The Prognostic Significance of WNT Pathway in Surgically-treated Colorectal Cancer: β-Catenin Expression Predicts for Disease-free Survival

KONSTANTINOS KAMPOSIORAS[1], ATHINA KONSTANTARA[2], VASSILIKI KOTOULA[3], SOTIRIS LAKIS[4], GEORGE KOUVATSEAS[5], EVANGELOS AKRIVIADIS[6], ELENI VRETTOU[3], DIMITRIOS DIONYSOPOULOS[7], DIMITRIOS KRIKELIS[7], KYRIAKI PAPADOPOULOU[3], ELPIDA CHARALAMBOUS[4], SOFIA CHRISAFI[4], CHRISTOS KONSTANTARAS[2] and GEORGE FOUNTZILAS[7]

[1]*Department of Medical Oncology, University Hospital of Larissa, Larissa, Greece;*
[2]*Department of Surgery, St. Luke's Hospital, Thessaloniki, Greece;*
[3]*Department of Pathology, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece;*
[4]*Laboratory of Molecular Oncology, Hellenic Foundation for Cancer Research, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece;*
[5]*Health Data Specialists Ltd., Athens, Greece;*
[6]*Fourth Department of Medicine, Hippokrateion Hospital, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece;*
[7]*Department of Medical Oncology, Papageorgiou Hospital, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece*

Abstract. *Background: The wingless-type MMTV integration site family of proteins (WNT) pathway is highly involved in colorectal cancer development. The aim of this study was to explore the prognostic significance and clinicopatological correlations of this pathway in a cohort of surgically-treated patients with non-metastatic colorectal cancer in relation to the site of expression of pathway proteins. Materials and Methods: Immunohistochemical expression of nuclear cyclin D1, membranous E-cadherin and P-cadherin, membranous and nuclear β-catenin in the invasive front (IF), the tumor center (TC), as well as their mean, were assessed in 106 paraffin-embedded tissue samples. Adenomatous Polyposis Coli (APC), Axin-2 (AXIN2), cyclin-D1 (CCND1), Matrix Metalloproteinase-7 (MMP7), Secreted Frizzled Related Protein (SFRP) 1, 2 and 4 and WNT5A were evaluated by RT PCR. Results: Membranous β-catenin expression was statistically reduced in the IF. Cyclin-D1 was reduced in tumors arising closer to the rectum. Reduced nuclear expression of cyclin-D1 in the IF was associated with lymphatic, venous and perineural invasion. Loss of membranous β-catenin in the TC was more common among N2 tumors. Higher SFRP4 mRNA was associated with advanced T stage. In univariate analysis, membranous expression of β-catenin in TC and IF, and their mean, was associated with longer disease-free survival (DFS). In multivariate analysis, tumor stage and mean β-catenin expression were prognostic for longer DFS (hazard ratio=0.33; p=0.01). β-Catenin expression in the IF remained significant when the mean expression was not included in the multivariate analysis (hazard ratio=0.41; p=0.028). Conclusion: Mean membranous expression of β-catenin, as well as that in the IF, is prognostic for longer DFS in patients with non metastatic colorectal cancer.*

Correspondence to: Konstantinos Kamposioras, MD, Department of Medical Oncology, University Hospital of Larissa, 41 110 Larissa, Greece. Tel: +30 2413502028, e-mail: kambkons1@yahoo.gr

Key Words: Colorectal cancer, WNT, β-catenin, prognosis, E-cadherin, SFRP, invasive front.

Colorectal cancer (CRC) remains a major cause of mortality, despite the implementation of screening policies and early detection (1). Even in surgically-treated patients, the rate of relapse remains high, despite the use of adjuvant chemotherapy and radiotherapy. Although clinicopathological characteristics seem to distinguish a subset of patients that are at high risk of relapse, the need for molecular markers that would help discriminate the high-risk population is still unmet.

The wingless-type MMTV integration site family of proteins (WNT)/β-catenin pathway is highly implicated in the development of gastrointestinal malignancies (2). A key molecule in the canonical WNT pathway is β-catenin, which

is tightly regulated through complex intra- and extracellular interactions (3). Briefly, in non-stimulated cells Axin (AXIN), adenomatous polyposis coli (APC) , glycogen synthase kinase-3 (GSK3), and casein kinase-1 (CK1) form a 'destruction complex' which mediates the phosphorylation and eventual degradation of cytoplasmic β-catenin *via* the APC-dependent ubiquitin-proteasome pathway (3). Activation of the pathway through the binding of ligands to the WNT receptors frizzled and low-density lipoprotein receptor-related protein and/or dysfunction of any of the constituents of the destruction complex leads to nuclear translocation of β-catenin, interaction with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and finally transcription regulation of various effector molecules such as c-myelocytomatosis oncogene protein (c-MYC), metalloproteinase-7 (MMP7) (4, 5) and cyclin-D1, which is a major cell-cycle regulator in human cancer (6). Further regulation is conferred by secreted frizzled-related proteins (SFRPs) which act as WNT antagonists for both β-catenin and non-canonical WNT signaling (7).

Besides the WNT pathway, β-catenin has a crucial role in intracellular interactions through its connection with adhesion molecules, namely E- and P-cadherin. Loss of cell–cell contact is one of the first events in tumor metastasis and the loss of membranous E-cadherin and β-catenin is an adverse prognostic marker in early colorectal cancer (8, 9). On the other hand, P-cadherin seems to have an adverse prognostic impact, promoting liver metastasis in CRC (10), although this is not well-documented (11). The pathway is further regulated by WNT5a, potentially, through its ability to increase the adhesive capacity of the epithelial cells and renders a good prognostic signature in early-stage CRC (12).

It is now well-recognized that there are differences in the expression of various proteins in the invasive front (IF) of the tumor, *i.e.* the peripheral zone of the tumor which is in contact with the host normal tissue, as compared with the tumor center (TC) (13). The underlying biological hypothesis is that cells in the IF undergo epithelial–mesenchymal transition and are cells that will characterize the metastatic potential of the tumor (14).

The aim of the current study was to explore the differential expression of the WNT members and their effectors according to their location in the tumor, identify their prognostic role in a cohort of non-metastatic surgically-treated colorectal cancer cases and reveal correlations with the various clinicopathological parameters.

Materials and Methods

Patients. This is a retrospective-prospective translational study based on a series of fully-characterized patients with colorectal cancer. The case series consisted of 106 consecutive patients with CRC treated with curative intent between February 2005 and June 2007 by the same surgical team (A.K, C.K.). 5-Fluorouracil/oxaliplatin-based adjuvant chemotherapy and radiotherapy were administered postoperatively. CRC was defined according to the TNM AJCC-UICC classification (15). Data regarding patient demographics, clinicopathological parameters and outcome were retrieved from the medical records as documented in the Hellenic Cooperative Oncology Group (HeCOG) database. The clinical and translational protocol was approved by the Bioethics Committee of Aristotle University of Thessaloniki School of Medicine (2/2-3-2012). All patients signed an informed consent for the provision of biological material for future research purposes.

Immunohistochemistry (IHC). Hematoxylin-eosin (H&E)-stained slides from formalin-fixed paraffin-embedded (FFPE) tissue blocks were reviewed for confirmation of diagnoses and material adequacy regarding tumor, adjacent normal mucosa or evidence of pre-existing adenomas. Tumor tissue specimens were arrayed into a recipient paraffin block using a manual arrayer (MTA-1; Beecher Instruments, Sun Prairie, WI, USA). Each case was represented by two cores, 1.5 mm in diameter from both central and peripheral tumor zones, the latter corresponding to the tumor IF. The anatomic limit of tumor extension into the bowel wall layer with respect to the IF was noted. Tissue cores from normal mucosa or residual adenoma were transferred to separate blocks. Each tissue-microarray (TMA) block consisted of 35-58 tissue cores, while various cancerous and non-neoplastic tissues were also added, for the purpose of serving as assay controls.

