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CD133 and nuclear β-catenin: The marker combination to detect high risk cases of low stage colorectal cancer

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ABSTRACT

Nuclear β-catenin and CD133 are linked with two hallmarks of colon cancer, wingless-type mouse mammary tumour virus integration site (WNT)-pathway dysregulation and colon cancer stem cells (Co-CSCs), respectively. Both molecules may be related, as Co-CSCs were proposed to require activated WNT-signalling and as CD133 was postulated as a WNT/β-catenin target gene. Herein, we investigated the expression of these markers on serial sections of 162 stage IIA colonic adenocarcinomas. We found that the expression of these molecules is statistically independent and that they mark distinct but overlapping subpopulations of the tumour cells. Moreover, we show that their combined evaluation can identify colon cancer cases with vastly reduced survival (hazard ratio (HR) 13.4, 95% confidence interval (CI): 4.7–38.2) and a high risk of tumour progression (HR 6.8, 95%CI: 3.1–15.0). In conclusion, the independence of these markers may on the one hand have implications for their presumed value to identify Co-CSCs; on the other hand it allows their combined analysis to become a powerful tool to identify high risk cases of stage IIA colon cancer.

1. Introduction

Two major discoveries currently lead the research in the field of colon cancer oncology. First is the finding that most colon cancers are characterised by pathway dysregulation of wingless-type mouse mammary tumour virus integration site (WNT)-signalling which is mainly based on mutations that either inactivate adenomatous polyposis coli (APC) or stabilise β-catenin.[1,2] This leads to nuclear accumulation of β-catenin and to ensuing overexpression of its oncogenic target genes, such as *c-myc* and *cyclin-D1*,[3,4] and in this manner it is thought to substantially contribute to the malignant phenotype of the colon cancer cell. Although the tumour cells are of monoclonal origin,[5] most tumours still display a heterogeneous expression pattern for nuclear β-catenin so that in spite of WNT-pathway dysregulation, an intratumoural regulation of β-catenin is still preserved in most colon cancers.[6,7] We previously demonstrated that this preserved regulation can be immunohistochemically assessed by the presence of cancer cells with and without nuclear accumulation of β-catenin in the same tumour and is a predictor of improved patient survival and lower risk of tumour progression in colon cancer.[8]

The second discovery is that, in colon cancer, only a small subset of the tumour cells, referred to as tumour-initiating cells or cancer stem cells, has the ability to drive tumour growth and that colon cancer is thus subject to the cancer stem cell hypothesis.[9–11] In these studies such colon cancer

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stem cells (Co-CSCs) were found to express the surface marker CD133, while most cells of the bulk tumour population were negative for this marker. Although CD133 is not exclusively expressed on Co-CSCs,[10] it therefore allows their high enrichment and is currently the most widely accepted marker for Co-CSC characterisation. In a previous study we demonstrated that high CD133 expression correlates with lower survival of colon cancer patients and thus found evidence for the clinical impact of Co-CSCs.[12] Taken together, both β-catenin and CD133 can be viewed as molecules that directly relate to central mechanisms in colon cancer biology and both are of clinical relevance when evaluating their expression in tumour samples.

A link between both the molecules emerges from the postulation that, aside from CD133 expression, Co-CSCs are characterized by an activated WNT-signalling pathway and thus by expression of nuclear β-catenin.[13] Just recently, this postulation has been supported by the finding that a subset of colon cancer initiating cells, when grown in spheroid cultures, expresses both markers.[14] Moreover CD133 has been identified as a potential target gene of WNT-signalling.[15] Therefore, as does CD133, nuclear β-catenin is also assumed to mark colon cancer stem cells.

If both the markers thus characterise the Co-CSC population, *in situ* analysis should find a correlation of their expression, and by using either marker a similar value to predict patient survival and tumour progression in colon cancer should be observed. To test this hypothesis, we herein comparatively investigated the expression of CD133 and nuclear β-catenin on serial tumour sections of 162 stage IIA (UICC) colon cancer cases.

2. Materials and methods

2.1. Study design and human tissues

Only patients with moderately differentiated G2 (WHO), stage IIA (UICC) colonic adenocarcinomas that underwent surgical resection at the Ludwig-Maximilians-Universität München between 1994 and 2004 were enrolled in this study. None of these patients received adjuvant therapies. Paraffin-embedded tissue samples of primary tumours were available in 162 cases. The age of the patients ranged from 34 to 93 years (median 70 years). Followup data were available from the Tumorregister München. For cancer-specific survival analysis, colon cancer attributed deaths were defined as clinical endpoints while for disease-free survival, tumour progression after surgical resection was the clinical end-point which included either tumour recurrence or metastasis. For inclusion models, age, gender and tumour side[16] were considered as covariables. Clinicopathological characteristics of the study population are summarised in Table 3. This study was approved by the local ethics committee of the Medical Faculty of the Ludwig-Maximilians-Universität München.

