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Comparison of Beta-catenin with TGF-beta1, HIF-1alpha and Patients' Disease-free Survival in Human Colorectal Cancer

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Abstract Beta-catenin accumulation is suppressed by TGF-beta1 (transforming growth factor beta1) in intestinal epithelium suggesting negative feedback between these two factors. Besides that, beta-catenin interacts with HIF-1alpha (hypoxia-inducible factor-1alpha) at the promoter region of HIF-1 target genes. Our study was aimed at comparison of beta-catenin with HIF-1alpha, TGF-beta1, Ki67 and survival of sporadic colorectal cancer patients. Expressions of beta-catenin, TGF-beta1, HIF-1alpha, Ki67 were evaluated in triads of specimens of each primary tumor of 72 sporadic colorectal cancers with immunohistochemistry due to limited availability of tissue material. Disease-free survival was analyzed in case of all 100 beta-catenin stained tumors, in 85 cancers stained for HIF-1 and in 72 neoplasms with TGFbeta1 staining. Beta-catenin, TGF-beta1 and HIF-1alpha accumulated in 72 colorectal cancer cells. Beta-catenin correlated both with HIF-1alpha and TGF-beta1 in all colorectal cancers ($p<0.009$, $r=0.307$ and $p=0.003$, $r=0.342$, respectively) and in subgroups of different clinico-pathological profile. Beta-catenin failed to correlate with Ki67. In case of beta-catenin, TGF-beta1 and HIF-1alpha, disease-free survival curves failed to show any statistically significant differences between groups of marker negative tumors, cancers with low expression and neoplasms with higher protein expression. Positive correlations between beta-catenin and TGF-beta1 may indicate ineffective attempts of TGF-beta1 to reduce intracellular level of beta-catenin in colorectal cancer. Associations between beta-catenin and HIF-1alpha reflect previously detected interactions between HIF-1alpha with beta-catenin and are confirmative for presence of such reactions in human colorectal cancer.

Keywords Beta-catenin · TGF-beta1 · HIF-1alpha · Patients' disease free survival · Colorectal cancer

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Introduction

A mediator of Wnt pathway, beta-catenin is recognized a stimulator of cell proliferation and is detected in variety of neoplasms. Beta-catenin undergoes nuclear translocation, plays a role of nuclear transcriptional factor and unblocks proliferation of intestinal cells, if beta-catenin is not able to anchor APC protein (adenomatous polyposis coli) in cytoplasm due to APC mutations [1]. However, mutations of catenin beta-1 (CTNNB1) were reported to be found in minority of sporadic colorectal cancers [2]. Nevertheless, due to mutations of other genes for signaling proteins of Wnt pathway, not only nuclear accumulation of beta-catenin is a common, constitutive change in chain of protein expression alterations in the adenoma-carcinoma sequence, but also beta-catenin appears to be a central molecular link for crucial factors that play roles during onset and advancement of colorectal cancer. TGF-beta1 (transforming growth factor beta-1) was primarily described as anti-oncogenic inhibitor of nuclear beta-catenin signaling [1]. Although there is accumulation of TGF-beta1 both in primary tumors of colorectal

