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RESEARCH

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?

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Abstract β-catenin and E cadherin are both membrane-associated proteins which are essential regulators and providers of cellular adhesion. In the metastatic cascade of malignant tumours, detachment of tumour cells from each other is a very important step. It has been shown in several tumour types, that reduced expression of these proteins is important. The aim of our study was to clarify the expression profile of these proteins, and correlate the findings with the metastasizing potential of early stage colon and rectal cancers. Formalin fixed and paraffin embedded samples from 79 Dukes B2 stage colorectal cancer were examined using a tissue microarray approach. The expression of β-catenin and E-cadherin proteins was determined immunohistochemically. Our findings indicated that there is a tendency for metastatic spread in cases when membranous expression of β-catenin is lost (p=0.062). Similarly metastases in negative cases developed more rapidly, than in positive ones (p=0.05). Survival rate was worse in the negative cases. The risk of metastasis in rectal cancer was significantly higher in the β-catenin membranously negative than positive groups (p=0.024) and in case of β-catenin nuclear expression the risk was also higher (p=0.047). Reduced E-cadherin expression also correlated with development of metastatic disease, but this association was statistically not significant. The immunohistochemical analysis of 79 cases shows that in Dukes B2 stage colorectal tumours clarification of β-catenin and E-cadherin expression patterns is reliable for predicting the metastatic potential of early stage rectal cancer and hence the method may have relevant implications in the therapeutic management of these cancers.

Keywords β-catenin and E cadherin · Colorectal cancer · TMA · Metastasis

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Introduction

Colorectal cancer is estimated to be the second leading cause of cancer death worldwide [1]. Currently, selected therapy and identification of prognostic groups are based on classical clinicopathologic data, i. e., tumour grade and stage. However, Dukes B2 stage patients exhibit varying survival outcomes and there are several contradictions *vis-á-vis* post-operative treatment. Several molecular factors have been and are being considered currently to help selection of optimal treatment [2–4].

The role of β-catenin and E-cadherin proteins is well known in cellular adhesion [5]. E-cadherin, a member of the cell adhesion protein family, is a 120 kDa molecular weight transmembrane glycoprotein encoded by the CDH1 gene which is located on chromosome 16q22. The extracellular domain interacts in a calcium dependent mode with the E-cadherin molecules of neighbouring cells. Intracellularly α, β and γ catenins form complexes with E-cadherin and mediate linkage to the actin cytoskeleton. The cadherin-catenin complex is found at sites of zonula adherentes. The p120 protein also interacts with the intracellular domain of E-cadherin; however, the role of this protein is not defined yet. Tumour cell dissociation is an important step in the metastatic cascade. There is ample evidence to prove that the diminished expression of catenins and cadherins is critical in the metastatic cascade. In addition to its role in cellular adhesion β-catenin protein is also an important component of the so called WNT signalling pathway [6, 7]. The non-degraded β-catenin can translocate to the nucleus, where it acts as a transcriptional activator in conjuction with the TcF/LEF transcription molecule family. Thus it activates the transcription of c-myc, cyclin D1, matrix metalloprotease 7 and CD44 genes [8]. The APC protein is a negative regulator in the WNT signalling pathway. In physiological conditions, in non-stimulated cells the APC/GSK3b/axin destruction complex binds the cytoplasmic β-catenin which becomes phosphorylated and eventually is degraded via the APC dependent ubiquitin-proteosome pathway [9]. Mutations of the APC gene result in nuclear accumulation of β-catenin, since these mutations allow β-catenin to escape from the proteosomal degradation. Subsequently, the nuclear translocation of β-catenin leads to constitutive expression of the c-myc and cyclin D1 genes [8]. According to previous studies, the nuclear accumulation of β-catenin and the reduced membranous expression of E-cadherin play a key role in colorectal tumour progression [10–12].

The aim of our study was to investigate the prognostic significance of the expression of the β-catenin and E-cadherin in Dukes B2 stage colorectal cancer immunohistochemically. We intended to clarify whether and how altered expression patterns of these proteins do influence the metastatic potential of a given tumour.

