sample sizes and, due to their retrospective nature, lack many of the important clinical characteristics of the patients studied. Further, the methods used to assess expression have been different and the results conflicting at time.

In contrast, the NRG/RTOG 9704 samples are from patients who participated in a phase 3 randomized clinical trial, who have been fully characterized prospectively and were followed up rigorously. In addition, an objective quantitative and reproducible method of assaying β-catenin was used. The quantification is performed by an automated computer software, therefore avoiding human inconsistencies. Finally, assay performers were blinded to all clinical outcomes, eliminating potential bias.

The findings presented from this current study highlight the importance of Wnt signaling in PDAC biology. It has been previously reported[6] that GSK3β, a protein which is part of a complex that

ubiquitinates β-catenin, and thus acts as a negative regulator of β-catenin, is a strong and clinically meaningful prognostic biomarker in PDAC. Herein it is shown in samples from patients in the same clinical trial, high levels of cytoplasmic β-catenin are prognostic for better DFS and OS. In the current study, the assay does not distinguish between intracytoplasmic and membranous β-catenin. Other investigators who have studied this differential distribution[20] have shown that in PDAC, β-catenin localizes to the membrane as well as to the cytoplasm, and that when membranous staining was absent, so was cytoplasmic staining. This observation is important because membranous β-catenin is "transcriptionally silent" and it raises the possibility that what is detected as cytoplasmic is mostly non active. However, the data presented here also shows that high levels of cytoplasmic active β-catenin (non-phosphorylated) are prognostic for better survival, which is counter intuitive. While confusing, this finding is robust and is in complete agreement with most of the published literature on this topic.

In summary, cytoplasmic β-catenin is shown to be an important independent prognostic factor in PADC. It reduces the risk of death by 37% after adjustment for known prognostic factors such as CA-19-9. The magnitude of this effect is clinically significant and could easily explain inconsistencies in clinical trials by increasing as yet unrecognized heterogeneity within trial arms. The authors believe it is important to validate this finding in an independent high quality database as soon as possible.

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Figure Legends.

Figure 1: Cytoplasmic β-catenin dichotomized at the median. Kaplan-Meier curve of overall survival based on cytoplasmic β-catenin expression. Kaplan-Meier curve of disease-free survival based on cytoplasmic β-catenin expression. Abbreviations: HR, hazard ratio; CI, confidence interval.

Figure 2: Cytoplasmic Active β-catenin dichotomized at the median. Kaplan-Meier curve of overall survival based on cytoplasmic active β-catenin expression. Kaplan-Meier curve of disease-free survival based on cytoplasmic active β-catenin expression. Abbreviations: HR, hazard ratio; CI, confidence interval.