Labeling of 3-μm TMA sections with antibodies against E-cadherin, P-cadherin, β-catenin and cyclin-D1 (CCND1) was carried out with the Bond Polymer Refine Detection system (DS9800; Leica Biosystems Newcastle Ltd, United Kingdom Microsystems). For E-cadherin, the staining protocol was preset according to the manufacturer's instructions. 3,3-Diaminobenzidine (DAB) was applied as a chromogen and hematoxylin as a counterstain. In cases of inadequate or missing tissue cores, whole sections from the original blocks were used instead. The key parameters of the standardized staining protocols used in this study are summarized in Table I. All staining was evaluated by a single observer (S.L.) who had no knowledge of clinical characteristics and survival data. Cytoplasmic reactivity, when present, was recorded but not considered for statistical evaluations. For E-cadherin and P-cadherin, the fraction of cells with a positive membrane reaction was accounted for, when staining was clearly discernible as being above the level of background cytoplasmic effect. For the semi-quantification of positive cases, both the intensity and percentage of positive cells were noted. The average intensity was evaluated applying a 4-tier system with 0 for no staining, 1 for weak, 2 for moderate and 3 for strong, the latter corresponding to the intensity of normal squamous epithelium of pharyngeal tonsils. For CCND1, the proportion of positive cells was recorded separately for each intensity grade due to the substantial variation in staining intensity observed within each core. Suprabasal squamous epithelial cells (tonsil) served as a positive control (intensity grade 2). The extent of immunoreactivity for all markers was assessed with the H-score. The H-score was generated by adding the percentage of strongly-stained nuclei (3×), the percentage of moderately stained nuclei (2×), and the percentage of weakly stained nuclei (1×), giving a score of range 0-300. Membranous reactivity for β-catenin was evaluated as for E-cadherin. The percentage of cells with nuclear positivity was recorded separately when a uniform brown nuclear reaction of any intensity was present. Since there is no validated scoring system for interpreting IHC staining for any of the markers included in this study, cut-off values were defined *post-hoc*, based on analyses of data distribution in frequency histograms.

Table I. *Immunohistochemistry Methods*.

Marker	Clone	Manufacturer	Epitope Retrieval	Protein block	Antibody dilution/incubation
P-Cadherin	56C1	Thermo Scinetific	20' EDTA, pH 9	10'	1:200, over night
β-Catenin	17C2	Novocastra	20' Citrate, pH 6	10'	1:350, over night
E-Cadherin	36B5	Leica Microsystems	20' EDTA, pH 9	no	ready to use, 15'
Cyclin-D1	SP4	Spring Bioscience	20' Citrate, pH 6	no	1:80, 20'

mRNA expression. Tissue samples were obtained during surgery in the operating room from surplus tumor as determined by the pathologist on service, cut into pieces of maximally 5×5 mm, added to cryovials, immediately snap frozen in liquid nitrogen, and stored at –80˚C until use. The exact tissue samples available for gene expression analysis are depicted in Figure 1. Frozen tissue pieces were minced on disposable sterile plastic surfaces placed on dry ice between two scalpels and transferred into a lysis buffer containing 500 μg/ml proteinase K for overnight lysis at 56˚C. Tissue lysates were then processed for total RNA with TRIZOL-LS (Invitrogen/Life Technologies, Paisley, UK), according to the manufacturer's instructions. Following UV measurements, 4-5 μg of total RNA were reverse-transcribed with random hexamers and SuperScript® III Reverse Transcriptase (all reagents from Invitrogen/Life Technologies, Carlsbad, California, USA). cDNAs were normalized at 25 ng/μl and kept at 20˚C until use. Relative mRNA expression was assessed with quantitative Polymerase Chain Reaction (qPCR) and appropriate fluorogenic, fluorescein amidite (FAM), dye-labelled probes in an ABI7900HT system under default conditions.

The following Taqman-MGB assays (Applied Biosystems/Life Technologies) were selected for the gene targets under investigation (data in parentheses refer to assay ID; Genbank reference; amplicon location; size): *MMP7* (Hs01042796_m1; NM_002423.3; exons 4-5; 64 bp); *SFRP1* (Hs00610060_m1; NM_003012.4; exons 2 -3; 130 bp); SFRP2 (Hs00293258_m1; NM_003013.2; exons 1-2; 129 bp); *SFRP4* (Hs00180066_m1; NM_003014.3; exons 5-6; 109 bp); *WNT5A* (Hs00998537_m1, NM_001256105.1, NM_003392.4; exons 4-5; 61 bp), *AXIN2* (Hs00610344_m1; NM_004655.3; exons 4-5; 82 bp), *CCND1* (Hs00765553_m1; NM_053056.2; exons 3-4; 57 bp) and *APC* (Hs01568269_m1; NM_000038.5, NM_001127510.2, NM_001127511.2; exons 12-13, 14-15, 15-16, 93 bp).

Samples were processed in 10 μl reactions (50 ng cDNA/reaction) with TaqMan® Universal PCR Master Mix and run in duplicates in 384-well plates. As a positive control, a commercially available reference RNA (TaqMan® Control Total RNA, cat. no 4307281, Applied Biosystems/Life Technologies) was also used in each run. Moreover, as an endogenous control and for the normalization of cycle threshold (CT) values, an assay targeting β-glucuronidase (GUSB) mRNA was used (Hs00939627_m1; NM_000181.3; exons 8-9; 96 bp). Hence, for each gene target, duplex qPCR reactions were performed, whereby the VIC dye-labelled TaqMan Assay for *GUSB* was used together with the respective FAM dye-labelled TaqMan assay in the same reaction. *GUSB* was preferred over other commonly applied endogenous controls because no pseudogenes have as yet been reported for this gene.

Relative quantification (RQ) was assessed in a linear mode as (40-dCT), whereby dCT=(avg CT target) – (avg CT *GUSB*). Exclusion criteria for sample RQ analysis were *GUSB* CT values higher than 36, whereas PCR assay stability was evaluated among runs with the reference RNA.

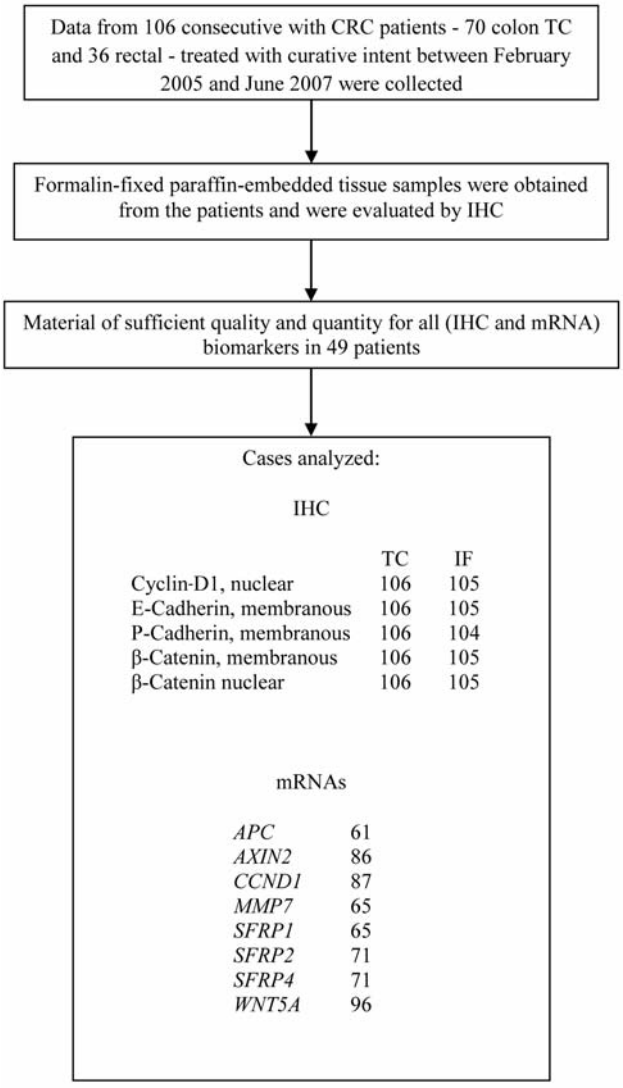


Figure 1. *Reporting recommendations for criteria in tumour marker prognostic studies (REMARK) flow chart*.

