

Prognostic Significance of Wnt-1, β -catenin and E-cadherin Expression in Advanced Colorectal Carcinoma

Aleksandra Stanczak · Rafal Stec · Lubomir Bodnar · Wojciech Olszewski · Marzena Cichowicz · Wojciech Kozlowski · Cezary Szczylik · Tadeusz Pietrucha · Maciej Wieczorek · Monika Lamparska-Przybysz

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Abstract Wnt/ β -catenin pathway plays an important role in initiation and progression of colorectal oncogenesis. The aim of this study was to determine expression and localization of E-cadherin, β -catenin and Wnt-1 proteins in colorectal tumors. Expression of β -catenin, E-cadherin and Wnt-1 was determined by immunohistochemistry on advanced colorectal cancers. Abnormal expression of E-cadherin, β -catenin, Wnt-1 was observed. Additionally, we revealed correlations between levels of studied proteins and histoclinical data. In multivariate analysis nuclear β -

catenin, higher carcinoembryonic antigen serum level before treatment, female sex and tumor localized in colon or rectum were independent unfavorable prognostic factors. These findings support the hypothesis that Wnt/ β -catenin pathway plays an important role in advanced colorectal carcinoma.

Keywords β -catenin · E-cadherin · Wnt-1 · Wnt signaling pathway · Advanced colorectal carcinoma · Prognostic factor

Abbreviations

APC	Adenomatous polyposis coli
β TrCP	Ubiquitin ligase protein
<i>CTNNB1</i>	β -catenin gene
<i>CDH1</i>	E-cadherin gene
CEA	Carcinoembryonic antigen
CI	Confidence interval
CKI	Casein kinase I
CRC	Colorectal carcinoma
DFS	Disease free-survival
FAP	Familial adenomatous polyposis
FOLFIRI	5-fluorouracil leucovorin and irinotecan
FOLFOX4	5-fluorouracil leucovorin and oxaliplatin
GSK3 β	Glycogen synthase kinase 3 β
K-RAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
HR	Hazard ratio
MMP7	Metalloproteinase 7
OS	Overall survival
R	Spearman's rank correlation coefficient
TCF/Lef	T-cell factor/lymphoid enhancer factor
Wnt-1	Wingless-type MMTV integration site family member 1

A. Stanczak · T. Pietrucha
Department of Medical Biotechnology,
Medical University of Lodz,
Lodz, Poland

A. Stanczak (✉) · M. Wieczorek · M. Lamparska-Przybysz
Innovative Drugs Research & Development Department,
Celon Pharma Ltd.,
41A Mokra St. Kielpin,
05-092 Lomianki, Poland
e-mail: aleksandra.stanczak@celonpharma.com

R. Stec · L. Bodnar · C. Szczylik
Department of Oncology,
Military Institute of the Health Services in Warsaw,
Warsaw, Poland

W. Olszewski
Department of Pathology, Institute of Oncology,
Warsaw, Poland

M. Cichowicz · W. Kozlowski
Department of Pathology,
Military Institute of the Health Services in Warsaw,
Warsaw, Poland

Introduction

Colorectal carcinoma (CRC) is one of the most common human cancers. In 2008, 1233000 new CRC patients were diagnosed worldwide and about 608000 deaths caused by colorectal cancer were estimated making it the fourth most common cause of death from cancer [1]. Five-year survival for CRC patients amounts 54% in Europe, but in Poland it is lower and amounts 38.8% [2]. Additionally, observed five-year survival for patients with stage I is 74.0%, 66.5% in patients with stage IIA disease, 73.1% in patients with stage IIIA disease and only 5.7% in patients with stage IV disease [3].

There are many factors influencing colorectal cancer prognosis. According to College of American Pathologists fundamental prognostic factors are: extent of tumor (pT category), regional lymph node metastasis (pN category), blood or lymphatic vessel invasion, residual tumor following surgery and serum level elevation of carcinoembryonic antigen (CEA) before treatment [4]. Other clinical and pathological factors are also extensively studied, for example histological type or microvessel density [5, 6]. Moreover, molecular markers are becoming to be important variables that have an impact on CRC patient prognosis. Variety of proteins are extensively explored in colorectal carcinoma.