Figure 1

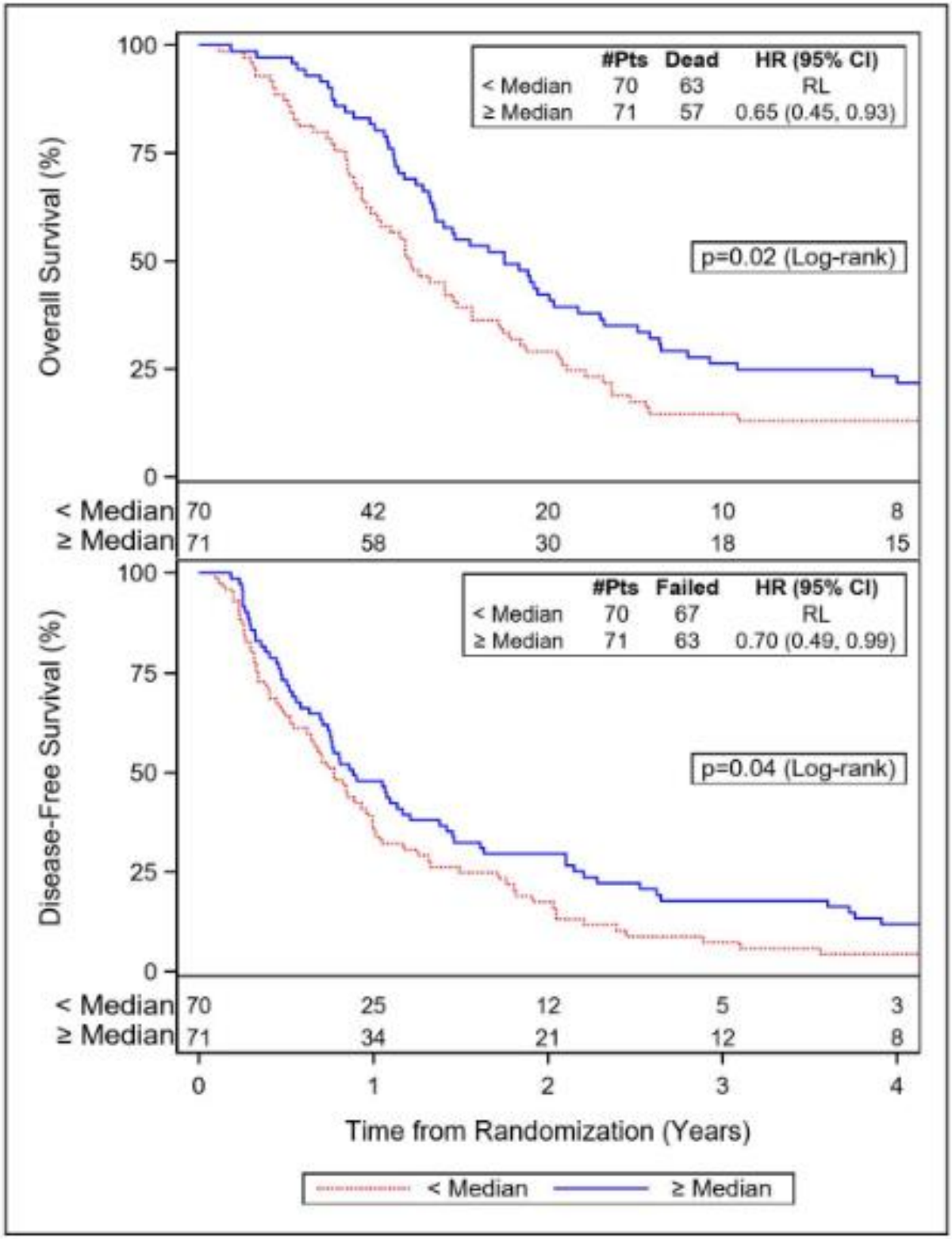
Overall Survival (%)
100
75
50
25
0
#Pts Dead HR (95% CI)
< Median 70 63 RL
≥ Median 71 57 0.65 (0.45, 0.93)
p=0.02 (Log-rank)
< Median 70 42 20 10 8
≥ Median 71 58 30 18 15
Disease-Free Survival (%)
100
75
50
25
0
#Pts Failed HR (95% CI)
< Median 70 67 RL
≥ Median 71 63 0.70 (0.49, 0.99)
p=0.04 (Log-rank)
< Median 70 25 12 5 3
≥ Median 71 34 21 12 8
0 1 2 3 4
Time from Randomization (Years)
< Median
≥ Median