Statistical analysis. Continuous variables are described using the median value estimate and the range, while categorical variables are reported as frequencies and percentages. The different clinicopathological variables were tested for association with the differential expression of the WNT pathway molecules. IHC

Table II. *Clinical characteristics of patients included in this study.*

		Cancer group		
		Colonic	Rectal	Total
Patients	N	70	36	106
Age at diagnosis, years	Median	71	72	71
	Min-Max	31-88	48-83	31-88
No. of LNs examined	Median	14.0	10.5	13.0
	Min-Max	5-56	4-29	4-56
Gender	Female	29 (41.4)	12 (33.4)	41 (38.6)
	Male	41 (58.6)	24 (66.6)	65 (61.4)
Histological type	Adenocarcinoma	65 (92.8)	35 (97.2)	100 (94.4)
	Intestinal	1 (1.4)		1 (1)
	Mucinous	4 (5.8)	1 (2.8)	5 (4.8)
pT Stage	T1-T2	14 (20)	12 (33.3)	26 (24.5)
	T3-T4	56 (80)	24 (67.6)	80 (75.5)
pN Stage	N0	41 (58.6)	22 (61.2)	63 (59.4)
	N1	17 (24.2)	8 (22.2)	25 (23.6)
	N2	12 (17.2)	6 (16.6)	18 (17)
Astler-Coller stage	A	5 (7.2)	4 (11.2)	9 (8.4)
	B1/B2	37 (52.8)	18 (50)	55 (51.8)
	C1/C2	28 (40)	14 (38.8)	42 (39.6)
Differentiation grade	Well	8 (11.4)	4 (11.2)	12 (11.4)
	Moderate	42 (60)	20 (55.6)	62 (58.4)
	Poor	14 (20)	11 (30.6)	25 (23.6)
	NA	6 (8.6)	1 (2.8)	7 (6.6)
Angiolymphatic invasion	None	25 (35.8)	18 (50)	43 (40.6)
	Lymphatic only	25 (35.8)	10 (27.8)	35 (33)
	Venous*	12 (17.2)	5 (13.8)	17 (16)
	NA	8 (11.4)	3 (8.4)	11 (10.4)
Perforation	No	59 (84.2)	33 (91.6)	92 (86.8)
	Yes	6 (8.6)	1 (2.8)	7 (6.6)
	NA	5 (7.2)	2 (5.6)	7 (6.6)
Perineural invasion	No	36 (51.4)	19 (52.8)	55 (51.9)
	Yes	17 (24.3)	7 (19.4)	24 (22.6)
	NA	17(24.3)	10 (27.8)	27 (25.5)
Tumor location#	Left	41 (58.6)		
	Right	28 (40)		
	NA	1 (1.4)		
Carcinoma arising in adenoma	No	52 (74.2)	26 (72.2)	78 (73.6)
	Yes	12 (17.2)	8 (22.2)	20 (18.8)
	NA	6 (8.6)	2 (5.6)	8 (7.6)
Adjuvant treatment	No	30 (42.8)	13 (36.2)	43 (40.6)
	Yes	31 (44.2)	20 (55.6)	51 (48.2)
	NA	9 (12.8)	3 (8.4)	12 (11.4)

*Irrespective of lymphatic invasion; #applicable only for colon cancer; NA: not assessed.

expression of nuclear CCND1, membranous E-cadherin and P-cadherin, membranous and nuclear β-catenin by in the IF and the TC, as well as the mean of the two values, were assessed by H-score. *APC, AXIN2, CCND1, MMP7, SFRP1, 2* and *4* and *WNT5A* were evaluated by RT-PCR. The associations were examined using the Fisher's exact test or the Chi-square test for categorical variables and the Kruskal–Wallis test for continuous variables. Disease-free survival (DFS) was measured from the date of diagnosis until verified disease progression, death or last contact, whichever occurred first, and overall survival (OS) from diagnosis until death from any cause or date of last contact. Time-to-event distributions were estimated using Kaplan-Meier curves. Unsupervised hierarchical clustering (using the Ward's minimum variance method) was conducted using IHC and mRNA markers separately and combined, in order to identify distinct groups correlated with prognosis. In univariate analysis, significance was determined at the level of 5% and in multivariate at 15% (two-sided).

The clinical and tumor characteristics assessed were patient gender and age, site (colon *vs*. rectal and right *vs*. left/sigmoidal colon), T-stage, lymph node (N-) stage, Astler-Coller stage, grade of differentiation, vascular, lymphatic and perineural invasion, histological

type, presence of precursor lesion (adenoma), administration of adjuvant chemotherapy or not. The study complied with reporting recommendations for criteria in tumour marker prognostic studies (16). A (REMARK) flow chart is shown in Figure 1.

Results

Clinicopathological characteristics. The study series included 106 patients with colorectal cancer, 70 with colonic and 36 with rectal cancer. No statistical difference of the clinicopathological parameters related to tumor location was observed. The median age was 71 (range=31-88) years; there was a male preponderance. The majority of the tumors were moderately-differentiated, non perforated adenocarcinomas of stage B2 or C, (T3, N0 or N1). Half of them exhibited vascular invasion and absence of perineural invasion. In almost 20% of the cases, the tumors had developed in a pre-existing adenoma. Half of the patients received adjuvant chemotherapy with 5-flurouracil/oxaliplatin-based regimens (Table II).

Immunohistochemistry analysis. IHC expression of the different markers were assessed by H-score in the TC and IF. Characteristic staining patterns are shown in Figure 2. Additionally, an overall value per case was used for calculations considering the mean expression of the two tumor zones, as shown in Table III. The cut-off value used for membranous E- and P-cadherin, β-catenin and nuclear CCND1 was 50%. Membranous expression of β-catenin was significantly lower in the tumor IF as compared to the TC (mean score: TC=142, IF=122; p=0.0009). No such difference was observed for the other studied markers, including nuclear β-catenin. There was a statistically significant positive correlation between the two cadherins (E and P; p<0.0001), and for each of them with membranous β-catenin (p<0.0001 and p=0.0059, E- and P- cadherin, respectively).

As compared to rectal carcinomas, colonic carcinomas had higher expression of CCND1 both in peripheral as well as central tumor areas. The right colon also appeared to express higher levels of CCND1 as compared to the left colon, indicating altogether a loss of cyclin-D1 in carcinomas arising closer to the hindgut (Table IV).

Reduced nuclear expression of CCND1 in the IF was associated with lymphatic, venous (p=0.025), and perineural invasion, whereas the latter was also related to mean score of CCND1 expression (p=0.018 and 0.034, respectively). Loss of membranous β-catenin at the TC was more common among N2 tumors (p=0.009).

mRNA expression. Statistically significant associations between clinical and tumor characteristics and mRNA variables are presented in Table V. Carcinomas arising in adenomas had lower expression of *SFRP2* and *SFRP4* (p=0.016 and 0.011, respectively), while higher expression of *SFRP4* was associated with advanced T-stage (p=0.001).