cancers and adjacent inflammatory cells, TGF-beta1 signaling is abolished in most colorectal cancers [3–5]. TGF-beta1 signaling can have a prognostic significance in colorectal cancer. Namely, Bacman et al. reported that decrease of both TGF-beta receptors in tumour-associated stroma was associated with shorter survival of colon cancer patients, while our preliminary study depicted positive correlation between higher expression of TGF-beta1 in tumor- adjacent inflammatory cells and longer survival of patients [1, 3, 6]. Wnt is required for malignant transformation of colorectal epithelium, while HIF1alpha (hypoxia-inducible factor-1alpha) is responsible for cancer angiogenesis and acquiring of metastatic capability [7]. Wnt signaling is so important in colorectal cancer that it is reported that most of colorectal epithelial malignancies are set on by uncontrolled acceleration of the Wnt/beta-catenin stimulation of cell proliferation [8]. However, ceasing of downstream protein of Wnt signaling, VHL (von Hippel-Lindau (VHL) tumor suppressor) is associated with stabilization of HIF1alpha in late stages of cancer progression [7]. Wnt pathway was shown to be inactivated by HIF-1alpha by its interaction with beta-catenin and resultant hypoxia-induced inhibition of tumor cell growth [9]. HIF-1alpha binds human protein acetyltransferase hARD1 (human Arrest Defective 1) breaking complex of hARD1 and beta-catenin. In result HIF-1 counteracts acetylation, subsequent activation of beta-catenin and further repression of TCF4 action by direct binding of HIF-1 with beta-catenin, in LiCl-stimulated HEK293 (Human embryonic kidney cell line) and cancer cell lines with Wnt signaling switched on [9]. These findings suggest HIF-1 alpha and beta-catenin could mediate divergent and even opposite signaling in which stabilization and activation of one protein is accompanied with destabilization and inactivation of the other one. These molecular interactions gain more attention as pharmacological agents like artesunate suppressed beta-catenin dependant transcription via expulsion of beta-catenin from nucleus to membranous location and subsequently inhibited proliferation via down-regulation of Ki67 in human colorectal cancer [10]. In addition, HIF-1alpha interacts with beta-catenin at the promoter region of HIF-1 target genes so that HIF-1alpha prevents binding between T-cell factor-4 and beta-catenin and favors inhibition of cell cycle [11]. HIF-1alpha was found to correlate both with anti-apoptotic protein Bcl-xL and proapoptotic Bax in colorectal cancer [12]. Moreover, HIF-1alpha significantly associated with TGF-beta1 in this kind of neoplasm in our previous study [3].

Aims

The aim of our study was comparison of beta-catenin with HIF-1alpha, TGF-beta1, Ki67 and disease-free survival of sporadic colorectal cancer patients.

Methods

Material

Expressions of beta-catenin, HIF-1α TGF-beta1 and Ki67 were detected in 72 sporadic colorectal cancers with appliance of immunohistochemistry. The studied primary tumors were surgically removed without prior chemo- or radiotherapy. This research was preformed according to the ethical standards laid down in the latest revision of Declaration of Helsinki from 2004 (the approval by the ethics committee of Medical University of Bialystok). All the subjects gave an informed consent for inclusion in the study. The biopsy samples were preserved with 10% buffered formalin solution for 48 h and underwent standard histopathological evaluation with determination AJCC/UICC TNM stage (American Joint Committee on Cancer/International Union Against Cancer Tumor Node Metastasis), histopathological type and grade of histological differentiation (G). pT1 and pT2 neoplasms were separated into one group (pT1+pT2) from pT3 and pT4 tumors that constituted another group (pT3+pT4). All available patients with enough colorectal cancer tissue were selected for this immunohistochemical evaluation in the period of 5 years.

Staining

Immunohistochemical protocols were applied to detect studied proteins with specific anti-beta-catenin (sc-1496, dilution of 1:100), HIF-1alpha (sc-10790, dilution of 1:150) and anti-TGF-beta1 (sc-146, dilution of 1:300) (Santa Cruz Biotechnology, Inc. USA.) anti-Ki67 (clone MIB1, dilution of 1:100)(DAKO). Antibodies against beta-catenin, TGF-beta 1 and HIF-1alpha was incubated with tissue samples overnight at 4°C after one-hour-long pre-incubation with blocking serum, while Ki67 antibody (clone MIB1, DAKO) was incubated at dilution of 1:100 half an hour in room temperature after standard retrieval of antigen. Color visualization of beta-catenin and Ki67 was accomplished with LSAB method and 7-minutes-long DAB exposure. Color reaction was developed with EnVision method with 5 min for TGF-beta1 and 7 min for HIF-1alpha of exposure to DAB (diaminobenzidine). Sections were counterstained with haematoxylin. Usage of primary antibodies was excluded during preparation of negative controls. Beta-catenin, TGF-beta1 and HIF-1alpha stained specimens of breast cancer served as positive controls.