2.2. Immunohistochemistry

Immunohistochemical staining was done on 5 μm whole standard tissue sections of formalin-fixed, paraffin-embedded tumour samples. As primary antibodies, anti-CD133 rabbit monoclonal antibody (clone C24B9, Cell Signaling Technologies, dilution 1:100) and prediluted anti-β-catenin mouse monoclonal antibody (clone 14, Ventana Medical Systems) were used. Staining was performed on a Ventana Benchmark XT autostainer with the XT ultraView DAB Kit (Ventana Medical Systems). All slides were counterstained with Hematoxylin (Vector). To exclude unspecific staining, system controls were included.

2.3. Scoring of CD133 and nuclear β-catenin expression

Five medium power microscopic fields per tumour and staining were evaluated, considering tumour margin and tumour centre in each case. Two different scoring approaches were applied. First, positivity and negativity of both the markers were evaluated. Thereby, tumours with any nuclear accumulation of β-catenin were considered as nuclear β-catenin positive while all others were considered as nuclear β-catenin negative. For CD133, positivity was defined as either apical membranous staining or staining of shed cellular debris in the tumour glands[12] while negativity meant complete absence of any staining. Then a second scoring approach was chosen, to account for previously reported prognostic categories: Nuclear β-catenin expression was considered as unregulated, if virtually all tumour cells were nuclear β-catenin positive or all tumour cells lacked nuclear β-catenin accumulation. Regulated nuclear β-catenin expression meant coexistence of tumour cells with and without nuclear β-catenin expression in the same tumour.[8] CD133 expression levels were low if none or less than 50% of the tumour glands were CD133 positive, or high if more than 50% of the tumour glands were CD133 positive. Positivity of the glands for CD133 was again defined by the presence of apical staining of the tumour cells or staining of intraglandular debris, as previously shown.[12] For both the markers, the intensity of staining was not taken into consideration.

2.4. Sample size and statistical analysis

For this study, 162 cases were available which complied with the enrolment criteria. Within this study population, 15 events of cancer-specific death and 28 events of tumour progression within 10 years were observed, in accordance with known survival rates for this tumour stage.[17] The hazard ratios that could be detected at a power of 0.8 with $\alpha = 0.05$ were thus calculated as 4.2 and 2.9 for survival and progression, respectively.[18] Therefore, large effects on survival and progression could be detected which was assumed to be appropriate for this study design. Frequency data were analysed using the $\chi 2$ test. Cancer-specific survival and disease-free survival were calculated from the date of primary surgical resection to the date of colon cancer associated death and to the date of recorded cancer progression (metastasis or recurrence), respectively. Survival analysis was done applying the Kaplan–Meier method, using the log-rank test for comparison of groups. The Cox regression model was used for multivariate analyses. Statistical procedures were performed using SPSS version 16.0 (SPSS Inc.). *P*-values <0.05 were considered as statistically significant.

3. Results

3.1. CD133 and nuclear β-catenin expression are independent in colon cancer

To analyse the relation between nuclear β-catenin and CD133 expression in colon cancer, immunohistochemically stained serial tumour sections were evaluated for both markers using two different scoring systems. Firstly, evaluating either presence or absence of nuclear β-catenin and CD133, no correlation between these markers was found (Table 1, $p = 0.84$). Presence of both nuclear β-catenin and CD133 was the most frequently observed combination (56.2%) while absence of both markers was least common (5.6%). Secondly, assessing nuclear β-catenin regulation and CD133 expression level, also no significant correlation between these phenotypes was observed (Table 2, $p = 0.97$). In this manner, regulated nuclear β-catenin combined with low CD133 was the commonest phenotype (43.8%) while unregulated nuclear β-catenin paired with high CD133 was least frequent (11.1%). Taken together, CD133 and nuclear β-catenin are independent markers, without significant statistical interference in stage IIA colon cancer.