Particular interest of scientists is focused on a multi-functional protein - β -catenin. Along with E-cadherin it forms adherent junctions mediating epithelial cell adhesion and it is the key protein in canonical Wnt signaling pathway [7]. In the absence of Wnt ligands, β -catenin abundantly occurs in adherent complexes, while its level in the cytoplasm is very low. Free cytosolic β -catenin is phosphorylated in a complex formed by adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3 β (GSK3 β) and casein kinase I (CKI). Phosphorylated β -catenin is ubiquitinated by ubiquitin ligase protein (β TrCP) and then degraded in the proteasom. Binding one of the Wnt ligands to the receptors Fzd/LRP5/6 triggers signal inactivating the degradation complex. Then β -catenin is stabilized and goes to the nucleus, where it binds T-cell factor/lymphoid enhancer factor (TCF/Lef) and activates gene expression. Downstream target genes such as cyclin D1 [8], matrix metalloproteinase 7 (MMP7) [9], c-Myc [10], survivin [11] are responsible mainly for cell cycle and proliferation. Therefore, Wnt/ β -catenin pathway plays an important role in initiation and progression of colorectal carcinoma [12]. Inactivating APC mutations as well as changes in β -catenin gene (*CTNNB1*) often occur in colorectal tumors [13–15]. Additionally, changes of β -catenin and E-cadherin expression were observed in colorectal carcinoma [16–18].

The aim of this study was to determine the expression and localization of β -catenin, E-cadherin and Wnt-1 in

colorectal tumors and to assess the impact of expression of studied proteins on patients survival.

Material and Methods

Patients

Sixty-six unrelated patients with advanced colorectal carcinoma were enrolled consecutively from CRC patients attending Military Institute of the Health Services in Warsaw for palliative care between 2003 and 2008. None of them had a history of familial CRC. All patients were treated according to the routine protocols for CRC patients. All patient underwent radical resection of colorectal cancer while metasectomy was not performed in these group of patients according to guidelines of Polish Society of Clinical Oncology [19]. The study was approved by Local Ethical Committee at the Military Institute of the Health Services, in Warsaw.

Patients characteristic is summarized in Table 1. Out of 66 cases, there were 22 women and 44 men. The mean age was 71 years (range 59–83). Good performance status (WHO 0–2 or Karnofsky \geq 80%) was displayed by 59 pts (86.4%) or 63 pts (95.5%), respectively. The majority (81.8%) had T3 tumors and 46 pts (69.7%) had lymph node involvement. All the patients had metastasis, most of which was to the liver (57.6%). 25 (37.9%) patients have been treated with adjuvant chemotherapy. Most of the tumors were adenocarcinomas and they were well or moderately differentiated (87.9% and 80.3%, respectively).

Immunohistochemistry

Formalin-fixed, paraffin-embedded primary tumors were obtained from all patients. Tissue slides were routinely stained with hematoxylin and eosin. For immunohistochemistry analysis the slides were subjected to antigen retrieval in Target Retrieval Solution, pH 9 (DAKO) with PT Link (DAKO). Tissues were incubated with mouse monoclonal antibody anti- β -catenin (dilution 1:100, clone β -Catenin-1, DAKO), mouse monoclonal antibody anti-E-cadherin (dilution 1:100, clone NCH-38, DAKO) or rabbit polyclonal antibody anti-Wnt-1 (dilution 1:100, Thermo Scientific). Negative controls were incubated with mouse or rabbit IgGs (DAKO). Detection was done with EnVision TM+system (DAKO). The expression was scored by two independent observers who had no knowledge of the clinical data. All membranous, cytoplasmic and nuclear staining were evaluated. Staining was graded into four groups: +++ strong, ++ moderate, + weak, 0 negative. Tumors were regarded as immunopositive if 10% of tumor cells showed immunoreactivity.

Table 1 Characteristics of patients (n=66)