Table III. *Comparisons of scores for tumor center (TC) and invasive front (IF) (paired t-test).*

Variable	Mean score			
	IF	TC	*p*-Value	Overall mean score
Cyclin-D1 HScore	42.8	46.4	0.3114	44.4
Membranous E-cadherin H-Score	114.7	128.6	0.1206	121.5
Membranous P-cadherin H-Score	102.1	110.9	0.1317	107.1
Membranous β-catenin H-Score	122.0	142.0	0.0009	132.0
Nuclear β-catenin, %	13.8	14.6	0.7471	14.3

Survival analysis. Univariate analysis. DFS and OS analysis was performed in 93 patients. The median follow-up was 66.1 (range=0.6-87.4 months). The 4-year DFS and OS for the entire cohort was 78.5% and 83.9%, respectively. There were no OS and DFS differences among patients with colonic and rectal cancer and those who did or did not receive chemotherapy.

Stage C tumor (p=0.0008), lymph node involvement (p=0.0004), male gender (p=0.037), as well as perineural (p=0.034) and venous invasion (p=0.0028), were prognostic for short DFS (Table VI).

Membranous expression of β-catenin in both tumor compartments, as well as its mean expression, was associated with longer DFS. In addition, the simultaneous underexpression of E-cadherin and β-catenin was an adverse prognostic feature (Figure 3).

On the other hand, no molecular marker was prognostic for OS. Astler-Coller C-stage (p=0.0099), N2-stage (p=0.0049), and perineural invasion (0.048) were predictive for worse outcome (Table VII).

No prognostic significance was revealed either in DFS nor OS for mRNA and clustering analysis.

Multivariate analysis. The mean expression of membranous β-catenin alongside Astler-Coller stage was significant in multivariate analysis (Table VIIIa). The prognostic significance of membranous β-catenin expression in the IF was highlighted when the multivariate expression was performed without taking into consideration the overall expression (Table VIIIb). In this case, venous invasion remained significant in the analysis.

Discussion

In this cohort of patients with non-metastatic, surgically-treated colorectal cancer, the prognostic value of a variety of WNT members was explored. Membranous expression of β-catenin was statistically significant for longer DFS in both univariate and multivariate analyses. We analyzed the results

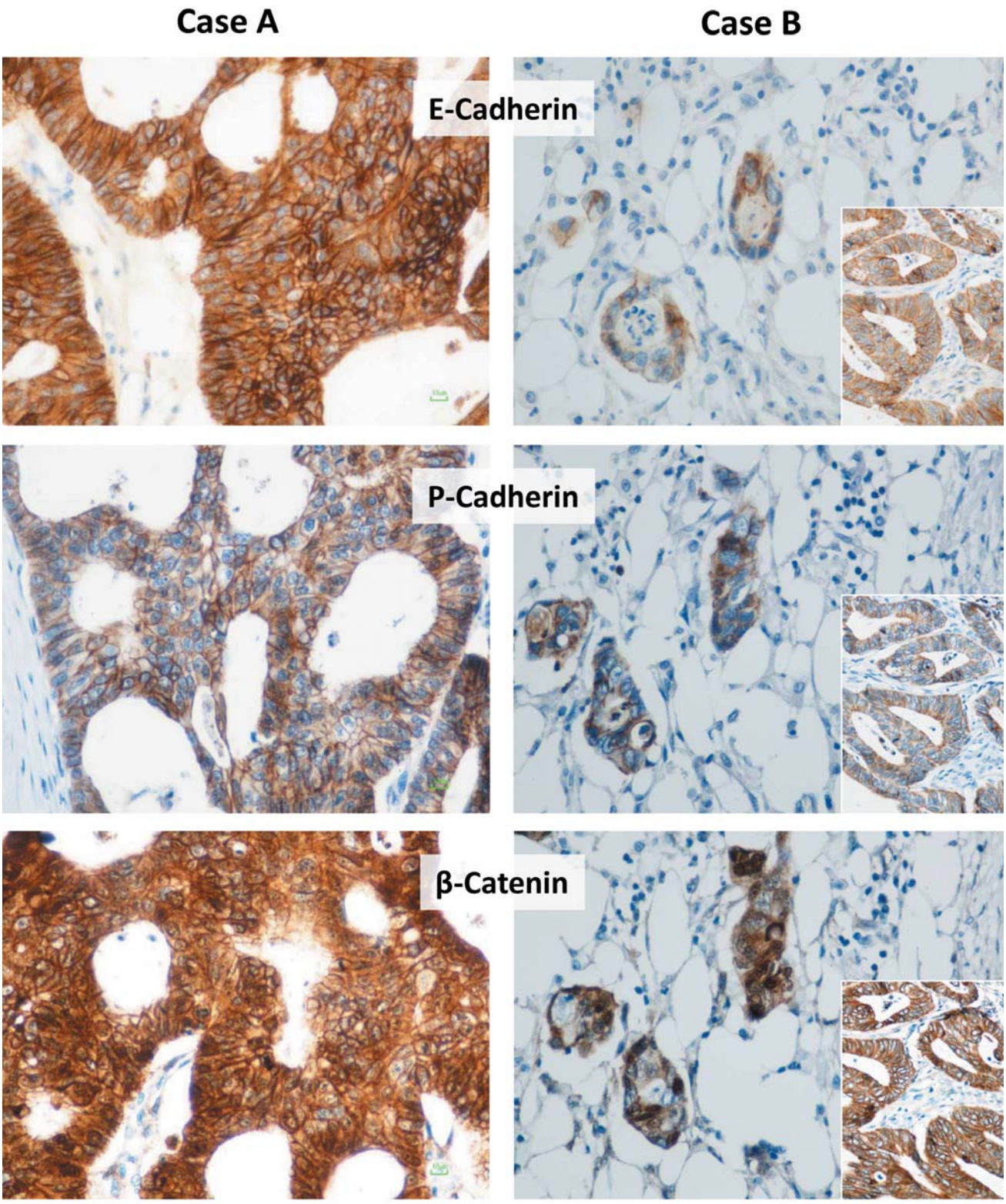


Figure 2. *Differential expression of E-cadherin, P-cadherin and β-catenin at the tumor invasive front. Two representative cases are shown. Coordinated underexpression for all three markers is noted in case B as compared to case A. In case B, there is a significant difference in E-cadherin and β-catenin expression between tumor zones. Insets: Central tumor areas. Disease-free survival in case A was longer than in case B (81.7 vs. 51 months, respectively).*

Table IV. *Associations between clinicopathological and immunohistochemistry (IHC) H-Scores for nuclear cyclin-D1 (CCND1) at the invasive front (IF) and tumor core (TC) and the membranous β-catenin.*

		Nuclear CCND1 TC				Nuclear CCND1 IF				Mean HScore			
		N	Median	Range	*p*-Value	N	Median	Range	*p*-Value	N	Median	Range	*p*-Value
Angiolymphatic invasion	None					43	50.0	0.0-175.0	0.025				
	Lymphatic only					34	12.0	0.0-180.0					
	Venous*					17	18.0	0.0-105.0					
	NA					11	30.0	0.0-275.0					
Perineural invasion	No					55	40.0	0.0-180.0	0.018	55	36.0	0.0-185.0	0.034
	Yes					23	10.0	0.0-150.0		23	13.5	0.0-157.5	
	NA					25	25.0	0.0-275.0		25	18.5	0.5-262.5	
Tumor location	Colon	70	31.0	0.0-250.0	0.008	70	35.0	0.0-275.0	0.009	70	34.3	0.0-262.5	0.004
	Rectal	36	11.5	0.0-160.0		35	10.0	0.0-150.0		35	14.5	0.0-152.5	
Tumor location	Right	28	39.0	2.0-250.0	0.022	28	40.0	2.0-275.0	0.014	28	40.0	3.5-262.5	0.008
	Left	41	30.0	0.0-215.0		41	30.0	0.0-175.0		41	30.0	0.0-165.5	
	Rectal	36	11.5	0.0-160.0		35	10.0	0.0-150.0		35	14.5	0.0-152.5	

		β-catenin TC			
		N	Median	Range	*p*-Value
pN Stage	N0	63	160.0	0.0-300.0	0.009
	N1	25	180.0	10.0-300.0	
	N2	18	90.0	0.0-200.0	

using the expression of β-catenin in the TC the IF and by using the mean of these values. All three parameters were significant in univariate analysis but the mean was prognostic in multivariate analysis. We suggest that the overall evaluation of membranous β-catenin expression can better-predict for DFS.