Next, to investigate a possibly intratumoural correlation of both markers, individual tumours with heterogeneous expression of these antigens were analysed. A subset of glandular differentiated tumour cells was found which expressed both nuclear β-catenin and CD133 (Fig. 1A and B). However, another subset that only expressed CD133 without nuclear β-catenin among the glandular differentiated cells (Fig. 1C and D) and a subset of nuclear β-catenin expressing cells, devoid of CD133 expression were also identified (Fig. 1E and F). Notably, the latter included undifferentiated tumour cells at the tumour margin. Thus, nuclear β-catenin and CD133 mark overlapping but uneven subpopulations of colon cancer cells.

3.2. Combined analysis of CD133 and β-catenin identifies high risk cases of stage IIA colon cancer

We then examined, whether the combined evaluation of CD133 expression and nuclear β-catenin regulation would be powerful in estimating the risk of tumour-associated death and tumour progression of colon cancer in our study population. The clinicopathological characteristics of this population are given in Table 3. The Kaplan–Meier method was employed and all four combinatory groups of high/low CD133 and regulated/unregulated nuclear β-catenin were compared. Cases characterised by lack of nuclear β-catenin regulation and high CD133 expression (Fig. 2) were associated with vastly reduced cancer-specific survival (5-year survival 47 ± 13% versus 95 ± 2%, $p = 5.0 \times 10^{-11}$) and disease-free survival (5-year survival 36 ± 12% versus 87 ± 3%, $p = 5.4 \times 10^{-9}$), when compared to those with all other marker combinations (Fig. 3), while this was of very high statistical significance. Meanwhile, differences observed between cases characterised by any of the remaining three marker combinations were non-significant (data not shown). In addition, no correlation of this marker combination and other clinicopathological variables was observed (Table 3).

Finally, the prognostic value of the CD133-high and unregulated nuclear-β-catenin marker combination was tested, using multivariate Cox-regression. As shown in Table 4, this marker combination was strongly associated with cancer-specific death ($p = 4.3 \times 10^{-7}$, HR = 13.4) and disease progression ($p = 1.9 \times 10^{-6}$, HR = 6.8) with very high relative risks. Taken together, the combined analysis of CD133 and nuclear β-catenin allows identification of high risk cases of moderately differentiated stage IIA colon cancer, independent of other clinicopathological variables.

Table 1 – No correlation of presence and absence of nuclear β-catenin and CD133 expression in colon cancer.

	CD133 absent	CD133 present	Total
Nuclear β-catenin absent	9 (5.6)	23 (14.2)	32 (19.8)
Nuclear β-catenin present	39 (24.1)	91 (56.2)	130 (80.2)
Total	48 (29.6)	114 (70.4)	

Correlation significance $p = 0.84$.
Odds ratio = 0.91.
Percent values given in parentheses.

Table 2 – No correlation of nuclear β-catenin regulation and level of CD133 expression in colon cancer.

	CD133 low	CD133 high	Total
Nuclear β-catenin regulated	71 (43.8)	28 (17.3)	99 (61.1)
Nuclear β-catenin unregulated	45 (27.8)	18 (11.1)	63 (38.9)
Total	116 (71.6)	46 (28.4)	

Correlation significance $p = 0.97$.
Odds ratio = 0.99.
Percent values given in parentheses.