Figure 2

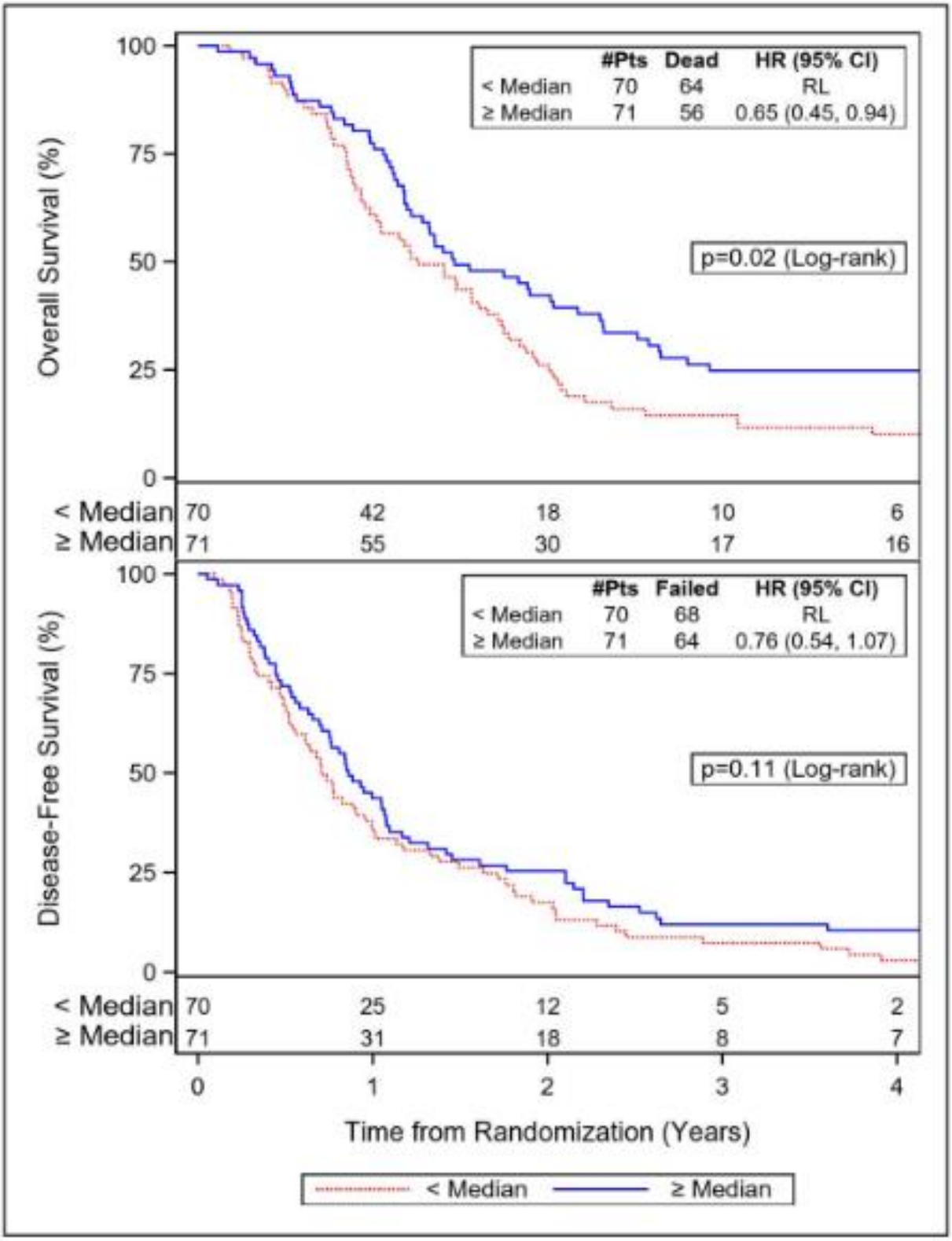
Overall Survival (%)
100
75
50
25
0
#Pts Dead HR (95% CI)
< Median 70 64 RL
≥ Median 71 56 0.65 (0.45, 0.94)
p=0.02 (Log-rank)
< Median 70 42 18 10 6
≥ Median 71 55 30 17 16
Disease-Free Survival (%)
100
75
50
25
0
#Pts Failed HR (95% CI)
< Median 70 68 RL
≥ Median 71 64 0.76 (0.54, 1.07)
p=0.11 (Log-rank)
< Median 70 25 12 5 2
≥ Median 71 31 18 8 7
0 1 2 3 4
Time from Randomization (Years)
< Median
≥ Median

Table 1 Patient, Tumor, and Treatment Characteristics of Eligible Patients with Cytoplasmic β-catenin (median) (n=141)