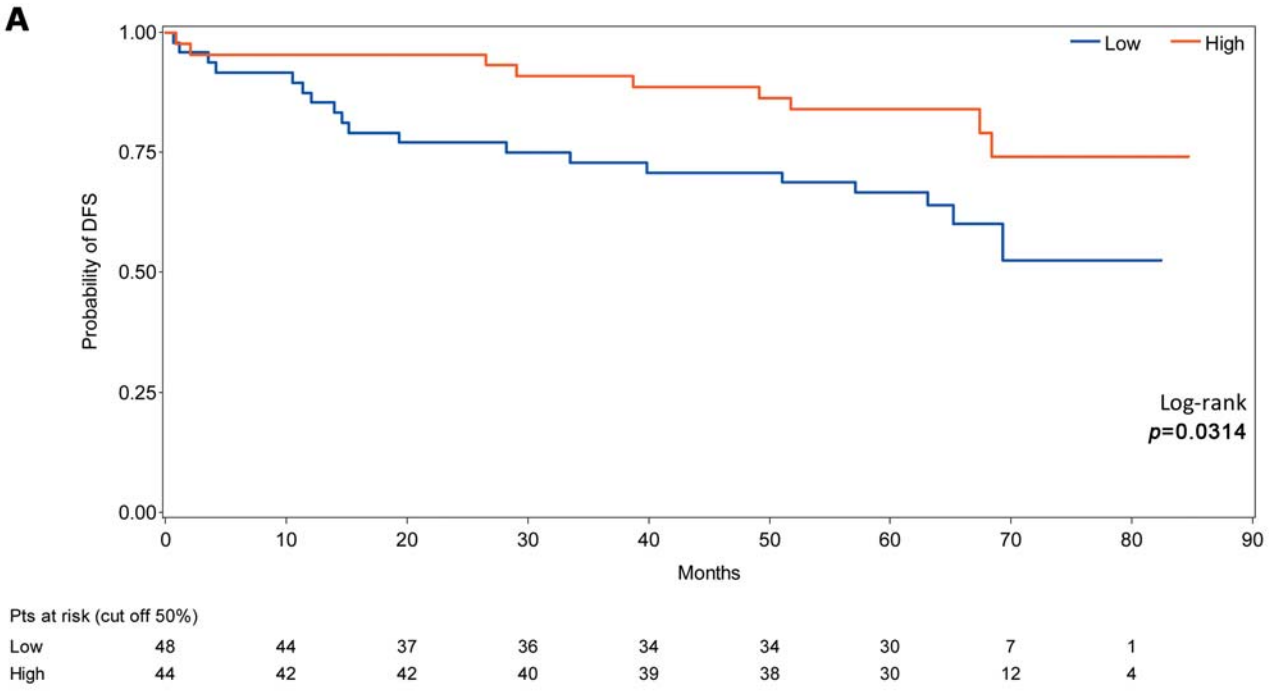
Studies comparing the differential expression of potential biomarkers between IF and TC tend not to correlate the mean expression of the marker with prognosis (17). Hence, in many cases the implication of the mean expression of the marker in the prognosis is not assessed. In our study, the expression of β-catenin in the IF seems to be similarly prognostic for DFS, when compared with TC alone. However, the mean expression remained significant in multivariate analysis when all three parameters were included. Therefore, the overall estimation of the marker should be assessed in a specimen, otherwise results should be considered cautiously.

The evaluation of different markers in the IF has been proposed to better depict the actual biologic behavior of the tumor since this is the site where the most aggressive cells with propensity to metastasize are located (18, 19). Loss of the membranous expression of β-catenin has been correlated with adverse features and outcome (8, 9). To our knowledge this is the only report to correlate loss of membranous expression of β-catenin in the IF with shorter DFS. However, the association with cancer-specific death has been previously reported (20). On the other hand, it was the expression in the TC that was correlated with lymph node involvement, indicating once more the need for meticulous evaluation of the various biomarkers.

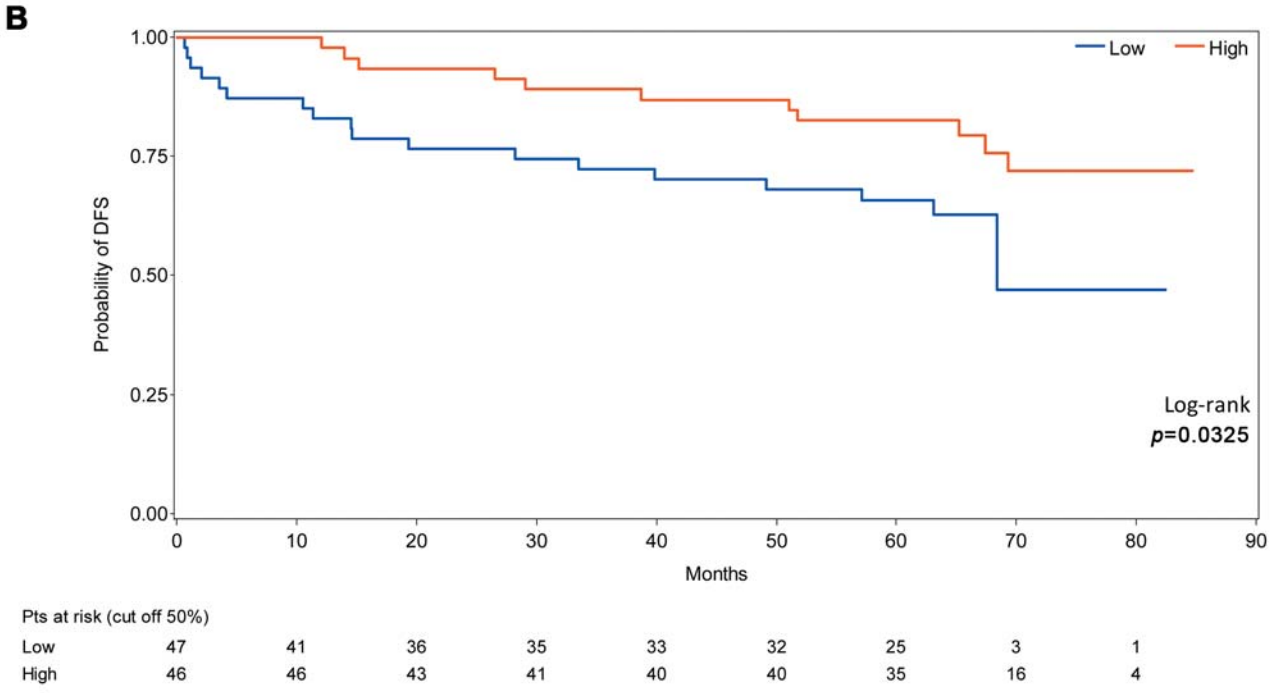
Expression of β-catenin has been characterized as being nuclear in the IF and to be correlated with poor prognosis (19, 21, 22). In our study, this was not confirmed. Nevertheless, nuclear overexpression in the IF has not been consistently reported (13, 20), while the impact of membranous expression in the IF has been highlighted in other malignancies (23, 24).