Characteristics	n=66
Age, years	
Median (range)	71 (59–83)
Gender	
Female	22 (33.3%)
Male	44 (66.6%)
WHO performance status before palliative treatment	
0	18 (27.3%)
1	41 (62.1%)
2	5 (7.5%)
3	2 (3.0%)
Karnofsky performance status before palliative treatment	
60%	1 (1.5%)
70%	2 (3.0%)
80%	5 (7.6%)
90%	43 (65.2%)
100%	15 (22.7%)
Histopathological type	
Adenocarcinoma	58 (87.9%)
Mucinous adenocarcinoma	1 (1.5%)
Unclassified	7 (10.6%)
Use of adjuvant chemotherapy	25 (37.9%)
Elevated CEA level before palliative treatment(>5 µg/l)	37 (56.1%)
Histological differentiation	
Well/moderately	53 (80.3%)
Poorly/undifferentiated	13 (19.7%)
Lymph node status	
N0	20 (30.3%)
N1	19 (28.8%)
N2	19 (28.8%)
N3	8 (12.1%)
Primary tumor size	
T2	3 (4.5%)
T3	54 (81.8%)
T4	9 (13.6%)
Primary tumor location	
Colon	23 (34.8%)
Sigmoid colon	24 (36.4%)
Rectum	19 (28.8%)
Location of metastases	
Liver	36 (57.6%)
Lungs	19 (28.8%)
Other	23 (34.8%)
Number of organs involved	
1	37 (56.1%)
≥2	29 (43.9%)
Palliative chemotherapy first line	
Capecitabine	26 (39.4%)
Irinotecan	5 (7.6%)
FOLFIRI	23 (34.8%)
FOLFOX4	12 (18.2%)

Statistics

A Spearman test for non-parametric variables was used to assess correlation between histoclinical data and expression of studied proteins. Overall survival (OS) was defined as time elapsed between date of diagnosis and date of death or the date of last follow-up. The end point of our analysis was established on August 2008. Median and life tables were computed using the product-limit estimate by the Kaplan and Meier method and the log-rank test was employed to assess the statistical significance, p values less than 0.05 were considered to indicate statistical significance. Univariate and multivariate proportional-hazards models (Cox) were fitted to the data to determine the importance of recognized explanatory variables. Factors that were significant in univariate analysis and factors that showed a trend towards significance were included in the multivariate model which evaluated factors potentially influencing OS [i.e. age (<70 vs. >70 years), T stage, type of histology, and performance status]. Multivariate analyses of overall survival were performed by Cox proportional-hazard regression using the forward stepwise method. Statistical calculation were performed using the STATISTICA for Windows Version 7.0 software.

Results

Expression of E-cadherin, β-catenin and Wnt-1 in CRC

Normal tissue was available from 32 (48.5%) patients. In all normal colonic epithelium E-cadherin and β-catenin were present in the cell membrane with strong, continuous staining. Adhesion proteins were not present in the cytoplasm nor in the nuclei of normal epithelial cells (Fig. 1 a, b). Wnt-1 was expressed in cytoplasm with strong staining. No other localizations of Wnt-1 were found in normal tissue (Fig. 1c).

In colorectal carcinoma, decreased expression and changes of localization of studied proteins were observed (Table 2, Fig. 1d–f). Declined level of membrane E-cadherin and β-catenin was observed in 31.8% (21/66) and 22.7% (15/66) of patients, respectively. Abnormal cytoplasmic E-cadherin was detected in 37.9% (25/66) of cases. In comparison to the normal epithelium, aberrant cytoplasmic and nuclear β-catenin was detected in 51.5% (34/66) and 31.8% (21/66) of patients, respectively. We observed that membrane E-cadherin was preserved in those cells where membrane β-catenin was present. On the other hand, in cancerous tissue cytoplasmic E-cadherin colocalized with β-catenin. 60.6% (40/66) of patients had decreased cytoplasmic Wnt-1 expression, while 26 (39.4%) patients had tumor cells with normal, strong Wnt-1 staining.