	<Median (n=70)	**≥ Median (n=71)**	**p-value†**
Age (years)			
< 65	44 (63%)	37 (52%)	0.20
≥ 65	26 (37%)	34 (48%)	
Sex			
Male	33 (47%)	43 (61%)	0.11
Female	37 (53%)	28 (39%)	
Race			
White	64 (91%)	63 (89%)	0.59
Other	6 (9%)	8 (11%)	
Primary Location			
Head	56 (80%)	59 (83%)	0.64
Everything else	14 (20%)	12 (17%)	
Karnofsky performance scale			
60-80	26 (37%)	28 (39%)	0.78
90-100	44 (63%)	43 (61%)	
T-Stage (surgical) *			
T1, T2	15 (21%)	19 (27%)	0.46
T3, T4	55 (79%)	52 (73%)	
N-Stage (surgical) *			
N0	22 (31%)	28 (39%)	0.32
N1	48 (69%)	43 (61%)	
AJCC Stage*			
I, II	19 (27%)	27 (38%)	0.17
III, IV	51 (73%)	44 (62%)	
Largest tumor dimension of primary			
<3 cm	20 (29%)	31 (44%)	0.062
≥3 cm	50 (71%)	40 (56%)	
Primary tumor status			

	<Median (n=70)	**≥ Median (n=71)**	**p-value†**
Complete resection with negative margins	37 (53%)	26 (37%)	0.017
Complete resection with positive margins	24 (34%)	22 (31%)	
Complete resection with unknown margins	9 (13%)	23 (32%)	
CA19-9	(n=63)	(n=67)	
< 90	55 (87%)	59 (88%)	0.90
≥ 90	8 (13%)	8 (12%)	
Treatment Arm			
Radiation + 5-FU	37 (53%)	36 (51%)	0.80
Radiation + Gemcitabine	33 (47%)	35 (49%)	

Data are n (%).

† p-value from Chi-square or Fisher's exact test

*AJCC, American Joint Committee on Cancer 5th edition

Table 2 Stepwise Multivariable Cox Proportional Hazards Models of Cytoplasmic β-catenin (median) (n=130/141)

Endpoint	**Variables**	**Hazard ratio (95% CI)**	**p value***
OS	Cytoplasmic β-catenin		
	< Median	1 (ref)	..
	≥ Median	0.63 (0.43-0.92)	0.017
	CA19-9		
	Continuous (unit increase=50)	1.09 (1.04-1.15)	0.0005
	Age		
	Continuous	0.98 (0.96-0.99)	0.015
	Race		
	White	1 (ref)	..
	Other	1.95 (1.08-3.51)	0.027
DFS	Cytoplasmic β-catenin		
	< Median	1 (ref)	..
	≥ Median	0.70 (0.48-1.01)	0.054
	CA19-9		
	Continuous (unit increase=50)	1.09 (1.03-1.14)	0.0013

Abbreviations: CI, confidence interval

*χ^2 using the Cox proportional hazards model for disease-free survival

Table 3 Patient, Tumor, and Treatment Characteristics of Eligible Patients with Cytoplasmic Active β-catenin (median) (n=141)

	<Median (n=70)	**≥ Median (n=71)**	**p-value†**
Age (years)			
< 65	44 (63%)	41 (58%)	0.54
≥ 65	26 (37%)	30 (42%)	
Sex			
Male	39 (56%)	39 (55%)	0.93
Female	31 (44%)	32 (45%)	
Race			
White	63 (90%)	65 (92%)	0.75
Other	7 (10%)	6 (8%)	
Primary Location			
Head	57 (81%)	60 (85%)	0.63
Everything else	13 (19%)	11 (15%)	
Karnofsky performance scale			
60-80	27 (39%)	26 (37%)	0.81
90-100	43 (61%)	45 (63%)	
T-Stage (surgical) *			
T1, T2	17 (24%)	19 (27%)	0.74
T3, T4	53 (76%)	52 (73%)	
N-Stage (surgical) *			
N0	25 (36%)	25 (35%)	0.95
N1	45 (64%)	46 (65%)	
AJCC Stage*			
I, II	23 (33%)	24 (34%)	0.91
III, IV	47 (67%)	47 (66%)	
Largest tumor dimension of primary			
<3 cm	24 (29%)	31 (44%)	0.25
≥3 cm	46 (71%)	40 (56%)	
Primary tumor status			
Complete resection with negative margins	37 (53%)	24 (34%)	0.0096

	<Median (n=70)	**≥ Median (n=71)**	**p-value†**
Complete resection with positive margins	25 (34%)	25 (35%)	
Complete resection with unknown margins	8 (13%)	22 (31%)	
CA19-9	(n=63)	(n=68)	
< 90	53 (87%)	62 (91%)	0.22
≥ 90	10 (13%)	6 (9%)	
Treatment Arm			
Radiation + 5-FU	36 (53%)	34 (48%)	0.67
Radiation + Gemcitabine	34 (47%)	37 (52%)	

Data are n (%).