Catenins form complexes with cadherins to mediate cellular adhesion and interactions of the neighboring cells. Loss of cadherins has been associated with an invasive potential and a metastatic tendency by the acquisition of a mesenhymal phenotype. Our results are in concordance with previous studies, as far as the interaction and the prognostic significance of these molecules is concerned. First of all E- and P-cadherin and β-catenin are statistically correlated, suggesting that they may form a functional apparatus (8). The simultaneous under-expression of β-catenin and E-cadherin rendered an adverse signature of shorter DFS, while this was not influenced by P-cadherin. Such finding has been reported already, indicating the combined expression of E-cadherin and β-catenin may be a useful prognostic marker (8).

Cyclin-D1 overexpression has been suggested as a prognostic and predictive marker in CRC, although conflicting results exist (25, 26). In our study loss of CCND1 was associated with lymphovascular and perineural invasion in the IF, but no prognostic significance was revealed. Moreover, CCND1 expression was significantly higher in colonic, as compared to rectal cancer in both the IF and TC, as well as by their mean. This is opposed to what Aamodt *et al*. have described, although gene amplification was higher in colonic and not in rectal cancer, as was the case with protein

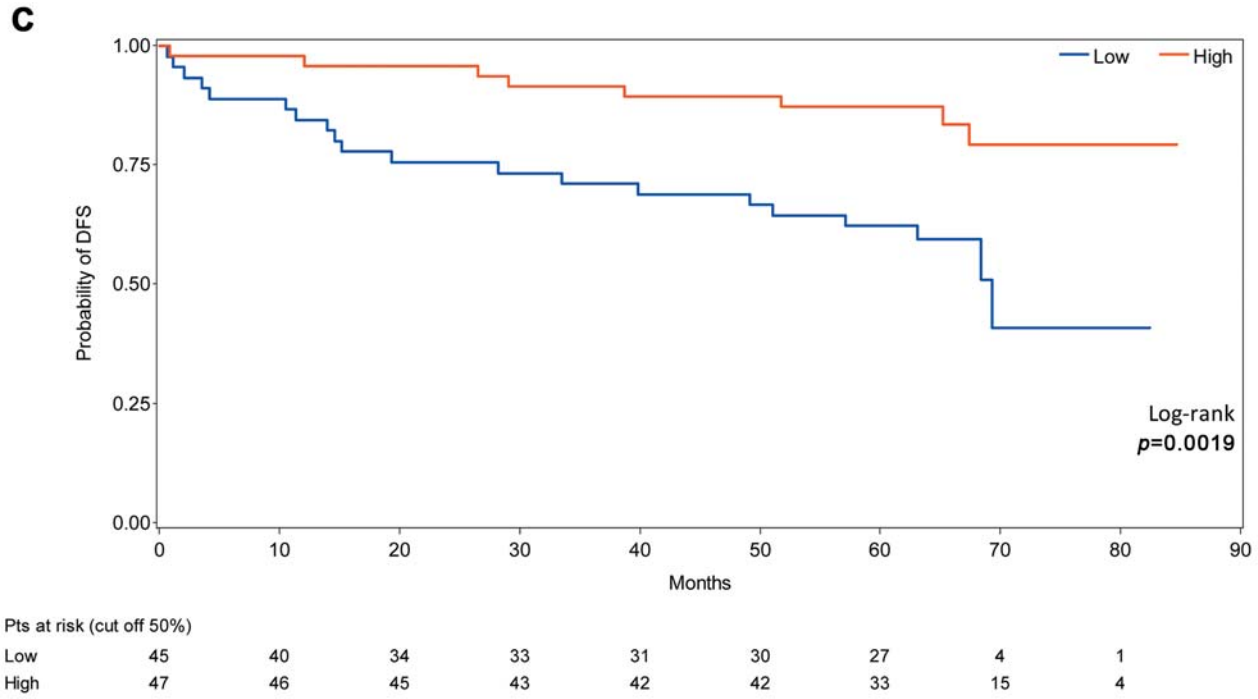


Pts at risk (cut off 50%)									
Low	48	44	37	36	34	34	30	7	1
High	44	42	42	40	39	38	30	12	4



Pts at risk (cut off 50%)									
Low	47	41	36	35	33	32	25	3	1
High	46	46	43	41	40	40	35	16	4

Figure 3. *Continued*



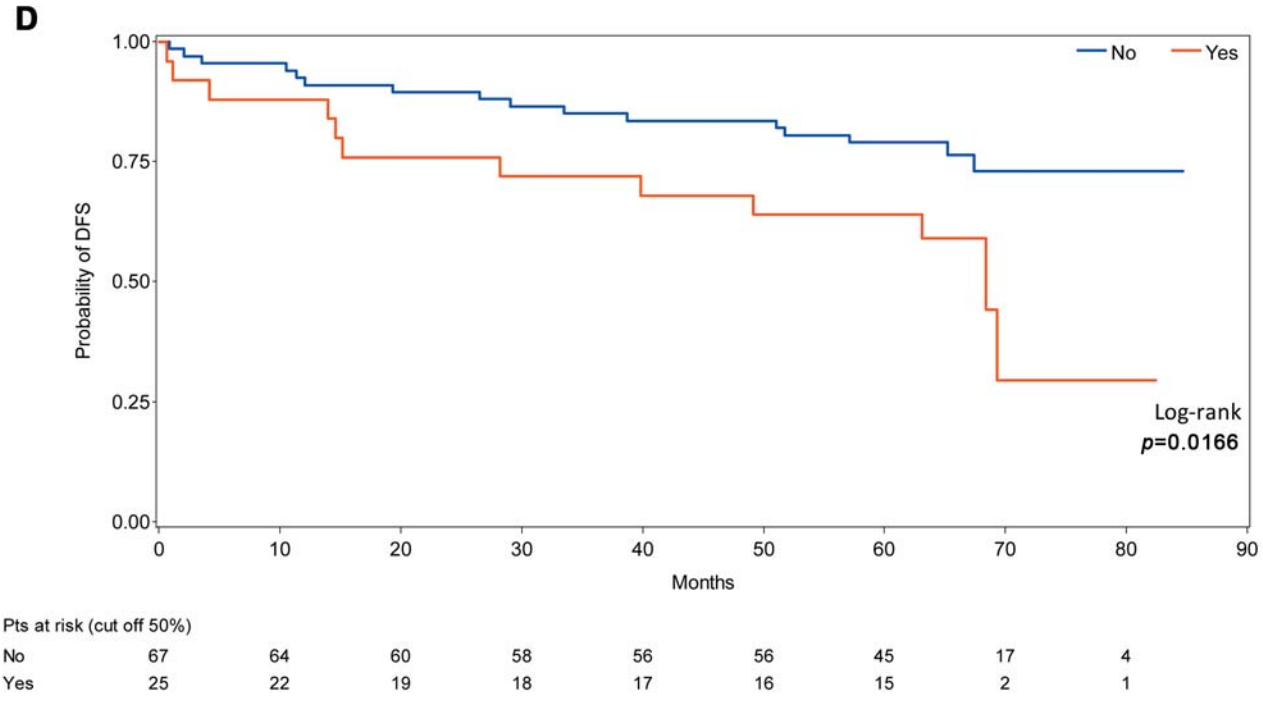


Figure 3. *Kaplan–Meier analysis of 4-year disease-free survival according to membranous β-catenin expression in the invasive front (IF) (A), tumor core (TC) (B), overall expression (C) and concurent underexpression of membranous β-catenin and E-cadherin (D).*

Table V. *Associations between clinicopathological and mRNA expression of secreted Frizzled related proteins (SFRPs).*

		SFRP4 RQ (40-DCT)				SFRP2 RQ (40-DCT)			
		N	Median	Range	*p*-Value	N	Median	Range	*p*-Value
Carcinoma arising in adenoma	No	50	36.7	28.2-42.5	0.011	48	34.7	24.3-42.9)	0.016
	Yes	15	31.3	26.7-40.4		16	29.5	26.6-40.3	
	NA	6	37.1	28.6-41.0		7	35.1	29.8-42.0	
pT-stage	T1-T2	17	31.3	28.2-40.4	0.001				
	T3-T4	54	37.0	26.7-42.5					

DCT: Delta cycle threshold; NA: non available; RQ: relative quantification.