This study population is Eastern-European (i.e., Hungarian) and heretofore no such population has been analyzed and reported on with regard to catenin-cadherin expression. All patients had the same early stage (pT3) disease, and received the same standardised adjuvant chemotherapy. Our major goal was to determine if there is a difference in β-catenin and E-cadherin expression between colon and rectal tumours and if so, does that difference have any functional/clinical significance. This seemed to be relevant since previous similar studies did not separate these two, anatomically different groups.

There is convincing literary evidence about the adequacy of the sc. TMA method for investigating various prognostic and predictive factors in many tumour types [13, 14]. Hence we opted for this methodology.

Materials and Methods

Patients and Specimens

Seventy-nine colorectal cancer cases were collected from tissue archives of the Department of Pathology, University of Debrecen, Medical & Health Sciences Centre [MHSC], covering the time period of 5 years (1996–2001). The fundamental selection criterion required that each tumour had to belong to Dukes B2 stage (pT3N0 TNM stage). All cases were selected using the original histopathology reports. Clinical data were gathered from the MHSC database on patients' clinical records. The patients' mean age was 65.8 years (range: 35–85 years). The group comprised of 39 women and 40 men. Mean follow-up time was 52 months. All the patients with colon cancer received standard postoperative 5-fluorouracil (5FU) adjuvant chemotherapy, while patients with rectal tumour received additional radiotherapy as well. None of the patients had preoperative treatment. If metastatic disease was diagnosed the patients received further chemotherapy. Three patients died within 6 weeks following surgery, mainly of septic or other complications. Other six patients had no follow-up data. Data from these nine patients were not included in the statistical analysis.

We divided the tumors into two groups according to the tumours' anatomical location: the colonic group included tumours located anywhere from between the beginning of the cecum to the sigmoid-rectal border. Tumors affecting the large bowel's most distal part comprised the "rectal tumour group". The clinicopathologic data are shown in Table 1.

Tissue Microarray (TMA) Construction

Tumorous samples were formalin fixed and paraffin embedded according to the Departmental routine protocol. All cases were reviewed for confirmation of diagnosis. Representative areas of paraffin blocks were identified on corresponding HE stained slides. From the representative areas 1 mm thick core biopsies were retrieved using a TMA master (3DHistech Budapest, Hungary) and positioned in a 10×5 recipient paraffin array block. From all tumours 3-3 representative cores were obtained. A total of 237 cores were built in TMA blocks from the 79 tumours. In 12 cases,

Table 1 Clinicopathological data of the patients

Number of patients	79
Age	65.8 (35–85)
Sex	
Male	40
Female	39
Tumour localisation	
Right sided colon	19 (24.1%)
Left sided colon	29 (36.7%)
Rectum	31 (39.2%)
Grade	
Well and moderately differentiated (G1-G2)	64 (81%)
Poorly differentiated (G3)	15 (19%)
Mean follow up time	52 months
5 year OS	59%
3 year DFS	64%

one or both immunohistochemical reactions failed in two or all three cores from the same tumour. These cores were either lost or severely damaged. Some were nonrepresentative due to necrosis or dominance of nonneoplastic tissue, in other cases the IHC reaction(s) were unequivocal or technically inadequate. From these tumour blocks new cores were punched from other representative areas and a new TMA block was built.

Immunohistochemistry (IHC)

Five micron sections from TMA blocks were used for immunohistochemistry (IHC). The slides were deparaffinated and rehydrated with xylol and graded alcohol. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 for 30 min. Antigen retrieval was accomplished in 10 mM citrate buffer (pH 6.0) in a microwave at 600 W for 5 min. Nonspecific binding was blocked with 1% BSA for 10 min. Subsequently, the slides were incubated with the primary antibody: β-catenin (Transduction Laboratories, Lexington KY) clone C19220, 1:100 and E-cadherin (Transduction Laboratories, Lexington KY) clone 36, 1:100 for 1 h, at 37°C. The slides were incubated with a biotinylated rabbit antimouse immunoglobulin as second antibody and subsequently treated with streptavidin peroxidase conjugate for 30 min at 37C temperature and DAB was used as chromogen substrate. After counterstaining with haematoxylin, the slides were dehydrated and mounted. Slides without the primary antibody were used as negative controls.