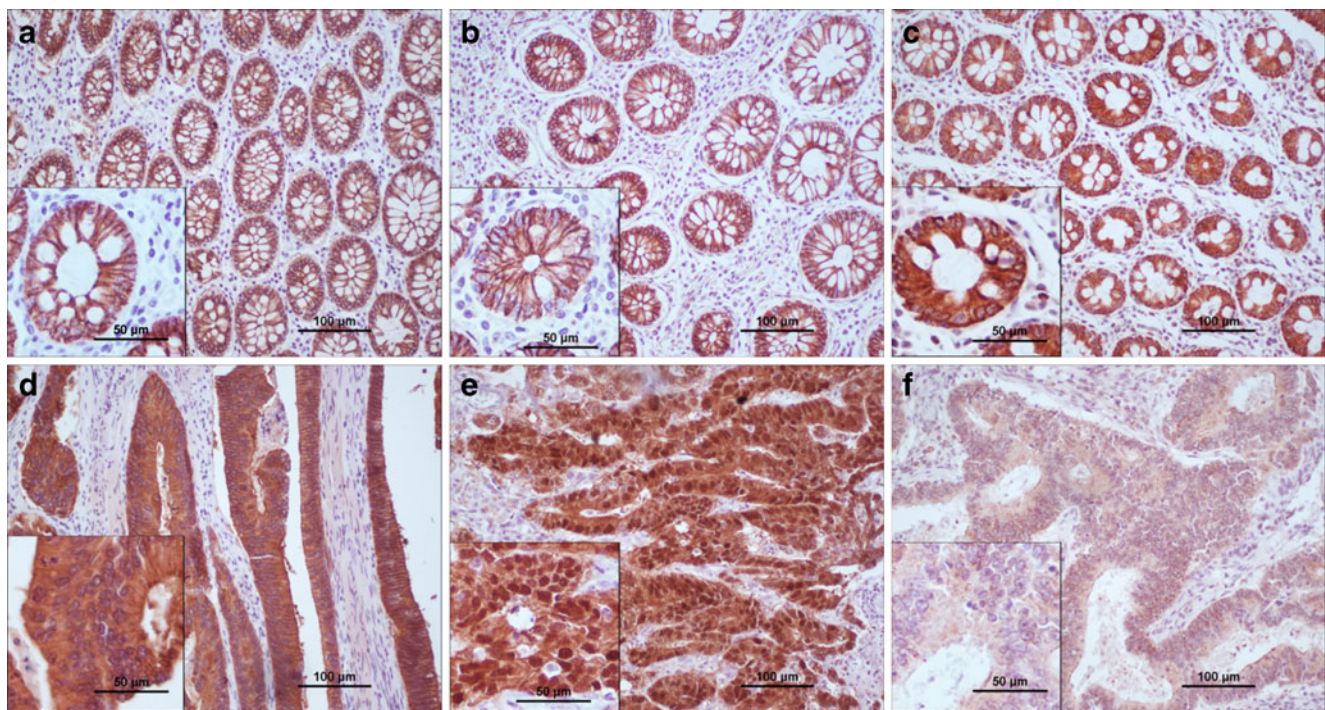


Fig. 1 Expression of E-cadherin, β -catenin and Wnt-1 in normal colonic epithelium and in colorectal carcinoma. E-cadherin displayed strong membranous immunoreactivity in normal epithelium (a), while in cancer cells decrease of membranous and increase of cytoplasmic E-cadherin was detected (d). High expression of β -catenin in the cell

membrane was found in normal cells (b). Weak membranous, moderate cytoplasmic and strong nuclear expression of β -catenin was observed in tumor tissue (e). In normal epithelial cells high cytoplasmic Wnt-1 expression was detected (c). In colorectal carcinoma, there was weak Wnt-1 immunoreactivity (f)

Correlation with Histoclinical Data

Expression of E-cadherin, β -catenin and Wnt-1 did not significantly correlate with tumor size, lymph node involvement nor histopathological type of tumor. Analysis of histoclinical data and expression of studied proteins revealed association between tumor localization and presence of β -catenin in nuclei. Cancer localized in rectum displayed greater nuclear localization of β -catenin than tumors localized in sigmoid and colon ($R\ 0.35$; $p=0.004$). No other correlation was found.

To investigate the association between expression of studied proteins and survival, log-rank test was performed (Table 3). There were no differences between patient survival and age, sex, performance status, tumor size, lymph node metastasis. As expected, elevated CEA level

before palliative treatment correlated with shorter survival. Median survival of patients having elevated CEA level was 24.3 months, where median survival of patients having CEA below $5\ \mu\text{g/l}$ was 46.4 months ($p=0.014$). In addition, patients with tumor localized in colon or rectum had worse prognosis than patients with tumor localized in sigmoid (20.9 vs. 47.9 months, $p=0.028$). Among analyzed proteins only nuclear β -catenin was found to be statistically associated with poor survival. Median survival of patients with colorectal carcinoma displaying nuclear β -catenin was two times shorter than CRC patients with normal β -catenin (19.2 vs. 39.8; $p=0.007$) (Table 3, Fig. 2).