Scoring and Statistical Analysis

This study was designed to be an extension of previously performed IHC studies on patients with colorectal cancers. The previous studies included 108 and 123 patients, while

the current study is performed on 72 patients in regard to relation of TGF-beta1 to HIF-1alpha and GLUT-1. Due to limited availability of slides for immunohistochemical evaluation from archives of paraffin blocks of cancer tissues, we could provide triads of microscopic slides for evaluation of all three markers in each case of only these 72 patients. Actually, these selected patients were cases with sporadic colorectal cancer, as there was no information about familiar incidence of colorectal cancer, and gene profile that would reveal familiar predisposition to colorectal cancer and enable classification to one of hereditary colorectal cancer syndromes. Nevertheless, gene profiling was beyond the scope of this study and actually closed project pointing on its limitations. Partially we can suspect colon cancer syndromes in relatively young individuals with colorectal cancer. Thus, younger patients below 45 years old were excluded from studied group for one of statistical analysis to be more comfortable in analysis of only sporadic colorectal cancers. Spearman rank correlation test was used to reveal binary associations between studied proteins. Border level of statistical significance was determined at $p=0.05$. Following 3 grades were issued in scoring system for cancer expression of investigated proteins: 0 less than 10% positive cancer cells in a microscopic slide of each case; 1 ranging from 10 to 50% positive cancer cells; 2 more than 50% positive malignant cells for Spearman rank test. TGF-beta1 and HIF-1alpha expressions were analyzed in Chi2 test in regard to clinical and pathological variables in our previous works [30, 37]. Expressions of studied proteins was subjected in three groups of negative, low and high expression of the marker to Kaplan Meier analysis to look for any relation of this marker with patients' survival rates. Disease-free survival was analyzed in case of all 100 beta-catenin stained tumors, in 85 cancers stained for HIF-1 and in 72 neoplasms with TGFbeta1 staining. Overall survival data were too small to employ essential amount of patients for Kaplan Meier analysis, so overall survival was not considered in this study. TGF-beta1 and HIF-1alpha expressions were analyzed in Chi2 test in regard to clinical and pathological variables in our previous works [3, 13].

Results

Beta-catenin was present in 91% (66/72) cases giving cytoplasmic and nuclear appearance (Fig. 1a, b). HIF-1alpha was expressed in 83% (60/72) in cytoplasmic and nuclear location (Fig. 1c); TGF-beta1 was present in cytoplasm in the same rate of tumors (60/72) (Fig. 1d). Nuclear Ki67 staining was evidently positive in more than 10% of malignant cells in each case of colorectal cancer in 86% (62/72) of studied tumors.

Beta-catenin correlated both with HIF-1alpha and TGF-beta1 in all colorectal cancers ($p<0.009$, $r=0.307$ and $p=0.003$, $r=0.342$, respectively). Besides that, beta-catenin associated significantly with HIF-1alpha in node positive cancers, adenocarcinoma type, colon tumors and male patients. Beta-catenin correlated with TGF-beta1 in node negative and node positive tumors, in a group of deeply invading cancers (pT3+pT4) as well as in moderately and poorly differentiated cancers, adenocarcinoma type, men, older patients over the age of 60 (Table 1). β-catenin failed to correlate with Ki67 in studied colorectal cancers ($p=0.387$,$r=-0.104$) in generally and in comparison of only nuclear expression of beta-catenin with Ki67 ($p=0.917$, $r=-0.013$). Tumoral beta-catenin TGFbeta1 and HIF-1alpha expression did not appear to show any statistically significant association with disease-free survival of the patients in Chart 1, 2 and 3. However, Kaplan Meier analysis using the overall survival data, was not preformed in this case as it would refer to unessential amount of patients in this evaluation.

Discussion

TGF-beta1 normally prevents accumulation of beta catenin and reduces cytoplasmic concentration of this protein in non-tumor intestinal epithelium. However, knock-down of APC causes switch of TGF-beta1 function from tumor suppression to tumor promotion in colorectal cancer [14]. In addition, TGF-beta1 was found to inhibit beta-catenin/Wnt signaling in human colon carcinoma cells in a Smad4/DPC4 independent manner [15]. Concerning that results on TGF-beta1 and beta-catenin depend on different conditions of cancer cell lines, it is important for us to study distributions of these proteins on tissues of human tumors. Most of experimental pathology studies try to prove active role of TGF-beta1 in suppression or promotion of cell growth [14, 15]. In our opinion, on the ground of these reports any remarks of TGF-beta1 mediated induction of cancer proliferation comes from impairment of growth suppressive pathway that engages TGF-beta1 and not from active stimulatory action of TGF-beta1. In our present study we show coexistent accumulations of both beta-catenin and TGF-beta1 that are positively correlated with each other in sporadic colorectal cancers. Currently, it cannot be excluded that overexpression of beta-catenin could be a stimulatory for mechanisms of TGF-beta1 induction. Anyway—no matter how high level of TGF-beta1 can be reached—TGF-beta1 remains incapable of inhibition of growth of abnormal intestinal cells. In light of our findings, it seems that generation of TGF-beta1 is not stopped despite abrogation of TGF-beta regulated growth inhibitory pathway. An indirect evidence for such an abrogation is