IHC Scoring

The membranous and nuclear β-catenin expression (IHC "decoration") was evaluated separately. In case of E-cadherin, only the membranous staining was considered as positive. Normal colonic mucosa exhibited strong membranous staining with both β-catenin and E-cadherin. For both membranous and nuclear positivity of β-catenin those cases in which <10% stained cells were observed the result was classified as negative. If the decorated cells' number fell between 11% and 50% staining was scored as: 1+. In those instances when positive staining occurred in cells between 51% and 100% the score 2+ was given. For E-cadherin if the number of decorated cells was <75% the reaction was classified as "negative". When the reactive cells' number fell between 76% and 90% the score was 1+; staining of 91–100% of the cells scored 2+. Only those cases with score 1+ and 2+ were considered as positive for the purpose of statistical data analysis.

Statistical Analysis

The clinical outcome could fit into the 3-year disease free survival (DFS) category and the 5-year overall survival (OS) category. Associations between categorical factors were studied with the Fisher exact test or the chi2 test as appropriate. The rate of recurrence or death was estimated using the Kaplan-Meier method. The multivariate logistic

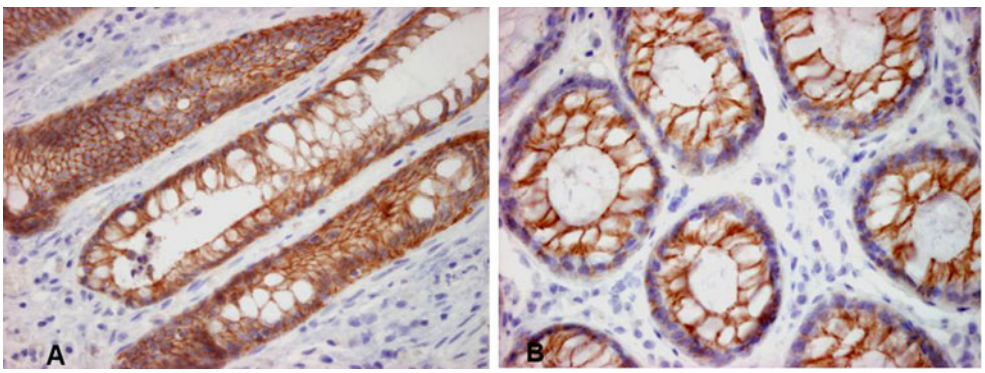

Fig. 1 **a** Membranous β-catenin staining of normal mucosa. **b** Membranous E-cadherin staining of normal mucosa

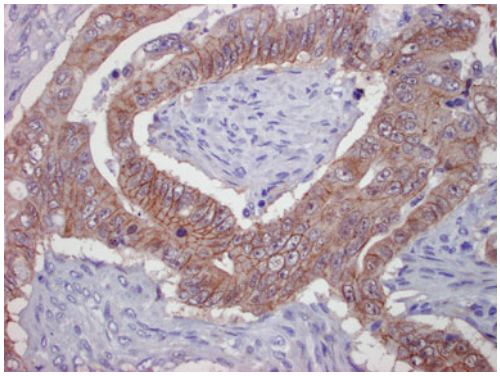

Fig. 2 Membranous β-catenin staining of colon cancer

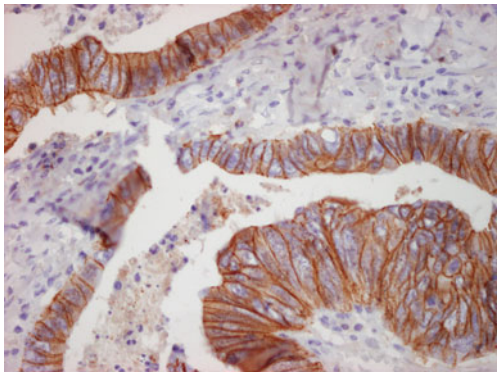

Fig. 4 E-cadherin staining of colon cancer

regression analysis was applied to detect independent metastasis development predictors. Statistical analysis was performed using the SPSS 15.0 statistical package (Chicago, IL). $P<0.05$ was considered as significant.