Table VI. *Univariate analysis of disease-free survival.*

Variable				DFS			95% CI			
		N	Failed	%Censored	Range	Median	LL	UL	% Event-free at T=48	*p*-Value
All patients		93	29	69	1-85	.	69	.	78.5%	.
β-catenin, membranous (H-Score of IF)	High	44	9	80	1-85	.	.	.	88.6%	0.0314
	Low	48	19	60	1-83	.	63	.	70.8%	
β-catenin, membranous (H-Score of TC)	High	46	11	76	12-85	.	.	.	87.0%	0.0325
	Low	47	18	62	1-83	68	63	.	70.2%	
β-catenin, membranous (Mean HScore)	High	47	8	83	1-85	.	.	.	89.4%	0.0019
	Low	45	20	56	1-83	69	51	.	68.9%	
Concurrent underexpression of E-cadherin and β-catenin	No	67	16	76	1-85	.	.	.	83.6%	0.0166
	Yes	25	12	52	1-83	68	40	.	68.0%	
Astler Coller stage	A	8	2	75	40-76	.	40	.	87.5%	0.0008
	B1/B2	49	9	82	1-85	.	.	.	93.9%	
	C1/C2	36	18	50	1-81	69	19	.	55.6%	
Angiolymphatic invasion	None	41	8	80	1-85	.	.	.	87.8%	0.0028
	Lymphatic only	31	9	71	14-83	.	67	.	80.6%	
	Venous*	12	8	33	1-69	47	2	69	50.0%	
	NA	9	4	56	11-69	.	11	.	66.7%	
Gender	Female	38	8	79	4-84	.	.	.	89.5%	0.0376
	Male	55	21	62	1-85	.	67	.	70.9%	
Perineural invasion	No	51	11	78	1-85	.	.	.	90.2%	0.0343
	Yes	18	9	50	1-79	69	15	.	61.1%	
	NA	22	8	64	4-75	.	27	.	68.2%	
pN-Stage	N0	56	11	80	1-85	.	.	.	92.9%	0.0004
	N1	24	11	54	4-81	69	29	.	62.5%	
	N2	13	7	46	1-66	15	4	.	46.2%	

Note: Kaplan-Meier estimates and *p*-value based on log-rank test. *Irrespective of lymphatic invasion; CI: Confidence interval; LL: lower limit; UL: upper limit; T: time; NA: not available.

expression (27). Nonetheless, no other expression difference was correlated with the tumor site contradicting previous reports indicating higher expression of nuclear β-catenin in rectal cancer (28).

SFRPs frequently act as negative regulators of the WNT pathway and are down-regulated during carcinogenesis, mainly through hypermethylation (29). Reduced expression of the cadherin/catenin complex during carcinogenesis through adenoma formation has been described (30), while conflicting results exist concerning the expression of SFRPs in colorectal carcinoma or adenomas as compared with normal tissue (29, 31). To our knowledge, this is the first report correlating different expression of SFRPs with different types of carcinogenesis. Indeed, loss of SFRP2 and -4 were correlated with carcinomas not arising from a pre-existing adenoma. Nevertheless, validation of this finding in a larger cohort comparing the expression in normal mucosa with carcinomas arising from adenomas or *de novo* is mandatory before any

Table VII. *Univariate analysis of overall survival.*

				OS			95% CI			
		N	Failed	%Censored	Range	Median	LL	UL	% Event-free at T=48	*p*-Value
All patients		93	22	76	1-85	.	.	.	83.9%	.
Astler Coller stage	A	8	2	75	42-76	.	42	.	87.5%	0.0099
	B1/B2	49	6	88	1-85	.	.	.	93.9%	
	C1/C2	36	14	61	1-81	.	51	.	69.4%	
Perineural invasion	No	51	8	84	1-85	.	.	.	92.2%	0.0487
	Yes	18	7	61	1-79	.	18	.	61.1%	
	NA	22	6	73	19-79	.	57	.	86.4%	
pN-Stage	N0	56	8	86	1-85	.	.	.	92.9%	0.0049
	N1	24	8	67	19-81	.	51	.	79.2%	
	N2	13	6	54	1-79	.	4	.	53.8%	

Note: KM estimates and *p*-value based on log-rank test. CI: Confidence interval; LL: lower limit; UL: upper limit; T: time; NA: not available.

further conclusion should be drawn. In addition, SFRP4 was correlated with depth of invasion, something that should be addressed with caution since in most cases it is the underexpression of this negative regulator that is correlated with adverse prognostic features (31, 32).

Despite the strengths of our study, some limitations should be addressed. In our cohort we observed no difference by the use of adjuvant chemotherapy. This is explained by the fact that this was not a homogenously-treated population and half of the non-treated patients should have received adjuvant chemotherapy either due to stage C or high-risk B2 disease. Nevertheless, the rest of the well-characterized prognosticators were also confirmed in our study: stage, lymph node involvement, vascular and perineural invasion, as well as male gender. The prognostic significance and correlation with different clinopathological parameters was not confirmed for many of the markers studied. This could be attributed to different methodology or cut-offs used. Indeed, no consensus exists about the best possible approach and a variety of methods to interpret results have been used.

Consequently, underexpression of membranous β-catenin as well as expression in the IF seems to identify a high-risk subset of patients, surgically-treated for colorectal cancer, with shorter DFS. Further evaluation in a larger cohort is mandatory to validate these results.

Table VIII. *Multivariate analysis of disease-free survival by mean of β-catenin H-score (a) and β- catenin in invasive front (IF) (b)_*

Variable	Hazard ratio	95% Confidence interval	Wald's *p*-Value
a.			
Astler Coller TNM A	0.47	(0.11-2.06)	0.3148
Astler Coller TNM B1/B2	0.29	(0.13-0.65)	0.0028
β-catenin membranous, Mean HScore	0.33	(0.14-0.77)	0.0100
b.			
Angiolymphatic invasion: Lymphatic only	1.19	(0.45-3.20)	0.7234
Angiolymphatic invasion: Venous	5.28	(1.97-14.13)	0.0009
β-catenin membranous, HScore of IF	0.41	(0.18-0.91)	0.0280

References

1 Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. CA Cancer J Clin *63(1)*: 11-30, 2013.

2 White BD, Chien AJ and Dawson DW: Dysregulation of Wnt/β-catenin signaling in gastrointestinal cancers. Gastroenterology *142(2)*: 219-232, 2012.

3 MacDonald BT, Tamai K and He X: Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev Cell *17(1)*: 9-26, 2009.

4 Brabletz T, Jung A, Dag S, Hlubek F and Kirchner T: β-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol *155(4)*: 1033-1038, 1999.

5 Matono H, Oda Y, Nakamori M, Tamiya S, Yamamoto H, Yokoyama R, Saito T, Iwamoto Y and Tsuneyoshi M: Correlation between beta-catenin widespread nuclear expression and matrix metalloproteinase-7 overexpression in sporadic desmoid tumors. Hum Pathol *39(12)*: 1802-1808, 2008.

6 Utsunomiya T, Doki Y, Takemoto H, Shiozaki H, Yano M, Sekimoto M, Tamura S, Yasuda T, Fujiwara Y and Monden M: Correlation of β-catenin and cyclin D1 expression in colon cancers. Oncology *61(3)*: 226-233, 2001.