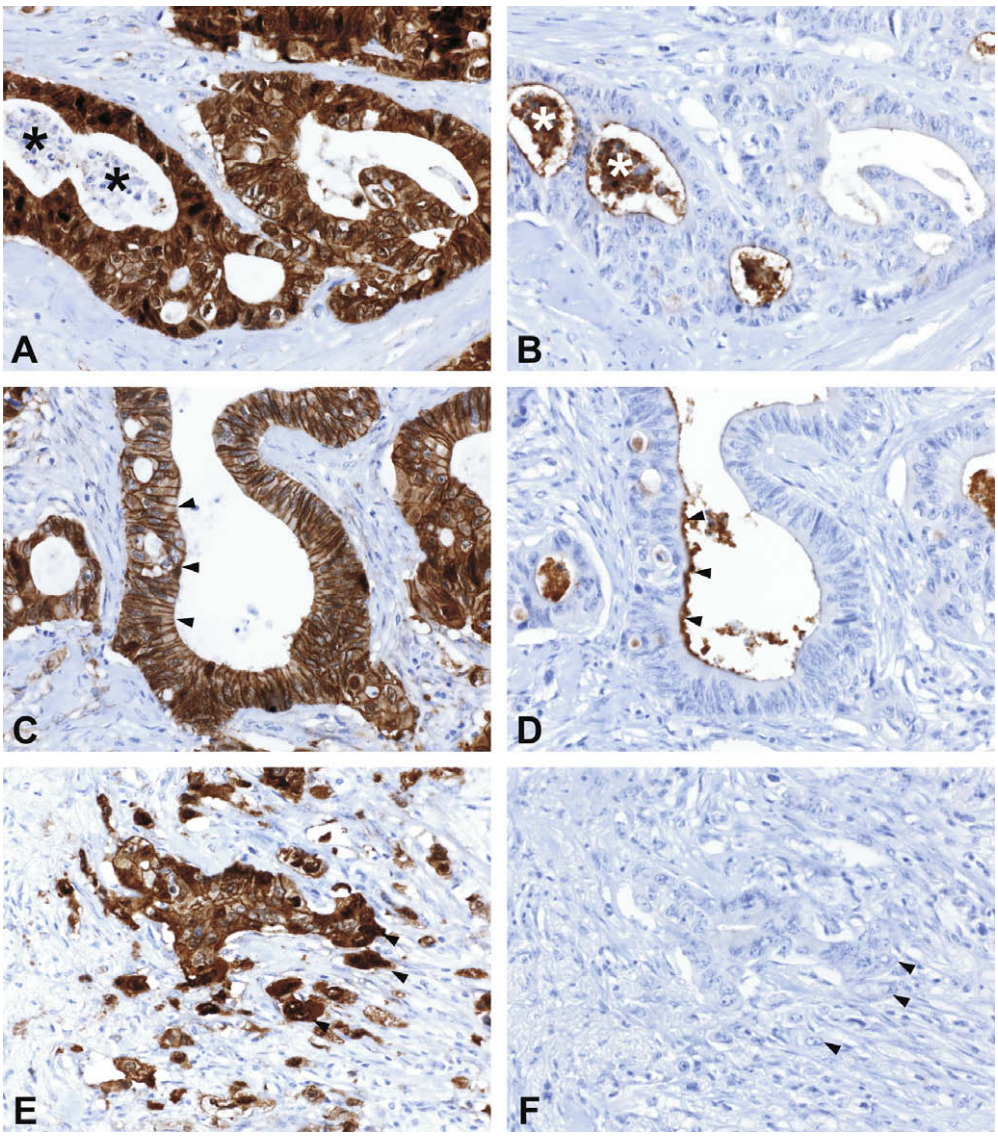

Fig. 1 – Immunohistochemical staining of β-catenin (left panel) and CD133 (right panel) on serial tumour sections of a colonic adenocarcinoma displays distinct but overlapping populations of cells expressing these markers. Glandular differentiated tumour cells with both strong expression of nuclear β-catenin (A) and apical expression of CD133 (B), as indicated by asterisks. In other areas of the tumour, absence of nuclear β-catenin expression (C) paired with presence of apical CD133 staining (D) (arrowheads). Undifferentiated tumour cells at the tumour margin were often positive for nuclear β-catenin (E) while always negative for CD133 (F), as indicated by arrowheads.

4. Discussion

In this study, we investigated the relation of nuclear β-catenin and CD133 expression in colon cancer, as these markers are closely linked with two hallmarks of this malignancy: Dysregulated WNT-signalling[1,2] and colon cancer stem cells (Co-CSCs),[9–11] respectively. We demonstrate that nuclear β-catenin and CD133 expression do not correlate and that these markers stain different but overlapping tumour cell populations.

When interpreting these results in regard to the cancer stem cell hypothesis, they are surprising, as both the molecules were proposed to mark Co-CSCs,[13,14] and as CD133 was suggested to be a WNT/β-catenin target gene.[15] However, these results comply with the recent observation that CD133 positive colon cancer cells only in part express nuclear β-catenin, when grown in spheroid cultures, which is assumed to select for tumour cells with stem cell like features.[14] Assuming that both markers are associated with the Co-CSC phenotype, our finding that a subpopulation of the tumour cells shows strong nuclear β-catenin expression while lacking expression of CD133 may suggest that nuclear β-catenin marks Co-CSCs but, as CD133, is unlikely to be highly specific for their phenotype. Vice versa, as the nuclear β-catenin expressing and CD133 negative tumour cell subset includes tumour buds[19] at the tumour margin, these cells may as well represent a subset of Co-CSCs[13] which are devoid of CD133, and may be accusable for contradictory findings on the spec-

Table 3 – Clinicopathological data of the investigated stage IIA colon cancer cases.