Results

Seventy-nine archival cases of Dukes B2 stage (pT3N0 TNM stage) colorectal cancer were analyzed. The 5-year OS rate was 59% and the 3-year DFS rate was 64%. The normal colonic mucosa exhibited strong membranous staining with both β-catenin and E-cadherin (Fig. 1.). Membranous expression of β-catenin was observed in 52 cases while 27 cases were negative (Fig. 2.). Nuclear expression of β-catenin was detectable in 36 cases while 43 cases were negative (Fig. 3.). Membranous staining with E-cadherin was observed in 46 cases, 33 cases were negative (Fig. 4.). The expression profiles for both proteins are shown in Table 2.

In the whole study population all those cases with tumour cells showing membranous expression of β-catenin had a better OS than those without it, however, this difference was not statistically significant, $p=0.280$ (Fig. 5.). In case of DFS there was no difference, $p=0.442$. The nuclear β-catenin expression or E-cadherin expression did not stratify patients with regard to OS or DFS. However, we observed that if either the β-catenin or E-cadherin showed membranous staining, the OS was better, compared to cases which were negative for both reactions, however this result did not reach statistical significance ($p=0.492$).

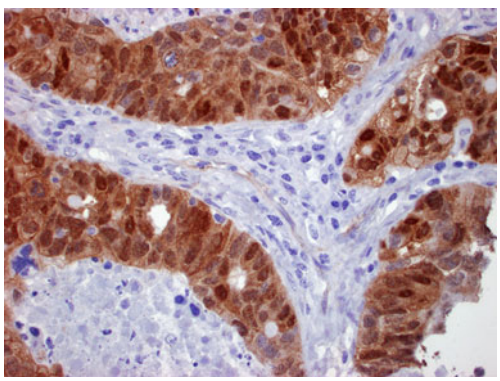

Fig. 3 Nuclear β-catenin staining of colon cancer

During the follow-up period 23 patients developed metastatic disease. The risk of metastases was higher in the β-catenin membranously negative group than in the positive group. In light of the statistical analysis ($p=0.062$, Table 3) this observation is best interpreted as a tendency which needs further corroboration. When β-catenin showed nuclear expression, the risk of metastases was higher and this result was statistically significant, $p=0.022$. No relationship between the expression of E-cadherin and development of metastatic disease could be identified. If neither β-catenin nor E-cadherin showed membranous staining, the metastatic disease was significantly more frequent, compared to cases in which both proteins exhibited membranous expression, $p=0.047$.

Forty-eight patients had tumour in the right- or left side of the colon. The patients' mean age was 67.6 (range: 48–83 years). Twenty-three women and 25 men were in this group of patients. Thirty-nine tumours were well or moderately differentiated while nine cases were poorly differentiated. The clinical outcome was (statistically) independent from β-catenin and E-cadherin expression patterns.

Twelve patients had metastatic disease in this group. There was no difference in the risk of metastases comparing

Table 2 β-catenin and E-cadherin staining and OS and DFS

	Membranous β-catenin staining	Nuclear β-catenin staining	E-cadherin staining	5 year OS	3 year DFS
All patients	65.8%	45.6%	58.2%	59%	64%
Colon group	72.3%	42.6%	61.7%	69%	73%
Rectum group	58.1%	51.6%	54.8%	42%	46%