† p-value from Chi-square or Fisher's exact test

*AJCC, American Joint Committee on Cancer 5th edition

Table 4 Stepwise Multivariable Cox Proportional Hazards Models of Cytoplasmic Active β-catenin (median) (n=130/141)

Endpoint	**Variables**	**Hazard ratio (95% CI)**	**p value***
OS	Cytoplasmic Active β-catenin		
	< Median	1 (ref)	..
	≥ Median	0.64 (0.44-0.94)	0.021
	CA19-9		
	Continuous (unit increase=50)	1.10 (1.05-1.15)	0.0001
	Age		
	Continuous	0.98 (0.96-0.99)	0.011
	Race		
	White	1 (ref)	..
	Other	1.94 (1.06-3.56)	0.032
DFS	Cytoplasmic Active β-catenin		
	< Median	1 (ref)	..
	≥ Median	0.87 (0.60-1.27)	0.48
	CA19-9		
	Continuous (unit increase=50)	1.10 (1.05-1.15)	0.0002
	Sex		
	Female	1 (ref)	..
	Male	0.67 (0.46-0.98)	0.037
	Race		
	White	1 (ref)	..
	Other	1.89 (1.02-3.51)	0.043
	Surgical Margin Status		
	Negative	1 (ref)	..
	Positive	1.01 (0.67-1.51)	0.97
	Unknown	0.60 (0.69-0.99)	0.046

Abbreviations: CI, confidence interval; OS, overall survival; DFS, disease-free survival