Variables	No. Patients	Nuclear β-catenin		CD133		Unregulated nuclear β-Catenin and CD133 high		
		Regulated	Unregulated	Low	High	Does not apply	Applies	*p*
All patients	162 (100.0)	99 (61.1)	63 (38.9)	116 (71.6)	46 (28.4)	144 (88.9)	18 (11.1)	
Age (y, median 70)								
⩽70	89 (54.9)	61 (37.7)	28 (17.3)	63 (38.9)	26 (16.0)	81 (50.0)	8 (4.9)	0.34
>70	73 (45.1)	38 (23.5)	35 (21.6)	53 (32.7)	20 (12.3)	63 (38.9)	10 (6.2)	
Gender								
Male	83 (51.2)	54 (33.3)	29 (17.9)	60 (37.0)	23 (14.2)	73 (45.1)	10 (6.2)	0.7
Female	79 (48.8)	45 (27.8)	34 (21.0)	56 (34.6)	23 (14.2)	71 (43.8)	8 (4.9)	
Tumour side								
Right	78 (48.1)	47 (29.0)	31 (19.1)	51 (31.5)	27 (16.7)	69 (42.6)	9 (5.6)	0.7
Left	83 (51.2)	52 (32.1)	31 (19.1)	65 (40.1)	18 (11.1)	75 (46.3)	8 (4.9)	
Unknown	1 (0.6)	0 (0.0)	1 (0.6)	0 (0.0)	1 (0.6)	0 (0.0)	1 (0.6)	
Tumour grade								
G2 (WHO)	162 (100.0)							
Tumour stage								
pT3, pN0	162 (100.0)							

Percent values are given in parentheses.

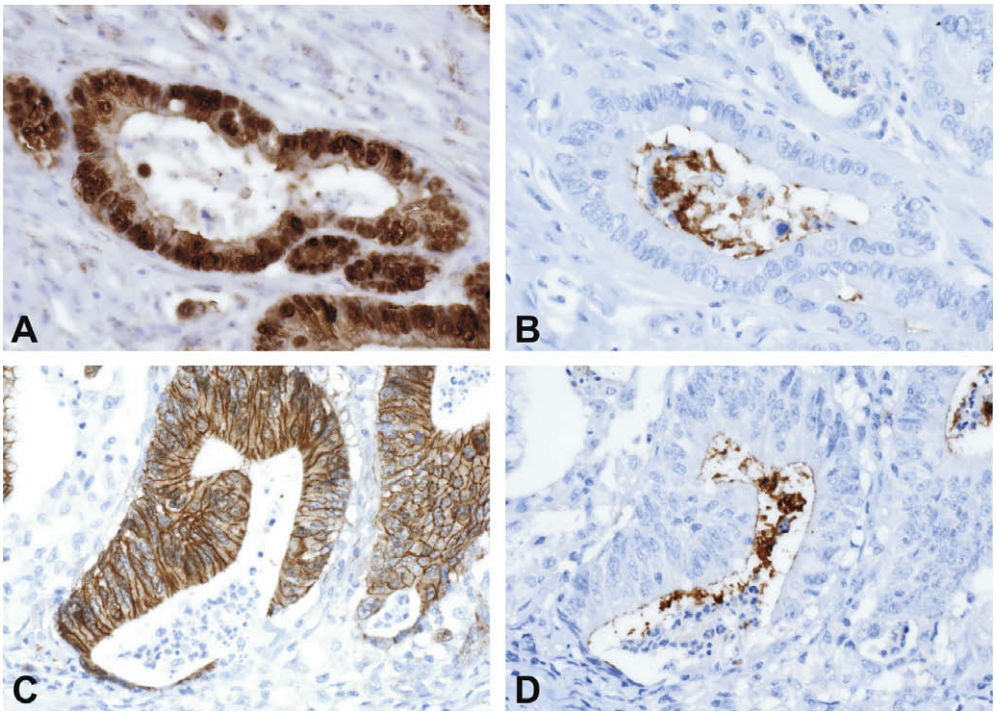

Fig. 2 – The high risk phenotypes of stage IIA colon cancer, as defined by CD133 and β-catenin immunohistochemistry. Tumours that have strong nuclear accumulation of β-catenin in virtually all tumour cells (A) or that do not show nuclear accumulation of β-catenin in any tumour cell (C) are considered as β-catenin unregulated. If these tumours express high levels of CD133 (B and D) they are the designated high risk phenotype of stage IIA colon cancer.

ificity of CD133 for the detection of Co-CSCs.[20] By assuming that tumour buds are less differentiated than glandular tumour cells,[19,21] the latter hypothesis finds additional support, as nuclear β-catenin expression thus marks an average less differentiated tumour cell population than CD133, which is invariably expressed on glandular differentiated tumour cells only.[12] Notably, these assumptions may only be appropriate for colon cancers that express these markers and are based on the hypothesis that nuclear β-catenin marks Co-CSCs which has not yet been demonstrated.