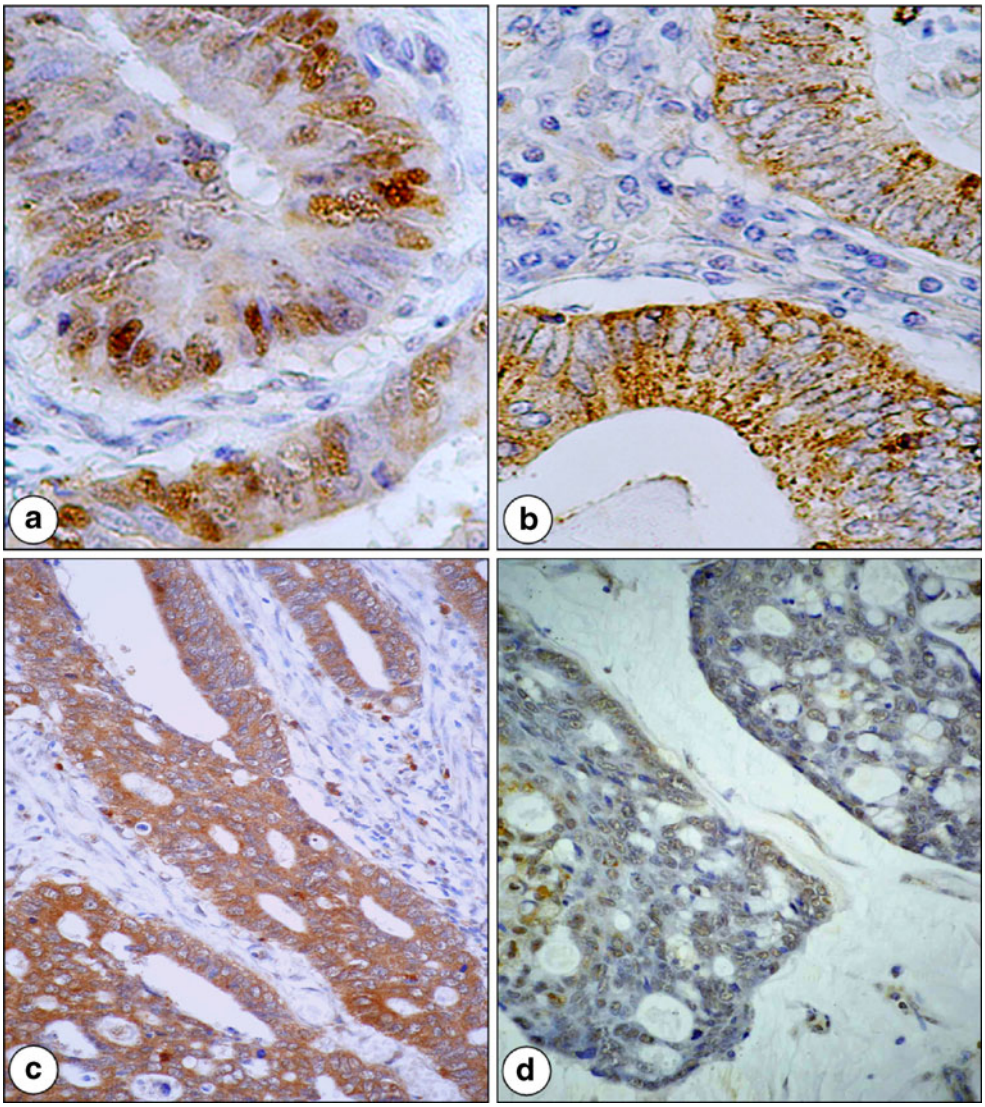

Fig. 1 **a**–**d** Expressions of beta-catenin, TGF-beta1, HIF-1alpha in colorectal cancer. **a** Nuclear immunoreactivity to beta-catenin in cancer tubes. Magnification × 400. **b** Cytoplamic staining for beta-catenin in colorectal cancer foci. Magnification × 400. **c** Strong anti- TGF-beta1 labeling in cytoplasm of colorectal cancer cells. Magnification × 200. **d** Mixed cytoplasmic and nuclear accumulation of HIF-1alpha. Magnification × 200