Factors that were significant in univariate analysis and factors that showed a trend towards significance ($p<0.1$) were included in the multivariate model which evaluated factors potentially influencing OS (Table 4). We found that,

Table 2 Expression of E-cadherin, β -catenin and Wnt-1 in colorectal tumors

Protein	Membrane		Cytoplasmic		Nuclear	
	Normal	Decreased	Present	Absent	Present	Absent
E-cadherin	45 (68.2%)	21 (31.8%)	25 (37.9%)	41 (62.1%)	0	0
β -catenin	51 (77.3%)	15 (22.7%)	34 (51.5%)	32 (48.5%)	21 (31.8%)	45 (68.2%)
Wnt-1	0	0	26 (39.4%)	40 (60.6%)	0	0

Table 3 Univariate analysis of overall survival (log-rank test)

Covariate	n (%)	Median (months)	p value
Age			
≤70 years	41 (62.1%)	31.8	0.741
>70 years	25 (37.0%)	29.9	
Gender			
Female	22 (33.3%)	23.3	0.070
Male	44 (66.7%)	35.4	
Histological differentiation level			
Good/average	53 (80.3%)	32.6	0.307
Low/unknown	13 (19.7%)	16.5	
Primary location			
Sigmoid colon	25 (37.9%)	47.9	0.028
Colon/rectum	41 (62.1%)	20.9	
WHO performance status			
0	18 (27.3%)	49.5	0.140
1–2	48 (72.7%)	28.1	
Karnofsky performance status			
≤ 80	8 (12.1%)	21.4	0.624
>80	58 (67.9%)	32.0	
Primary tumor size			
T1-2	3 (4.6%)	NA	0.530
T3-4	63 (95.4%)	31.8	
Lymph node status			
Cancer-free	20 (30.3%)	31.5	0.880
Involved	46 (69.7%)	30.1	
Location of metastases			
Liver	38 (57.6%)	24.7	0.068
Other	28 (42.4%)	47.9	
Number of organs involved			
1	37 (56.1%)	17.2	0.155
≥2	29 (43.9%)	37.0	
Pretreatment CEA level (μg/l)			
≤5	29 (43.9%)	46.4	0.014
>5	37 (56.1%)	24.3	
E-cadherin membranous			
normal	45 (68.2%)	30.9	0.963
decreased	21 (31.8%)	32.4	
E-cadherin cytoplasmic			
positive	25 (37.9%)	32.7	0.952
negative	41 (62.1%)	27.6	
β-catenin membranous			
normal	51 (77.3%)	31.7	0.649
decreased	15 (22.7%)	21.9	
β-catenin cytoplasmic			
positive	34 (51.5%)	27.4	0.707
negative	32 (48.5%)	39.9	
β-catenin nuclear			
positive	21 (68.2%)	19.2	0.007
negative	45 (31.8%)	39.8	
Wnt-1			
normal	26 (39.4%)	30.5	0.798
decreased	40 (60.6%)	30.2	

NA not available

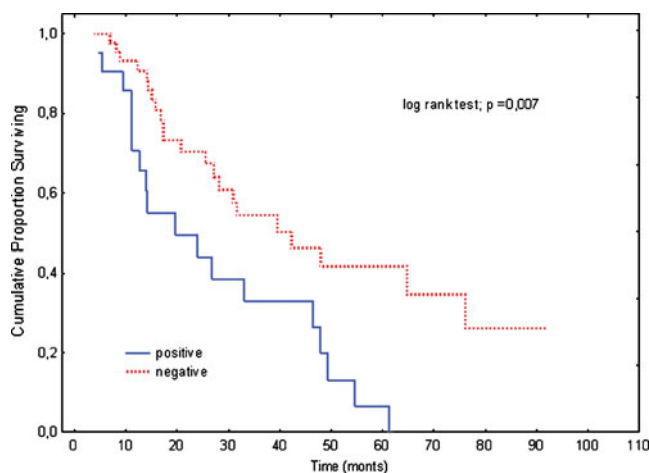


Fig. 2 Overall survival by nuclear β-catenin expression. The continuous line: nuclear β-catenin positive (n=21). The dotted line: nuclear β-catenin negative (n=45). The Kaplan-Meier, log rank test, p=0.007

higher CEA serum level before treatment (HR 2.75; 95% CI 1.15–6.59; p=0.023), female sex (HR 2.73; 95% CI 1.32–5.68; p=0.023) and tumor localized in colon or rectum (HR 3.11; 95% CI 1.42–6.82; p=0.005) were independent prognostic factors for shortened survival. Among studied proteins, nuclear β-catenin was confirmed unfavorable prognostic factor (HR 2.48; 95% CI 1.30–4.74; p=0.006).

