

β -catenin expression in relation to genetic instability and prognosis in colorectal cancer

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Abstract. In the carcinogenesis of colorectal cancer (CRC) genetic instability and dysfunction of the Wnt-signalling pathway play important roles. Most Wnt-signalling dysfunctions lead to the nuclear accumulation of β -catenin. The aim of the present study was to investigate whether nuclear accumulation of β -catenin is associated with prognosis and genetic instability. We used immunohistochemistry to study nuclear β -catenin expression in 67 CRCs. The expression was evaluated in the entire tumour section as mean values and in tumour budding at the invasive margin. We compared the results with chromosomal and microsatellite instability (CIN vs. MSI), p53 accumulation, and clinicopathological variables including survival. The nuclear accumulation of β -catenin was significantly associated with abnormal p53 expression and aneuploidy, typically for CIN, whereas no tumour with nuclear β -catenin expression at the invasive margin displayed MSI. The β -catenin expression pattern did not correlate significantly with CRC patient prognosis when including all stages. However, in the clinically most interesting prognostic group, Dukes' stage B patients, high nuclear accumulation of β -catenin was associated with a poor prognosis ($p=0.01$). Our results suggest that nuclear accumulation of β -catenin in CRC is related to CIN and may be of prognostic importance. However, larger studies are needed to verify these findings.

Introduction

Colorectal cancer remains the second leading cause of cancer deaths in Western Countries. In contrast to many other malignancies the survival rate has not changed significantly during the last three decades (1).

Colorectal cancer (CRC) develops along two distinct pathways: chromosomal instability (CIN) or microsatellite instability (MSI) (2-4). Up to 85% of colorectal tumours show chromosomal instability (CIN) which is accompanied by

accumulation of somatic mutations in tumour suppressor genes as *TP53* or the adenomatous polyposis coli (APC) and oncogenes such as β -catenin and *KRAS* (3,5,6). On the other hand, MSI is caused by an impaired capacity of the DNA mismatch repair system and only 10-15% of sporadic colorectal cancer show MSI. These cancers are mostly diploid and show no gross chromosomal changes (7).

In sporadic CRC the overall frequency of APC mutation is around 60%, leading to loss of degradation of its downstream target, β -catenin, the main effector of the Wnt pathway, causing nuclear translocation and constitutive overexpression (8-10).

Normally, APC forms a multimolecular complex with β -catenin, Axin/Conductin and GSK3 β , to target cytoplasmic β -catenin for proteasomal degradation. Since most APC mutations result in truncated proteins lacking most of the binding domains for β -catenin and/or Axin, no efficient degradation can take place, and thus β -catenin will accumulate (9,10). Although about 40% of the MSI colorectal carcinomas contain wild-type APC, they may have other defects in the Wnt pathway causing stabilization of β -catenin and thereby increased activity. For example dominant mutations in the β -catenin gene itself or the tumour suppressive Conductin/Axin-2 gene have been found which all omit efficient β -catenin degradation (6,8).

Aberrant nuclear accumulation of β -catenin leads to association with transcription factors including lymphoid enhancer factor-1 (LEF-1) or T cell factors (TCF) (11,12). These molecules are DNA-binding proteins driving together with β -catenin the transcription of multiple target genes such as *c-myc*, *cyclin D1*, *MMP-7*, *laminin-5 γ 2* implicated in proliferation, cell-cell attachment, and invasion (13-15). Collectively, aberrant Wnt-signalling and nuclear β -catenin accumulation may serve as the underlying basis for invasion, metastasis and poor prognosis (8,15).

The relationship between alterations in the Wnt-signalling pathway and concomitant accumulation of nuclear β -catenin as well as differences in the β -catenin expression between CIN and MSI have not been fully clarified and divergent results have been reported (16). However, in most sporadic CRC, high degree of MSI has been reported to correlate with low frequencies of APC and β -catenin mutations and better prognosis, while CIN is associated with poor outcome and disruption of the Wnt signalling pathway (17,18).

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The aim of the present study was to investigate whether nuclear accumulation of β -catenin is associated with genetic instability and prognosis. Therefore, we studied the expression of β -catenin in the clinically well characterized material of 67 CRC using immunohistochemistry and compared the results with data on DNA content, MSI/MSS status and the p53 expression status.