accompanying accumulation of beta-catenin which was visualized in this study. If TGF-beta1 inhibitory signaling was not abolished, beta-catenin would be degraded and would not be overexpressed. Beta-catenin thus would not be detected with immunohistochemical staining as in normal intestinal epithelium, which was negative for beta-catenin at margins of all studied tumors. Presuming that growth inhibitory function of TGF-beta1 is lost in colorectal cancers on the base of previous reports [14], we observed accumulation of inactive TGF-beta1 and simultaneous overexpression of beta-catenin. Resultant positive correlation of TGF-beta1 and beta-catenin means that beta-catenin and TGF-beta1 expressions can be mutually dependent in colorectal cancers.

Decrease of Ki67 proliferation index that was consistent with suppression of proliferation and promotion of apoptosis was accompanied by translocation of beta-catenin from nucleus to membranous location and reduction of beta-catenin mediated transcription after exposition of colorectal cancer cells to artesunate (ART) [10]. One would expect that positive correlation would be a relationship between driver of proliferation—beta-catenin and Ki67—a marker of proliferation. However, our study failed to detect additionally any correlation between beta-katenin and Ki67. In our opinion this lack of any significant relationship is reasonably justified in group of studied cases no matter how high are rates of positive beta-catenin staining and Ki67 index. It is well known that colorectal cancers universally show accumulation of beta-catenin. These beta-catenin accumulation is a triggering but not sufficient factor of unrestricted proliferation. Thus, some other conditions like normoxia and cellular supplementation with nutrients should be met to enable cell division which is marked with positive Ki67 staining. Similarly to our findings, several studies revealed abundant co-expression of Ki67 and beta-catenin in colorectal hyperplastic polyps,

Table 1 Comparison between beta-catenin and HIF-1alpha and TGF-beta1 in different clinicopathological groups of colorectal cancer. Spearman's correlation rank test

Groups of patients		Number	β-catenin – HIF-1α		β-catenin – TGF-β1	
			p	r	p	r
All		72	<0.009	0.307	0.003	0.342
pN	pN (−)	30	0.18	0.253	<0.025	0.409
	pN (+)	42	<0.027	0.341	<0.049	0.306
pT	pT1+2	9	0.161	0.509	0.822	0.087
	pT3+4	63	0.300	0.0168	<0.002	0.387
G	G2	52	0.087	0.240	0.030	0.302
	G3	20	0.227	0.283	0.029	0.488
HP-type	Adc.	63	0.008	0.330	0.003	0.367
	Adc muc.	9	<0.001	1,000	0.159	0.512
Sex	Male	35	<0.003	0.489	0.003	0.489
	Female	37	0.178	0.226	0.189	0.221
Age	≤60	25	0.097	0.339	0.056	0.387
	>60	47	0.0567	0.28	0.029	0.32
Location	Rectum	34	0.218	0.215	0.577	0.099
	Colon	38	0.019	0.379	0.529	0.001

pN, lymph node involvement; pT, depth of tumor intramural growth; G, grading of cancer histological differentiation (G2, moderately differentiated cancers; G3, poorly differentiated cancers); HP type, histopathological type (Adc., adenocarcinoma; Adc. muc, mucinous adenocarcinoma); Location, tumor site

intrahepatic cholangiocarcinomas, hydatidiform moles, except for choriocarcinomas and non-small cell lung cancers that were characterized by down-regulation of beta-catenin and high index of Ki67 [16–19].