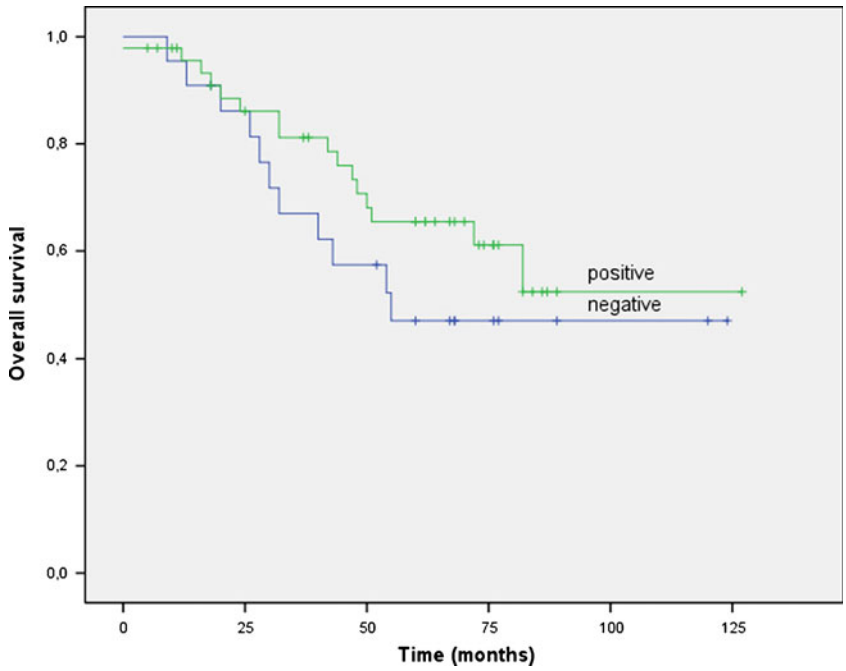

Fig. 5 Membranous β-catenin staining and OS, all patients

the groups with different protein expression patterns (Table 4.).

Thirty-one patients had rectal tumour. The patients' mean age was 63.1 (range: 35–85 year). Sixteen women and 15 men were in this group of patients. Twenty-five tumours were well or moderately differentiated while 6 cases were poorly differentiated. Membranous expression of β-catenin was observed in 18 cases (58.1%), 13 cases (41.9%) were negative. Nuclear expression of β-catenin was detectable in 16 cases (51.6%), 15 cases (48.4%) were negative (Fig. 3.). Membranous staining with E-cadherin was observed in 17 cases (54.8%), 14 cases (45.2%) were negative. Five-year OS was 42%, mean OS was 44.3 months. Three year DFS was 46%, mean DFS was 29.1 months. There was a trend towards improved DFS and OS in patients with membranous β-catenin expression, but it did not reach statistical significance, [p=0.087 and 0.085, respectively] (Figs. 6 and 7). There was no difference in OS and DFS either between nuclear β-catenin positive and negative cases, or between E-cadherin positive and negative patients.

In this group 11 patients had metastatic disease. The risk of metastases was significantly higher in cases which were β-catenin membranously negative than in those which were membranously positive (p=0.024). When β-catenin showed nuclear expression, the risk of metastases was also significantly higher (p=0.047). No relational pattern could be identified between the expression of E-cadherin and development of metastatic disease (Table 5.).

Multivariate logistic regression analysis was carried out with inclusion of patients' age, tumour grade and localisation, E-cadherin/β-catenin membranous and nuclear expression as statistical variables in order to identify those parameters which could be important predictors for assessing the risk of metastatic disease. Multivariate analysis did not help to detect any additional significant correlations between these variables compared to the results provided by univariate analysis (Table 6).

Finally, we also established that in those patients with metastatic disease (23 cases) there was a significantly better DFS in those with positive membranous staining for β-catenin than in the negative cases [36.58 months vs 14.55 months, respectively] (p=0.011). The OS was also better in the group of positive membranous staining for β-catenin (p=0.032). There was a trend towards improved DFS and OS in patients with membranous E-cadherin

Table 3 The development of metastatic disease and β-catenin and E-cadherin staining, all patients

	Metastatic disease	Non metastatic disease	*P* value
Membranous β-catenin staining	52.2%	74.4%	0.062
Nuclear β-catenin staining	65.2%	36.2%	0.022*
E-cadherin staining	52.2%	59.6%	0.369
E-cadherin and β-catenin membranous staining	26.1%	51%	0.047*.