Since both the molecules relate to central biological characteristics of colon cancer, as mentioned above, a link of their expression to the course of the disease could be expected and in agreement with this hypothesis, we previously demon-

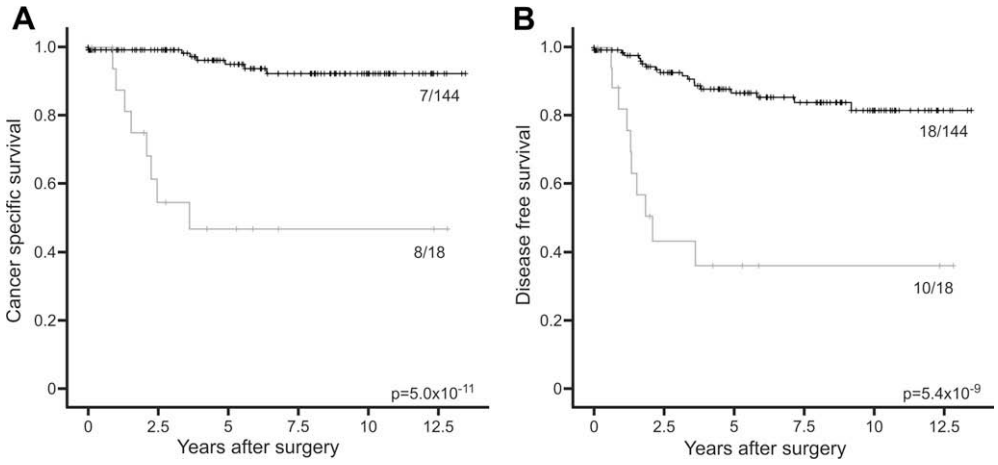

Fig. 3 – Kaplan–Meier survival analysis. Colon cancers characterized by unregulated nuclear β-catenin expression and high CD133 expression (grey curves) associate with very low cancer-specific survival (A) and disease-free survival (B) when compared to tumours without this marker combination (black curves). Both the findings are highly significant as indicated by *p*-values (log-rank test). Ratios on curves indicate the number of events over the number of patients per group.

Table 4 – Multivariate analysis of cancer-specific and disease-free survival.

Variables	Cancer-specific survival			Disease-free survival		
	HR	(95% Confidence interval)	*p*	HR	(95% Confidence interval)	*p*
Age (y)						
⩽70	1			1		
>70	2.4	(0.7–8.0)	0.15	1.3	(0.6–2.9)	0.45
Gender						
Male	1			1		
Female	1.2	(0.4–3.8)	0.77	0.7	(0.3–1.5)	0.32
Tumour side						
Right	1			1		
Left	1.3	(0.5–3.5)	0.64	0.8	(0.4–1.8)	0.65
Unregulated nuclear β-Catenin and CD133 high						
Does not apply	1			1		
Applies	13.4	(4.7–38.2)	4.3×10^{-7}	6.8	(3.1–15.0)	1.9×10^{-6}

strated the usefulness of both markers to predict low patient survival while the observed effect using either marker was overall comparable.[8,12] Here we demonstrate that these markers do not statistically interfere, and that their combined evaluation is highly potent to identify stage IIA colon cancer cases which harbour an exorbitantly increased risk to be fatal and to progress after primary surgical management, while both the findings are highly statistically significant and robust in multivariate analysis. As these cases show a 5-year survival rate of only 47% (±13%), their risk even becomes comparable to that of stage IIIC colon cancer with a known 5-year survival of approximately 44%.[17] Given the controversy about adjuvant treatments in stage II colon cancer cases,[22–24] this marker combination may thus well be used to select for those patients who may benefit from increased clinical attention and more rigorous as well as adjuvant therapeutic approaches.

In conclusion we demonstrate that CD133 and nuclear β-catenin are independent markers in stage IIA colon cancer and that their combined analysis is a powerful tool to identify high risk cases of this malignancy. However, as these results were derived from a single institution, further independent studies will be needed for their confirmation. Additionally, it may be of interest to determine the molecular and functional properties of such high risk colon cancers in comparison with their low risk counterparts.

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There are no financial interests in coherence with this study from any of the authors.

Conflict of interest statement

None declared.

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