Beta-catenin is known to be expressed from the very start of adenoma formation, therefore one would consider no utility of analysis of beta-catenin as a predictive marker for survival in CRC. Some reports underlined that there was no significance in differences of beta-catenin expression in regard to staging [20] and altered expression of beta-catenin failed to significantly associate with intravasation of colorectal epithelial cells in primary colorectal cancer [21]. Anyway, besides that beta-catenin abnormalities can be found in variety of tumors such as germinoma and teratoma [22], beta-catenin positive metastases could imply a role of beta-catenin in spreading of malignancies as in

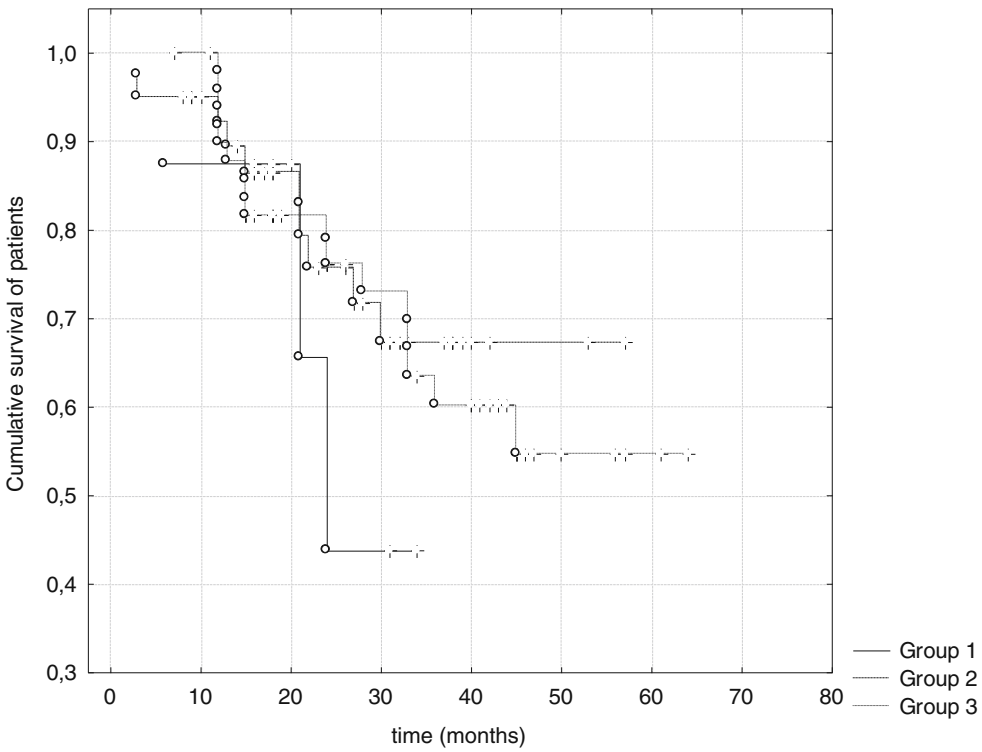

Chart 1 Beta-catenin expression and disease-free survival of colorectal patients (n=100). 0—beta-catenin-negative tumors (n=8), 1—tumors with low expression of beta-catenin (n=40). 2—tumors with high expression of beta-catenin (n=52)