Materials and methods

Patient samples and tumour data. Seventy-seven patients with primary colorectal cancer were retrospectively included in this study, but 10 were excluded due to no evaluable tissue material [loss of tissue material in the antigen retrieval procedures or lack of β -catenin staining (see below) in non-tumour cells], leaving 67 patients for further analyses. The age of the patients ranged from 37 to 88 years and the male to female ratio was 3:2. Each tumour was classified according to tumour localisation within the bowel (right colon, left colon and rectum), stage, grade and growth pattern at the invasive margin (pushing or infiltrating). All tumours were operated on with a curative intent and were classified as radically removed by both the surgeon and the pathologist. Regarding ploidy, MSI and p53 expression (see below), evaluation of less than 67 tumours was possible, explaining the smaller number in these groups (Tables III and IV). This study was approved by the Ethics Committee at Umeå University, Umeå, Sweden.

Immunohistochemical procedures. CRC specimens had been fixed in formalin and embedded in paraffin wax, according to routine procedures. For this study, 4- μ m sections were prepared, dried, deparaffinised and rehydrated before microwave treatment in citrate buffer (pH 6.0). Antigen visualisation was performed using a semiautomatic staining machine (Ventana ES, Ventana Inc., AZ, USA). A polyclonal β -catenin antibody (C2206; Sigma Chemical Co., St. Louis, MO, USA) was applied at a dilution of 1:750 and the slides were then counterstained with haematoxylin. Similar staining procedures were applied for p53. The antibody used was anti-p53 (Ab-6, Oncogene Research Inc., NY, USA) at a dilution of 1:400.

Evaluation of immunohistochemical staining. Slides were interpreted by one investigator, unaware of the results of the other analyses. All slides were independently reviewed twice and intraobserver disagreements (<10%) were reviewed a third time followed by a conclusive judgment. Evaluation of the antibody was performed using a semiquantitative scale.

The entire tissue sections for each tumour were scanned to estimate the mean value of β -catenin positive nuclei using a three graded scale (-), (<5%); (+), (5-50%); and (++), (>50%). At the invasive margin tumour budding (<10 cells) was evaluated according to the same semiquantitative scale. Concerning p53, the tumours were assigned to one of the two categories: abnormal expression, more than 25% of the cancer cells were stained; and normal expression, 0-25% of the cancer cells were stained (19).

DNA ploidy analysis. DNA-staining was performed according to Vindelof *et al.* (20). A FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, Inc., San Jose, CA)

was used and the DNA index (DI) was calculated. A tumour was denominated diploid (DI=1) when only one peak was detected and aneuploid when two (or more) separate peaks were found.

Microsatellite instability

DNA extraction. Tissue for extracting DNA was available from 62 out of 67 tumours. DNA was extracted from snap-frozen tumour and corresponding normal tissue collected immediately after curative surgical removal of the tissue. A minority of normal control tissue, 6 out of 67, had to be extracted from formalin-fixed and paraffin-embedded material when snap-frozen normal tissue was missing.

From snap-frozen tissue, DNA was extracted using the NucleonTM ST kit (Amersham Life Science, England) according to the manufacturer's instructions. From paraffin-embedded tissue, 5-10 μ m sections (depending on the size of the tissue available) were deparaffinised in xylene and then subjected to centrifugation, followed by incubation of the pellet in alcohol in order to remove any remnant of the xylene solution. The retrieved material was then incubated overnight in buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.50% Tween-20 and proteinase K (0.4 mg/ml) at a temperature of 55°C. In order to inactivate proteinase K, the tumour material was incubated for 8 min at a temperature of 95°C. The samples were analysed for microsatellite status using PCR.

PCR analysis and interpretation. Five different microsatellite markers were used to decide the MSI status of the tumours. They included two markers covering (CA)_n repeats (D2S123 and D17S250) and three poly-A repeat specific markers (BAT25, BAT26 and BAT34C4). According to Boland *et al.* (21), a tumour was scored as high-frequency MSI (MSI-H) if at least two out of five markers showed instability, while those with one out of five markers were scored as low-frequency MSI (MSI-L) (21). Tumours with no markers showing instability were defined as microsatellite stable (MSS). The PCR reaction was performed using standard conditions with one of the primers in each pair being fluorescence-dye labelled. The PCR products were analyzed by use of an ABI 377 Auto-mated Genetic Analyzer with the help of GeneScan software.