Statistical significance (*) $p<0.05$

Table 4 The development of metastatic disease and β-catenin and E-cadherin staining in the colon group

	Metastatic disease	Non metastatic disease	*P* value
Membranous β-catenin staining	66.6%	71.8%	0.504
Nuclear β-catenin staining	58.3%	37.5%	0.184
E-cadherin staining	58.3%	59.4%	0.607

expression, but it was not significant statistically (29.5 months vs 22.7 months respectively; p=0.851). The loss of membranous β-catenin staining was associated with faster appearance of metastases (27.16 months vs 13.18 months, respectively; p=0.05). The loss of membranous staining of E-cadherin also lead to faster appearance of metastases, but this result was not statistically significant (24 months vs 16.63 months, respectively; p=0.16).

Discussion

The last two decades have witnessed a rapid accumulation of studies aiming at the clarification of the exact role of β-catenin and E-cadherin expression in colorectal carcinogenesis. It is now believed that there are two pathogenetically distinct pathways for the development of colorectal cancer [15]. Most cases are characterised by chromosomal instability, the rest are featured by mutation(s) of the mismatch repair genes and microsatellite instability. In the first group, there is an accumulation of well known gene defects during carcinogenesis. One of the most important defects affects the APC-β-catenin system and this defect seems to occur in the early stage of carcinogenesis. The recently published results are neither uniform nor are they consistent about the prognostic and predictive value of these proteins. According to most reports, in case of membranous staining of E-cadherin and β-catenin, better survival can be expected and the occurrence of metastatic disease is less common [16–20]. The nuclear expression of β-catenin seems to have potentially adverse prognostic significance in colorectal cancer [21].

From our results the following conclusions may be drawn. Considering the total patient-population en masse, the OS seems to be worse when the membranous expression of the β-catenin is lost than in those cases where it is retained. This difference, however, was not statistically significant. In the whole population, the development of metastatic disease is more frequent, when the β-catenin membranous staining is lost. In case of nuclear β-catenin staining, metastatic disease is also more frequent, this correlation is significant.

Furthermore, by dividing the patients into two groups according to the tumour localisation, additional remarkable differences become obvious.

In the group of patients with colonic tumours, there is no association between alterations of the expression of β-catenin and E-cadherin on the one hand and OS or DFS on the other. In these patients no difference in the risk of metastases is detectable when comparing groups with different β-catenin and E-cadherin expression patterns.

It is noteworthy that recent publications have emphasized a worse OS and DFS for patients with rectal cancer.