*χ^2 using the Cox proportional hazards model

Supplemental Materials

Figure S1 Consort Diagram

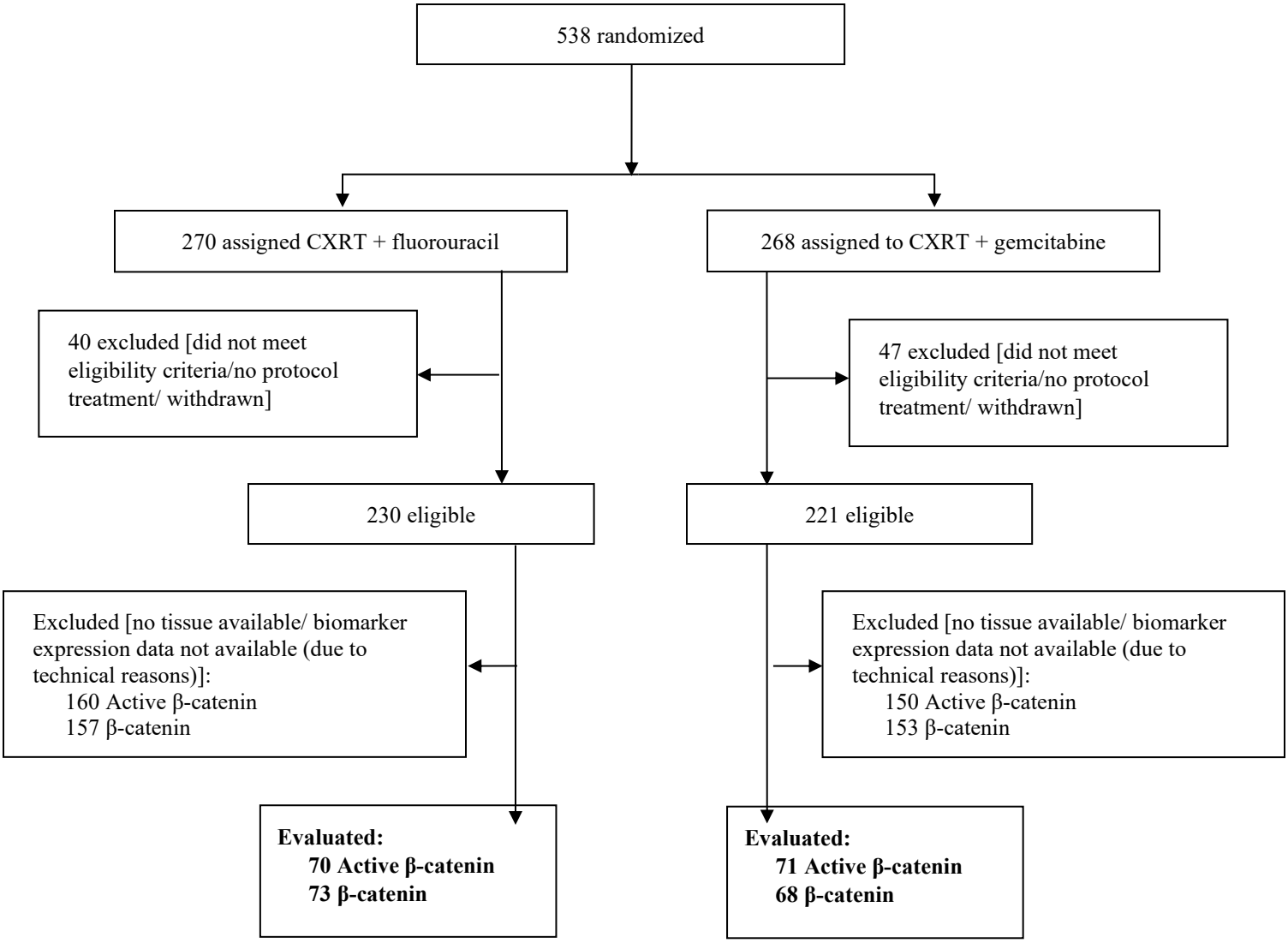

Table S1: Primary and secondary antibodies used, their dilutions, and the order in which they were stained for b-catenin quantitative immunofluorescence assays.

Primary Ab	Company	Clone	Cat.#	OPAL Fluorophore	Akoya Catalog #
Met	Cell Signaling	D1C2	8198	620	FP1495001KT
Non-phospho (Active) beta-Catenin	Cell Signaling	D13A1	8814	520	FP1487001KT
Pan-Cytokeratin	Dako	AE1/AE3	M351501	570	FP1488001KT
Beta-Catenin	Cell Signaling	D10A8	8480	520	FP1487001KT

Primary Ab	Dilution	Time (min)	Temp (Celsius)	Secondary	Time (min)	Fluorophore (dilution)	Ab Denature Conditions
Met	1:100	36	37	Rabbit	20	620 (1:50)	8min @90°C
Active b-Catenin	1:500	32	37	Rabbit	20	520 (1:50)	8min @90°C
Pan-Cytokeratin	1:100	60	RT/25	Mouse	16	570 (1:50)	
Beta-Catenin	1:50	32	37	Rabbit	20	520 (1:50)	8min @90°C

Abbreviations: Ab, antibody; RT, room temperature

Table S2: Patient, Tumor, and Treatment Characteristics of Eligible Patients by No β-catenin Quantification vs. Has β-catenin Quantification (n=451)