Statistics. Differences between groups were examined using the Kruskal-Wallis test. Correlations between ordinals scale variables were examined using the linear-by-linear association test. The Kaplan-Meier method was used to estimate cancer-specific survival and comparison between groups was performed with the log-rank test. A p-value <0.05 was required for statistical significance and two-sided tests were performed for all analyses. Statistical analyses were done using SPSS version 11.0 (SPSS Inc., IL, USA).

Results

Immunohistochemistry. Twenty-two out of 67 tumours (33%) lacked nuclear expression of β -catenin (-). Thirty-two tumours (48%) expressed low levels (+) and 13 (19%) tumours expressed high levels (++) of nuclear β -catenin. At the invasive margin 36 out of 67 tumours (54%) expressed no nuclear

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 Expression of β -catenin (mean values) in relation to clinicopathological variables.

	β -catenin			p-value	Total (n)
	-	+	++		
Gender					
Male	13	17	9	0.73	39
Female	9	15	4		28
Age, years					
≤ 59	4	6	-	0.53	10
60-69	6	5	6		17
70-79	10	12	6		28
>80	2	9	1		12
Localization					
Right	12	12	2	0.10	26
Left	5	9	7		21
Rectum	5	11	4		20
Stage					
Dukes' A	-	7	-	0.34	7
Dukes' B	12	16	9		37
Dukes' C	10	9	4		23
Grade					
Well	-	1	-	0.30	1
Moderate	18	29	12		59
Poor	2	1			3
Tumour type					
Mucinous	10	6	1	0.01	17
Non-mucinous	12	26	12		50
Growth pattern					
Pushing	13	21	4	0.23	38
Infiltrating	9	11	9		29

β -catenin (-) in areas showing tumour budding, 10 (15%) expressed low (+), and 21 (31%) expressed high levels (++) of β -catenin. A significant correlation was found between the mean nuclear β -catenin expression and nuclear expression in tumour budding at the invasive margin ($p < 0.001$).

Clinicopathological parameters. The mean β -catenin nuclei expression in tumours did not correlate with gender, age, stage, grade, localisation or growth pattern but with tumour type (0.01) as detailed in Table I. Only 1 out of 17 mucinous tumours expressed high levels of nuclear β -catenin compared with 12 out of 50 of non-mucinous tumours. Nuclear β -catenin expression in tumour budding at the invasive margin did correlate with tumour type ($p = 0.03$) and growth pattern ($p = 0.020$), but not with other investigated clinicopathological parameters (Table II).

Genetic instability-ploidy and MSI. The mean values of nuclear β -catenin expression showed a significant correlation

Table II. Expression of β -catenin in tumour budding at the invasive margin in relation to clinicopathological variables.

	β -catenin			p-value	Total (n)
	-	+	++		
Gender					
Male	19	5	15	0.19	39
Female	17	5	6		28
Age, years					
≤ 59	7	-	3	0.82	10
60-69	7	2	8		17
70-79	16	5	7		28
>80	6	3	3		12
Localisation					
Right	16	6	4	0.21	26
Left	9	3	9		21
Rectum	11	1	8		20
Stage					
Dukes' A	5	1	1	0.44	7
Dukes' B	19	6	12		37
Dukes' C	12	3	8		23
Grade					
Well	-	1	-	0.15	1
Intermediate	31	8	20		59
Poor	3	-	-		3
Tumour type					
Mucinous	13	2	2	0.025	17
Non-mucinous	23	8	14		35
Growth pattern					
Pushing	25	5	8	0.020	25
Infiltrating	11	5	13		29

with ploidy ($p = 0.02$). All tumours expressing high amounts (++) of nuclear β -catenin were aneuploid (Table III). β -catenin expression in tumour budding at the invasive margin were also correlated with ploidy ($p = 0.02$), where 17 tumours out of 18 (94%) with high mean β -catenin levels were aneuploid (Tables III and IV).