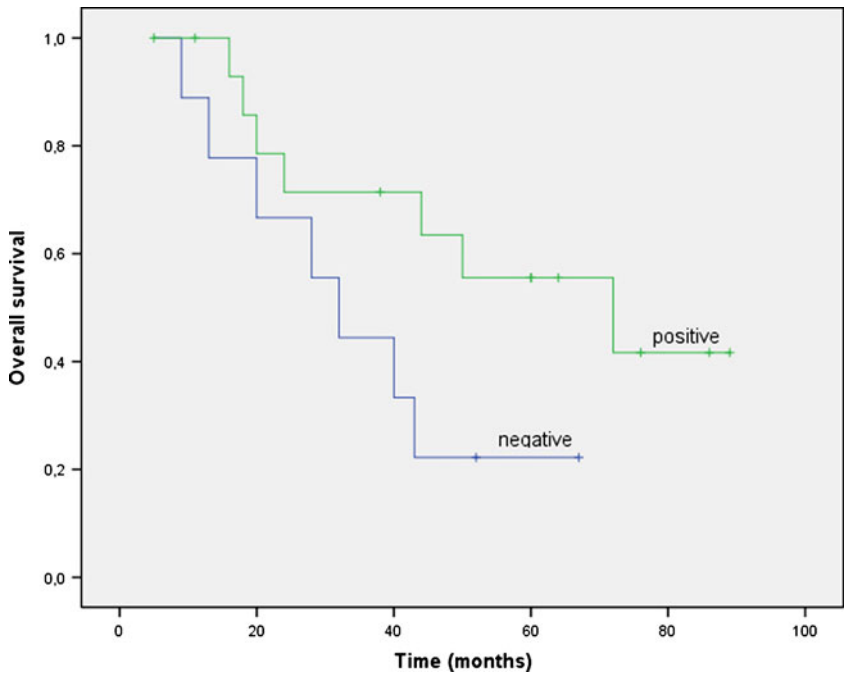

Fig. 6 Membranous β-catenin staining and OS in rectum group

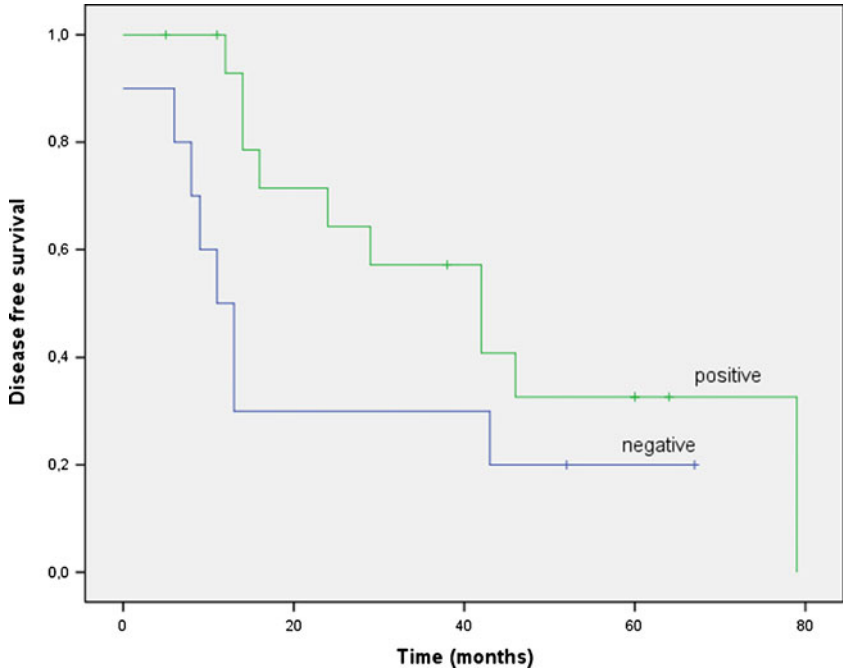

Fig. 7 Membranous β-catenin staining and DFS in rectum group

Hence we believe that our findings which confirm that the loss of β-catenin and E-cadherin expression is more common in this group does have clinical and biological significance. Negativity of membranous staining of β-catenin indicates worse OS. The difference in our study although statistically is not significant, clearly indicates an obvious susceptibility. Statistical significance may be reached if additional cases can be recruited. The risk of metastatic disease is significantly higher in the β-catenin membranously negative cases than in the β-catenin membranously positive rectal cancers. Our results implicate that when β-catenin shows nuclear translocation, the risk of metastases is also significantly higher and this strong association likewise has clinical relevance.

We could also demonstrate that if neither β-catenin nor E-cadherin show membranous staining, metastatic disease is significantly more frequent, compared to the situation when both proteins exhibit membranous expression.

Finally, we could also establish that in the group of patients with metastatic disease the loss of membranous staining of β-catenin leads to faster appearance of metastases and DFS is also worse. The loss of membranous staining of E-cadherin also seems to be associated with rapidly evolving metastatic disease. Statistical significance again may be arrived at by increasing the sample-number.

It is important to note that we could demonstrate reliably strong association between IHC results and clinical outcome only in the group of patients with rectal cancer. For the rest of the patients only tendencies seem to surface so far.

There are some notable discrepancies between our findings and various reports already available. These discrepancies might have a host of possible explanations.