7 Bovolenta P, Esteve P, Ruiz JM, Cisneros E and Lopez-Rios J: Beyond Wnt inhibition: New functions of secreted Frizzled-related proteins in development and disease. J Cell Sci *121(Pt 6)*: 737-746, 2008.

8 Filiz AI, Senol Z, Sucullu I, Kurt Y, Demirbas S and Akin ML: The survival effect of E-cadherin and catenins in colorectal carcinomas. Colorectal Dis *12(12)*: 1223-1230, 2010.

9 Tóth L, András C, Molnár C, Tanyi M, Csiki Z, Molnár P and Szántó J: Investigation of β-catenin and E-cadherin expression in Dukes B2 stage colorectal cancer with tissue microarray method. Is it a marker of metastatic potential in rectal cancer? Pathol Oncol Res *18(2)*: 429-437, 2012.
10 Sun L, Hu H, Peng L, Zhou Z, Zhao X, Pan J, Sun L, Yang Z and Ran Y: P-Cadherin promotes liver metastasis and is associated with poor prognosis in colon cancer. Am J Pathol *179(1)*: 380-390, 2011.
11 Van Marck V, Stove C, Jacobs K, Van den Eynden G and Bracke M: P-Cadherin in adhesion and invasion: opposite roles in colon and bladder carcinoma. Int J Cancer *128(5)*: 1031-1044, 2011.
12 Dejmek J, Dejmek A, Safholm A, Sjolander A and Andersson T: WNT-5a protein expression in primary Dukes B colon cancers identifies a subgroup of patients with good prognosis. Cancer Res *65(20)*: 9142-9146, 2005.
13 Karamitopoulou E, Zlobec I, Panayiotides I, Patsouris ES, Peros G, Rallis G, Lapas C, Karakitsos P, Terracciano LM and Lugli A: Systematic analysis of proteins from different signaling pathways in the tumor center and the invasive front of colorectal cancer. Hum Pathol *42(12)*: 1888-1896, 2011.
14 Kahlert C, Lahes S, Radhakrishnan P, Dutta S, Mogler C, Herpel E, Brand K, Steinert G, Schneider M, Mollenhauer M, Reissfelder C, Klupp F, Fritzmann J, Wunder C, Benner A, Kloor M, Huth C, Contin P, Ulrich A, Koch M and Weitz J: Overexpression of ZEB2 at the invasion front of colorectal cancer is an independent prognostic marker and regulates tumor invasion *in vitro*. Clin Cancer Res *17(24)*: 7654-7663, 2011.
15 Edge SB and Compton CC: The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol *17(6)*: 1471-1474, 2010.
16 McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM; Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics: REporting recommendations for tumour MARKer prognostic studies (REMARK). Eur J Cancer *41(12)*: 1690-1696, 2005.
17 Wang X, Zhang J, Fan M, Zhou Q, Deng H, Aisharif MJ and Chen X: The expression of E-cadherin at the invasive tumor front of oral squamous cell carcinoma: Immunohistochemical and RT-PCR analysis with clinicopathological correlation. Oral Surg Oral Med Oral Pathol Oral Radiol Endod *107(4)*: 547-554, 2009.
18 Cianchi F, Cuzzocrea S, Vinci MC, Messerini L, Comin CE, Navarra G, Perigli G, Centorrino T, Marzocco S, Lenzi E, Battisti N, Trallori G and Masini E: Heterogeneous expression of cyclooxygenase-2 and inducible nitric oxide synthase within colorectal tumors: correlation with tumor angiogenesis. Dig Liver Dis *42(1)*: 20-27, 2010.
19 Baldus SE, Mönig SP, Huxel S, Landsberg S, Hanisch FG, Engelmann K, Schneider PM, Thiele J, Hölscher AH and Dienes HP: MUC1 and nuclear beta-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. Clin Cancer Res *10(8)*: 2790-2796, 2004.
20 Matsuoka T, Mitomi H, Fukui N, Kanazawa H, Saito T, Hayashi T and Yao T: Cluster analysis of claudin-1 and -4, E-cadherin, and β-catenin expression in colorectal cancers. J Surg Oncol *103(7)*: 674-686, 2011.
21 Wang L, Cheng H, Liu Y, Wang L, Yu W, Zhang G, Chen B, Yu Z and Hu S: Prognostic value of nuclear beta-catenin overexpression at invasive front in colorectal cancer for synchronous liver metastasis. Ann Surg Oncol *18(6)*: 1553-1559, 2011.
22 Suzuki H, Masuda N, Shimura T, Araki K, Kobayashi T, Tsutsumi S, Asao T and Kuwano H: Nuclear β-catenin expression at the invasive front and in the vessels predicts liver metastasis in colorectal carcinoma. Anticancer Res *28(3B)*: 1821-1830, 2008.
23 Retterspitz MF, Mönig SP, Schreckenberg S, Schneider PM, Hölscher AH, Dienes HP and Baldus SE: Expression of {β}-catenin, MUC1 and c-met in diffuse-type gastric carcinomas: correlations with tumour progression and prognosis. Anticancer Res *30(11)*: 4635-4641, 2010.
24 Liu LK, Jiang XY, Zhou XX, Wang DM, Song XL and Jiang H: Upregulation of vimentin and aberrant expression of E-cadherin/β-catenin complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome. Mod Pathol *23(2)*: 213-224, 2010.
25 Ogino S, Nosho K, Irahara N, Kure S, Shima K, Baba Y, Toyoda S, Chen L, Giovannucci EL, Meyerhardt JA and Fuchs CS: A cohort study of cyclin D1 expression and prognosis in 602 colon cancer cases. Clin Cancer Res *15(13)*: 4431-4438, 2009.
26 Myklebust MP, Li Z, Tran TH, Rui H, Knudsen ES, Elsaleh H, Fluge Ø, Vonen B, Myrvold HE, Leh S, Tveit KM, Pestell RG and Dahl O: Expression of cyclin D1a and D1b as predictive factors for treatment response in colorectal cancer. Br J Cancer *107(10)*: 1684-1691, 2012.
27 Aamodt R, Jonsdottir K, Andersen SN, Bondi J, Bukholm G and Bukholm IR: Differences in protein expression and gene amplification of cyclins between colon and rectal adenocarcinomas. Gastroenterol Res Pract *2009*: 285830, 2009.
28 Kapiteijn E, Liefers GJ, Los LC, Kranenbarg EK, Hermans J, Tollenaar RA, Moriya Y, van de Velde CJ, van Krieken JH: Mechanisms of oncogenesis in colon *versus* rectal cancer. J Pathol *195(2)*: 171-178, 2001.
29 Qi J, Zhu YQ, Luo J and Tao WH: Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor. World J Gastroenterol *12(44)*: 7113-7117, 2006.
30 Hao X, Palazzo JP, Ilyas M, Tomlinson I and Talbot IC: Reduced expression of molecules of the cadherin/catenin complex in the transition from colorectal adenoma to carcinoma. Anticancer Res *17(3C)*: 2241-2247, 1997.
31 Feng Han Q, Zhao W, Bentel J, Shearwood AM, Zeps N, Joseph D, Iacopetta B and Dharmarajan A: Expression of sFRP-4 and beta-catenin in human colorectal carcinoma. Cancer Lett *231(1)*: 129-137, 2006.
32 Kinoshita T, Nomoto S, Kodera Y, Koike M, Fujiwara M and Nakao A: Decreased expression and aberrant hypermethylation of the SFRP genes in human gastric cancer. Hepatogastro enterology *58(107-108)*: 1051-1056, 2011.

Received July 11, 2013
Revised September 3, 2013
Accepted September 3, 2013