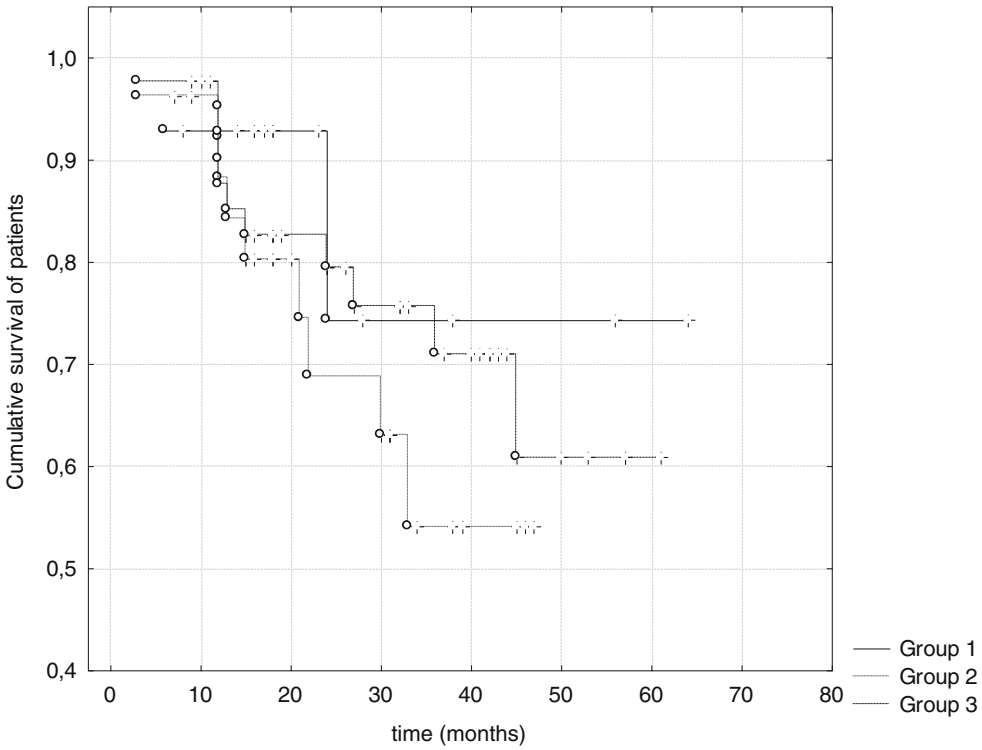

Chart 2 HIF1alpha expression and disease-free survival of colorectal patients (n=85). 0—HIF1alpha -negative tumors (n=14), 1—tumors with low expression of HIF1alpha (n=27). 2—tumors with high expression of HIF1alpha (n=44)

case of breast carcinoma meta to a recurrent myxoid liposarcoma [23]. Lately, interesting reports have been issued on prognostic significance of beta-catenin nuclear expression in colon cancers [24, 25]. Bivariate analysis of CD133 and nuclear beta-catenin was reported to be associated with decreased survival of patients due to ominous course of IIA colonic adenocarcinomas with co-expression of these both markers [24]. Earlier the same team of scientists elaborately described four detailed distinct patterns of nuclear beta-catenin expression to

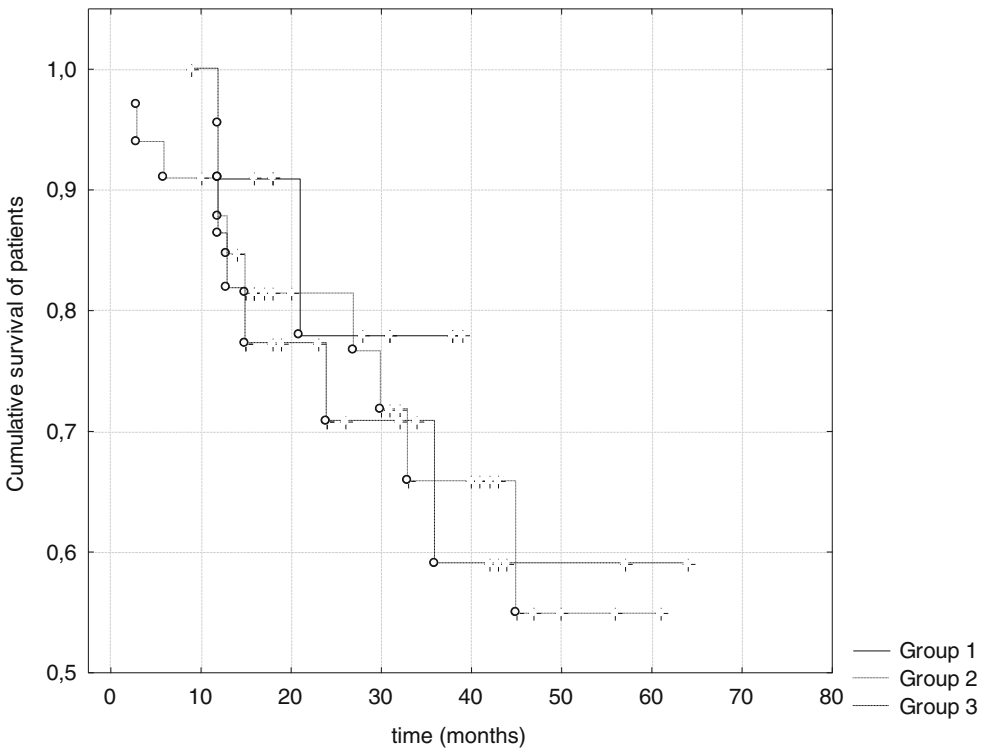

Chart 3 TGF-beta1 expression and disease-free survival of colorectal patients (n=72). 0—TGF-beta1 -negative tumors (n=11), 1—tumors with low expression of TGF-beta1 (n=38). 2—tumors with high expression of TGF-beta1 (n=23)