Several previous studies investigated clinicopathologically inhomogeneous patient cohorts, including both early or late stage tumors and a mixture of rectal- and colonic cancers. Other authors strictly selected the cases and investigated only stage I or II patients [22–24]. Moreover

Table 5 The development of metastatic disease and β-catenin and E-cadherin staining in the rectum group

	Metastatic disease	Non metastatic disease	*P* value
Membranous β-catenin staining	36.4%	80%	0.024*
Nuclear β-catenin staining	72.7%	33.3%	0.047*
E-cadherin staining	45.4%	60%	0.368

Statistical significance (*) $p<0.05$

Table 6 Multivariate analysis of the risks of metastasis, all patients

	Sig.	Exp (B)	95.0% C.I. for Exp(B)	
			Lower	Upper
Age	0,661	0,988	0,938	1,042
Grade	0,249	2,235	0,570	8,774
E-cadherin staining	0,970	1.022	0,332	3,140
Membranous β-catenin stainin	0,436	,581	0,148	2,276
Nuclear β-catenin staining	0,222	2,256	0,611	8,321
Localisation	0,200	2,070	0,681	6,293
Constant	0,920	1,219		

one group studied only early stage rectal cancer patients [25]. In our study we selected only early stage patients (i.e., Dukes B2) who had not received any preoperative treatment. Thus we consider our patient-cohort to be clinicopathologically rather homogeneous.

We used the increasingly popular TMA method in our study. This method was first described by Battifora [13], then was improved by Koononen and co-workers [26, 27]. Recently a number of studies have demonstrated the usefulness of this technique as a high throughput method in tumour proteomic investigations [28, 29]. The TMA method was also used by many working groups in the investigation of colorectal cancer [14, 24, 25, 30]. Some reports were published which studied the β-catenin- E-cadherin complex this way [7, 31]. Some papers have shown the validity of this method [32]. One of the problems with the TMA method is that only a small part of the tumours can be examined [12, 33]. We tried to avoid this problem by the use of multiple cores from different representative areas of the same tumour blocks.

In the previous reports we found no consistent scoring system for the evaluation of the expression of β-catenin and E-cadherin. Lack of standardisation of what constitutes "positive" or "negative", and the different cut-off value may affect concordance between the results of various studies.

Next to the above described methodical and patient selection differences within the complexity of colorectal carcinogenesis can also explain the incoherent results. According to different reports β-catenin and E-cadherin both play a crucial role in cellular adhesion [5]. It is well known that highly aggressive tumours, like signet ring gastric cancer or lobular breast cancer usually do not exhibit membranous E-cadherin expression [34, 35]. In these cases the high metastatic potential is mostly due to the loss of expression of E-cadherin. On the other hand tumour cells which transgress vessel-walls and migrate via circulation to distant sites also need adhesion molecules for attachment. Indeed, the absence of adhesion molecules is an advantage at the detachment of tumour cells, while, in contrast, it is a drawback at homing.

Nuclear expression of β-catenin is also an important event in colorectal carcinogenesis. The abnormality of the β-catenin- APC system mostly can be detected and generally so in early stage of tumorigenesis in the group of colorectal tumours with chromosomal instability.

Certain studies provided evidence that there are important genetic differences between the tumours of the right or left colonic side vs the rectum [15, 36]. We have observed correlations between the expression patterns of β-catenin/E-cadherin complex and clinical outcomes in the group of patients with rectal cancer.

In conclusion our results repeatedly confirm the critical role of β-catenin and E-cadherin complex in colorectal carcinogenesis. So far no specific targeted therapy acting via these molecules is at hand. Furthermore, in case of node-negative colorectal cancers, the necessity of adjuvant treatment is not unequivocal. In Dukes B2 stage rectal cancers the loss of membranous expression of β-catenin is a predictor of the development of distant metastasis. Hence we suggest, that in node negative patients with loss of β-catenin membrane staining, adjuvant chemotherapy might be considered necessary since the risk of metastatic spread is increased.

Our results emphatically call for a possibly multi-centred, large patient-pool based study. This requires a clinicopathologically homogenous patient population, well controlled IHC sampling and technique that can be standardized. Only this approach can further clarify the exact prognostic and predictive values of β-catenin and E-cadherin expression in colorectal cancer.

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Disclosure of conflict of interest The authors declare that they have no conflict of interest

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