	No β-catenin Quantification (n=310)	**Has β-catenin Quantification (n=141)**	**p-value†**
Age (years)			
< 65	183 (59%)	81 (57%)	0.75
≥ 65	127 (41%)	60 (43%)	
Sex			
Male	180 (58%)	76 (54%)	0.41
Female	130 (42%)	65 (46%)	
Race			
White	272 (88%)	127 (90%)	0.47
Other	38 (12%)	14 (10%)	
Primary Location			
Head	273 (88%)	115 (82%)	0.065
Everything else	37 (12%)	26 (18%)	
Karnofsky performance scale			
60-80	111 (36%)	54 (38%)	0.61
90-100	199 (64%)	87 (62%)	
T-Stage (surgical) *			
T1, T2	77 (25%)	34 (24%)	0.87
T3, T4	233 (75%)	107 (76%)	
N-Stage (surgical) *			
N0	102 (33%)	50 (35%)	0.59
N1	208 (67%)	91 (65%)	
AJCC Stage*			
I, II	95 (31%)	46 (33%)	0.67
III, IV	215 (69%)	95 (67%)	
Largest tumor dimension of primary			
<3 cm	135 (44%)	51 (36%)	0.14
≥3 cm	175 (56%)	90 (64%)	
Primary tumor status			
Complete resection with negative margins	125 (40%)	63 (45%)	0.66
Complete resection with positive margins	106 (34%)	46 (33%)	
Complete resection with unknown margins	79 (25%)	32 (23%)	
CA19-9	(n=255)	(n=130)	
< 90	218 (85%)	114 (88%)	0.55
≥ 90	37 (15%)	16 (12%)	
Treatment Arm			
Radiation + 5-FU	157 (51%)	73 (52%)	0.82
Radiation + Gemcitabine	153 (49%)	68 (48%)	

Data are n (%).

† p-value from Chi-square or Fisher's exact test

*AJCC, American Joint Committee on Cancer 5th edition

Table S3: Patient, Tumor, and Treatment Characteristics of Eligible Patients by No Active β-catenin Quantification vs. Has Active β-catenin Quantification (n=451)

	No Active β-catenin Quantification (n=310)	**Has Active β-catenin Quantification (n=141)**	**p-value†**
Age (years)			
< 65	179 (58%)	85 (60%)	0.61
≥ 65	131 (42%)	56 (40%)	
Sex			
Male	178 (57%)	78 (55%)	0.68
Female	132 (43%)	63 (45%)	
Race			
White	271 (87%)	128 (91%)	0.30
Other	39 (13%)	13 (9%)	
Primary Location			
Head	271 (87%)	117 (83%)	0.21
Everything else	39 (13%)	24 (17%)	
Karnofsky performance scale			
60-80	112 (36%)	53 (38%)	0.77
90-100	198 (64%)	88 (62%)	
T-Stage (surgical) *			
T1, T2	75 (24%)	36 (26%)	0.76
T3, T4	235 (76%)	105 (74%)	
N-Stage (surgical) *			
N0	102 (33%)	50 (35%)	0.59
N1	208 (67%)	91 (65%)	
AJCC Stage*			
I, II	94 (30%)	47 (33%)	0.52
III, IV	216 (70%)	94 (67%)	
Largest tumor dimension of primary			
<3 cm	131 (42%)	55 (33%)	0.52
≥3 cm	179 (58%)	86 (67%)	
Primary tumor status			
Complete resection with negative margins	127 (41%)	61 (43%)	0.54
Complete resection with positive margins	102 (33%)	50 (35%)	
Complete resection with unknown margins	81 (26%)	30 (21%)	
CA19-9	(n=254)	(n=131)	
< 90	217 (85%)	115 (88%)	0.53
≥ 90	37 (15%)	16 (12%)	
Treatment Arm			
Radiation + 5-FU	160 (52%)	70 (50%)	0.70
Radiation + Gemcitabine	150 (48%)	71 (50%)	

Data are n (%).

† p-value from Chi-square or Fisher's exact test

*AJCC, American Joint Committee on Cancer 5th edition

Table S4: Overall and Disease-Free Survival of Eligible Patients by No β-catenin Quantification vs. Has β-catenin Quantification (n=451)

Endpoint	Comparison	HR	95% C.I. LL	95% C.I. UL	p-value
OS	No β-catenin Quantification	1.00	..	..	..
	Has β-catenin Quantification	1.13	0.91	1.41	0.27
DFS	No β-catenin Quantification	1.00	..	..	..
	Has β-catenin Quantification	1.08	0.88	1.33	0.47
OS	No Active β-catenin Quantification	1.00	..	..	..
	Has Active β-catenin Quantification	1.17	0.94	1.45	0.16
DFS	No Active β-catenin Quantification	1.00	..	..	..
	Has Active β-catenin Quantification	1.18	0.95	1.45	0.13

Abbreviations: HR, hazard ratio; C.I., confidence interval; LL, lower limit; UL, upper limit