MSS status was found in 56 out of 62 tumours while 6 tumours were MSI-H. No tumour was classified as MSI-L. MSI was significantly associated with low mean levels of nuclear β -catenin ($p = 0.02$) and with low expression in tumour budding ($p = 0.03$). Five out of 6 (83%) MSI-H tumours were β -catenin negative, while no such β -catenin expression pattern was found in the other group of tumours (Tables II and III).

p53. Accumulation of p53 was significantly associated with nuclear β -catenin expression ($p = 0.003$) and with nuclear β -catenin in tumour budding ($p = 0.01$). Interestingly abnormal p53 accumulation was found in 13 out of 16 (81%) tumours with high mean value of β -catenin (++) and in 16 out of 21

Table III. Expression of β -catenin (mean values) in relation to ploidy, microsatellite instability status and p53 status.

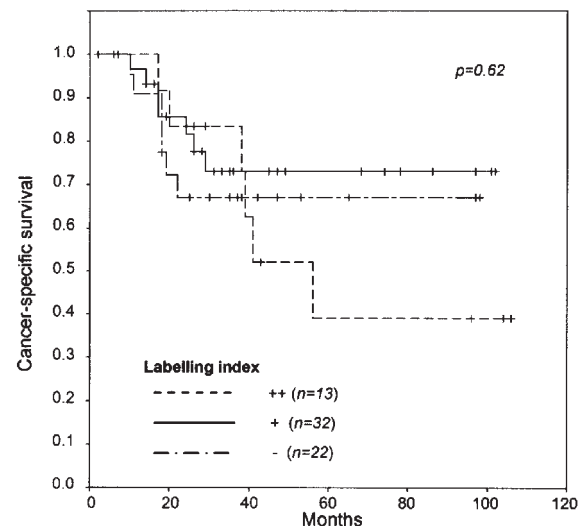
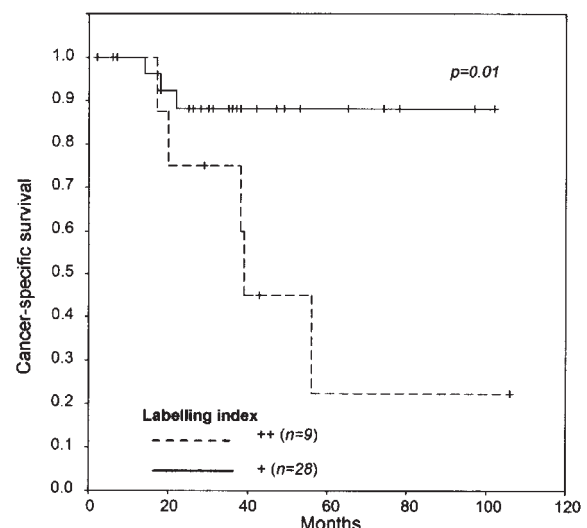
	β -catenin			p-value	Total (n)
	-	+	++		
Ploidy					
Aneuploid	10	17	11	0.02	38
Diploid	8	8	-		16
MSI					
None	16	27	13	0.02	56
High	5	1	-		6
p53					
$\leq 25\%$	16	11	3	0.003	30
$> 25\%$	6	20	10		36

Table IV. Expression of β -catenin in tumour budding at the invasive margin in relation to ploidy, microsatellite instability status and p53 status.

	β -catenin			p-value	Total (n)
	-	+	++		
Ploidy					
Aneuploid	17	4	17	0.02	38
Diploid	11	4	1		16
MSS/MSI					
MSS	28	7	21	0.03	56
MSI-L	-	-	-		6
MSI-H	6	-	-		
p53					
Normal	22	3	5	0.004	30
Accumulation	13	7	16		36

(76%) tumours with high expression in tumour budding at the invasive margin (Tables II and IV).


Survival analyses. Mean nuclear β -catenin expression levels did not correlate with patient survival when comparing all Dukes' stages ($p=0.62$) with all three expression levels of β -catenin (Fig. 1), nor when sub-grouping the β -catenin expression into the two sub-groups high vs. negative as well as low level ($p=0.44$). However, when separately analysing the clinically most important prognostic group, Dukes' stage B patients, nuclear β -catenin expression (high vs. low and negative) turned out to be significantly related to patient prognosis ($p=0.04$). Tumours with high nuclear β -catenin expression had a poorer prognosis vs. tumours with low or negative expression for β -catenin combined ($p=0.01$; Fig. 2).