Discussion

In this study we demonstrated that there are changes in the expression and localization of β-catenin, E-cadherin and Wnt-1 in advanced colorectal carcinoma. Additionally, we revealed correlations between levels of studied proteins and histoclinical data. We found that nuclear β-catenin, higher carcinoembryonic antigen serum level before treatment,

Table 4 Multivariate analysis of overall survival

Covariate	Hazard ratio (95% CI)	p value
Gender		
Female vs. male	2.73 (1.32–5.68)	0.007
Pretreatment CEA level (μg/l)		
>5 vs. ≤5	2.75 (1.15–6.59)	0.023
Primary location		
Colon/rectum vs. sigmoid colon	3.11 (1.42–6.82)	0.005
Nuclear β-catenin		
Positive vs. negative	2.48 (1.30–4.74)	0.006

Non significant correlations were not shown

female sex and tumor localized in colon or rectum could be independent unfavorable prognostic factors however the influence of treatment on survival cannot be excluded.

β -catenin is a protein responsible for cellular adhesion since it forms a complex with E-cadherin. On the other hand, this protein is a key component of Wnt pathway controlling proliferation and cell cycle. Disturbances in both functions of β -catenin may affect colorectal tumorigenesis. Changes of membrane β -catenin level may be caused by the decrease of membrane E-cadherin. Our results showing decreased level of membrane β -catenin and membrane E-cadherin are concordant with numerous earlier reports [16–18, 20, 21]. Additionally, we confirmed previous data concerning detection of E-cadherin in the cytoplasm of tumor cells [22, 23]. There are few studies documenting that abnormal E-cadherin expression was associated with poor prognosis [20, 22]. However, we did not corroborate those findings.

Genetic alterations, transcriptional changes or protein trafficking could disturb E-cadherin functions. Mutations of *CDH1* encoding E-cadherin are relatively rare in colorectal carcinomas [24, 25], but are more frequent in gastric or breast cancers [26, 27]. Furthermore Truant et al. showed that mRNA level of E-cadherin in colon cancer is not different than in normal tissue [28]. A possible explanation for reduced level of E-cadherin is that proper formation of cell-cell junctions could be disrupted by Src kinase. Elevated Src expression disturbs E-cadherin regulation in colon cancer cells [29]. However, detailed studies focused on control of E-cadherin expression in colorectal carcinoma are needed.

Decreased level of membrane E-cadherin could have an impact on invasiveness of CRC cells. Tumor cell release as a result of disruption of adherent junctions plays a crucial role in metastasis. Expression of E-cadherin observed in metastases varies. Some studies showed that level of E-cadherin decreased in metastatic liver as compared to primary tumor [21]. On the other hand, expression of E-cadherin increased in metastatic lymph nodes compared to primary tumor [30].

Furthermore, we detected β -catenin in cytoplasm and nuclei of tumor cells. Recent studies showed that, in advanced CRC, there was an increase of cellular and nuclear level of β -catenin [16, 21, 31, 32]. Stabilization of β -catenin in the cytoplasm, its transport to the nucleus and activation of gene expression is enabled by Wntless pathway. Wnt-1 is one of the ligands which trigger signaling cascade. There are a few inconsistent studies concerning Wnt-1 expression in colon, showing over-expression or lack of expression of Wnt-1 in tumor when compared to the normal colonic mucosa [33–35]. In our study, we showed that in normal colon epithelium there is a high expression of this ligand, whereas in the tumor tissues

there was a decrease of Wnt-1 expression. This result suggests other mechanism of β -catenin accumulation than Wnt-1 activation in analyzed cases of CRC.

Another plausible explanation of β -catenin stabilization is occurrence of inactivating APC mutations. Most colorectal carcinomas harbor mutations in *APC* or *CTNNB1* encoding β -catenin [13–15]. Inactivating mutations in *APC* was found in familial adenomatous polyposis (FAP) [36, 37], but they also occur in the sporadic CRC [37, 38]. The most frequent are the mutations in β -catenin binding domain [39]. Truncated APC is not able to bind the β -catenin and leads to its accumulation which provokes the activation of downstream target genes [15]. *CTNNB1* mutations occur in the region coding amino acids phosphorylated by GSK3 β [14]. Thus mutated β -catenin is rescued from phosphorylation and accumulates in the nucleus.