differentiate two main categories comprising tumors with or without intratumoral regulation of nuclear beta-catenin. Such a patterning was referred to cancer-specific survival and disease-free survival to reveal intratumoral distribution of nuclear beta-catenin as prognostic marker in colon cancer [25]. With all compliments and respects to authors' effort such a pattering seems to be too much time consuming and sophisiticated [25]. Similarly to our studies, unfortunately it is pending only on immunohistochemical semi-quantitive assessment which fails to be precise and sensitivity of immunohistochemical detection is low in comparison to molecular biology methods. On the other hand, authors quoted that most colon cancers harbor mutations of APC or beta-catenin, both of which may result in nuclear beta-catenin accumulation [25]. Thus, if impairment of gene structure is found at wnt-pathway in most of colorectal cancers, beta-catenin related discrimination would be surprising in survival of patients. In our opinion accumulation of beta-catenin appears to be early event of colorectal cancer carcinogenesis so that beta-catenin is significantly higher expressed in adenomas than in normal epithelium. Anyway, there was no significant difference in expression of beta-catenin in colorectal cancers in comparison with adenomas and high-grade intraepithelial neoplasia (HGINs) and this finding suggested that expression of beta-catenin was not remarkably altered in cancers versus adenomas [20]. Thus, it is not astonishing that beta-catenin failed to be significantly associated with disease-free survival of patients, so that it cannot be regarded as independent prognostic factor and a marker of aggressiveness of tumor in our study and previous report by Bondi et al. [26]. However, further studies are required in this field. Similarly, HIF-1 alpha that is a marker of tissue hypoxia and TGF-beta1 failed to be of significantly linked to disease-free survival of the patients in this work.

Moreover, Canavese at al. [27] concluded that expression of the E-cadherin-catenins complex in sentinel node is not associated with cancer dissemination to non sentinel ones. Thus, this protein complex does not constitute metastatic potential and is not a direct causative of formation of non sentinel nodal metastases. Instead of that, a phenotype of primary tumor simply determines expression of the similar immunohistochemical markers both in a maternal tumor and its secondary lesions in lymph nodes [27].

Concerning mixed nuclear-cytoplasmic immunoreactivity to beta-cateinin in our study, it should be mentioned that it is generally believed that it is the nuclear presence of beta-catenin that is pathologic and of importance. Anyway, cytoplasmic expression of beta-catenin is reported along with its nuclear location in variety of tumors [28–30].

Onset of beta-catenin accumulation occurs at early stage of adenoma-colorectal cancer progression. Thus beta-catenin overexpression can mark one of starting points of molecular pathway of colorectal carcinogenesis and can be classified to one of many causatives of malignant transformation but not the only one.

There are at least two reasons why statistically significant positive linkage characterizes relationship between beta-catenin and HIF-1 in our present study of colorectal cancer. First of all simultaneous accumulation of these two proteins would contribute to establishment of eventual statistical relationship. However, beta-catenin is differently induced than HIF-1 expression. Namely, beta-catenin overexpression is triggered by loss of TGf-beta1 function, while HIF-1 is induced by hypoxia of growing malignant tissue [1, 3]. More soundly positive correlation of HIF-1alpha with beta-catenin could be explained by ties of HIF-1alpha with beta-catenin at the promoter region of HIF-1 target genes, though [11].

To sum up, beta-catenin—a marker of early stage of colorectal carcinogenesis, as well as TGF-beta1 and HIF-1alpha appear to be devoid of prognostic significance for colorectal cancer in this study of disease free survival. However, further studies are needed in this field due to limitations of this work, which did not cover overall survival data.

Beta-catenin positively correlated with TGF-beta1 in colorectal cancer despite the expectation that relationship would rather take a form of negative relationship due to antagonizing functions of beta-catenin and TGF-beta1 in normal epithelium.

Beta-catenin significantly associated with HIF-1alpha to reflect in present study previously detected interactions between HIF-1alpha with beta-catenin at the promoter region of HIF-1 target genes.

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Competing interests None

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