Figure 1. Kaplan-Meier cancer-specific survival curves for 67 CRC patients, grouped according to their mean values of nuclear β -catenin staining patterns -, + and ++.Figure 2. Kaplan-Meier cancer-specific survival curves for 37 CRC patients with Dukes' B tumours, grouped according to their mean values of nuclear β -catenin staining patterns -/+ vs. ++.

Although not statistically significant, the overall prognostic trend was similar studying β -catenin expression in tumour budding at the invasive margin. Including all Dukes' stages the three expression levels of β -catenin were not significantly associated with prognosis ($p=0.28$), neither when analyzing high β -catenin expression vs. negative and low levels combined ($p=0.11$). In the group of Dukes' B patients a p-value of 0.092 was recorded (high vs. low and negative groups combined).

Discussion

Dysfunction in the Wnt-signalling pathway, and thereby nuclear accumulation of β -catenin, is thought to be an important step in the carcinogenesis of colorectal cancer and other malignant tumours (6). β -catenin expression due to APC-mutation and development of CIN are accepted elements in

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In the present study we used immunohistochemical staining to study nuclear β -catenin expression in tumours already characterized with respect to microsatellite and chromosomal instability as well as p53 accumulation. In order to optimise technical conditions and reproducibility, we used a semi-automatic immunohistochemical staining machine for all staining procedures.

Our results show significant association between high expression of nuclear β -catenin, aneuploidy and abnormal accumulation of p53, which is in line with previous reports (2,16). Furthermore, in the clinically most interesting prognostic group, Dukes' B tumours, we found that high nuclear β -catenin expression is significantly associated with a poor prognosis. Similar findings have been reported previously (23-25), whereas Roca *et al* have reported contradictory observations (26).

There is also evidence that the amount of tumour budding at the invasive margin is associated with increased risk of death in CRC (27,28) and budding probably mirrors tumour behaviour associated with invasiveness (29,30). β -catenin has been shown to be expressed mostly at the invasive margin in most CRCs and seems to correlate with the amount of budding (17,31). In analogy, high nuclear accumulation of β -catenin in these cell buds might give the tumour cells the ability to detach, migrate and disseminate (8). In the present patient material we have also evaluated the value of β -catenin expression levels at the invasive margin. The prognostic results are in agreement with mean tumour expression level of β -catenin, although not statistically significant. Thus the expression levels of β -catenin may be an insufficient marker for the biological behaviour of colorectal cancers. It has been shown that inactive or phosphorylated β -catenin correlates with a better prognosis (32). Consequently the activation status of β -catenin has the potential of adding more prognostic information and should be further studied.

In addition, tumours with p53 mutations as well as aneuploid tumours are known to be associated with a slightly poorer prognosis (33). In contrast, MSI tumours were significantly related with lack of nuclear β -catenin expression and normal p53 expression (16). Lack of nuclear β -catenin expression tended ($p=0.10$) to be correlated to right sided tumour localisation, which is in agreement with several studies and known clinicopathological features. Another known clinical feature is that MSI CRCs tend to be late stage at presentation, but to have a better prognosis than CIN CRCs (34). The results in our survival analyses is in accordance with such a view, though the number of MSI cases in the present study is too small to establish conclusive results. However, the important question remains, if the lack of nuclear β -catenin expression renders MSI tumours less aggressive or whether other alterations in the tumorigenesis inhibit promotion of late stage lesions. Absence of nuclear β -catenin expression in MSI CRCs is consistent with previous studies (6). These tumours have been shown to have more often other mutations in the Wnt-signalling pathway than in the APC-gene, for example in exon 3 of β -catenin gene or Axin (35,36).

In conclusion, we have shown that CIN CRCs are frequently associated with high nuclear β -catenin expression, DNA-aneuploidy and abnormal p53 expression, whereas low/negative nuclear β -catenin expression, normal p53 expression seem to occur in MSI CRCs. In the survival analyses a significant poor prognosis was recorded for Dukes' B tumours with high nuclear expression of β -catenin. The clinical use of β -catenin as a prognostic factor needs to be investigated in larger patient cohorts.

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