We observed correlation between nuclear β -catenin and tumors localized in rectal area. The association between β -catenin and histoclinical data is disputed subject. There are few inconsistent studies concerning correlation of nuclear β -catenin with tumor localization. Our results differ from those reported by other groups showing lack of association between tumor localization and presence of nuclear β -catenin in tumor cells [40, 41]. Instead we corroborate previous observations that cancer localized in rectum display higher level of nuclear β -catenin than tumors localized in sigmoid and colon [42, 43].

Our search for prognostic factors for CRC resulted in identification of tumor localization as unfavorable prognostic factor. We found that tumors localized in colon or rectum are associated with shorter survival, while tumors localized in sigmoid area are correlated with prolonged survival. Our results are concordant with Wray et al. who performed the study on a large patient cohort (82 926 colon cancer cases) and found an association between sigmoid tumors location and decreased colorectal cancer-specific mortality, what is in agreement with our study [44].

Another adverse prognostic factor for CRC identified in our study was the presence of nuclear β -catenin. Although some reports of nuclear β -catenin being a favorable

Table 5 Tumor location and nuclear β -catenin connection

Presence of nuclear β -catenin		
Tumor location	Correlation (R Spearman test; p value)	Multivariate analysis Hazard ratio (95% CI); p value
Rectum	0.35; 0.004	3.11 (1.42–6.82); 0.005
Colon	NS	
Sigmoid		NS

NS non significant

predictor of disease free survival (DFS) exist [32], our finding is in agreement with a large body of previously published evidence [17, 22, 30]. Such a discrepancy may result from different localizations of tumors analyzed in particular studies. We found that a high level of nuclear β -catenin in tumors localized in rectum was paralleled by poor clinical prognosis and that a low level of β -catenin in nuclei was accompanied by prolonged survival in cases of tumors localized in sigmoid but not in colon (Table 5). These findings suggests that nuclear β -catenin could be an adverse prognostic factor only for some tumor localization. Moreover, low nuclear β -catenin level coincides with poor clinical prognosis in patients with cancer localized in colon what suggests existence of additional, yet unidentified factors influencing survival.

The variety of physiological, anatomical and genetic features of distinct sites of a large bowel results in different tumor characteristics dependent on its localization. All of these may influence clinical outcome. The existence of two biologically distinct types of CRC - according to the tumor location in the proximal or distal parts of a large bowel - was proposed for the first time by Bufill [45]. In turn, Li et al. suggested that there are three biological types of CRC dependent on tumor localization in proximal colon, distal colon and rectum [46]. Among genetic factors observed in rectal cancers are: high incidence of chromosomal instability, Wnt/ β -catenin activation and high p53 expression [47, 48]. Conversely, microsatellite instability abundance and K-RAS mutations are associated with colon tumors [47, 48]. Diversity of molecular features of different CRC types results in a complex array of possible prognostic factors for colorectal tumors.

In our study, results of gender analysis may suggest female sex as the unfavorable prognostic factor in colon cancer. However, this observation is in conflict with other reports showing no association between survival and female sex [17, 32, 49]. Cautious interpretation of sex as a prognostic factor in our study is required due to the lack of sex-matched group and relatively small number of patients analyzed.

In analyzed cases, higher CEA serum level before treatment was associated with shorter survival what is in agreement with observation that CEA is elevated in many disorders, particularly in cancers including the colon, rectum, breast and lung [50]. In the management of colorectal carcinoma, CEA level is the most useful clinical marker [50] and assessment of CEA concentration serves as a screening test for CRC [51].

Numerous new markers for colorectal cancer are discovered. Detailed list of factors measured in serum, tissue or stool are described elsewhere [52]. Despite a variety of analysis, there are only few prognostic factors for advanced colorectal carcinoma [4]. For better CRC

management more markers are needed. Although molecular markers become more and more important in cancer therapy, additional studies are needed to confirm their usefulness.

Conclusion

In summary, our study demonstrated that in advanced CRC there are disturbances in expression and localization of β -catenin, E-cadherin and Wnt-1. We showed the link between tumor localization and pattern of β -catenin expression and additionally we revealed an association between tumor localization and patient outcome. These finding suggests that nuclear β -catenin could be an adverse prognostic factor only for some tumor localization. A limitation in our study was the number of patients and controls included. Further investigations on a larger population are needed. These finding support the hypothesis that Wnt/ β -catenin pathway plays an important role in advanced colorectal carcinoma.

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