β-Catenin and its relation to VEGF and cyclin D1 expression in pT3 rectosigmoid cancers

pT3 rektosigmoid kanserlerde β-katenin ile VEGF, siklin D1 ekpresyonu ilişkisi

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Background/aims: *β-catenin is a critical component of the Wnt signaling pathway that regulates cell proliferation and differentiation. Wnt signaling leads to the stabilization of cytosolic β-catenin and to translocation to the nucleus, where it binds with T-cell factor and promotes the transcription and changes in target gene expression, including vascular endothelial growth factor and cyclin D1. The aim of this study was to assess the expression of cyclin D1 and vascular endothelial growth factor and to correlate them with β-catenin expression and some clinicopathologic parameters.* ***Methods:*** *In this study, we analyzed paraffin-embedded specimens from 42 patients with pT3 rectosigmoid cancer for β-catenin, vascular endothelial growth factor and cyclin D1 expression using immunohistochemistry.* ***Results:*** *Thirty-six (85.7%) and 24 (57.1%) tumors expressed vascular endothelial growth factor and cyclin D1, respectively. Nuclear expression of β-catenin was detected in only 26.1% of tumors. It was revealed that cytoplasmic β-catenin expression was significantly related to vascular endothelial growth factor expression (p=0.011). No association was found between nuclear or cytoplasmic β-catenin and cyclin D1 expression. No significant association was seen between β-catenin, vascular endothelial growth factor or cyclin D1 expression and some investigated clinicopathologic features.* ***Conclusions:*** *Our results may contribute to knowledge regarding the functional interaction between β-catenin and vascular endothelial growth factor. We suggest that the overexpression of cyclin D1 in rectosigmoid cancers may be more complicated than purely upregulation by β-catenin. Further larger studies on Wnt/β-catenin and target gene activity and protein expression are necessary to better understand and define their roles in the pathogenesis of colorectal carcinoma.*

Key words: β-catenin, vascular endothelial growth factor, cyclin D1, Wnt signaling, colorectal carcinoma

Amaç: *β-katenin hücre çoğalması ve başkalaşımını düzenleyen Wnt mesaj yolunun önemli bir unsurudur. Wnt mesajı sitosolik β-catenin'in stabilizasyonuna yol açar ve nukleusa yerleştirir, burada T hücre faktörüne bağlanarak vasküler endotelyal büyüme faktörü ve siklin D1 genlerini içeren hedef genlerde transkripsiyonu ilerleterek gen ekspresyonunu değiştirir. Bu çalışmanın amacı siklin D1 ve vasküler endotelyal büyüme faktörü ekspresyonunu değerlendirerek ve bunların β–katenin ekspresyonu ve bazı klinikopatolojik parametrelerle ilişkisini belirlemekdir.* ***Yöntem:*** *Bu çalışmada pT3 sigmoid kanserli 42 hasta spesmenlerinin parafin bloklarında β-katenin, vasküler endotelyal büyüme faktörü ve siklin D1 ekspresyonu immunhistokimyasal yöntemle analiz edildi.* ***Bulgular:*** *Tümörlerin 36'sında (%85.7) vasküler endotelyal büyüme faktörü ve 24 ünde (%57.1) siklin D1 ekspresyonu vardı. Nukleer β-katenin ekspresyonu tümörlerin sadece %26.1'inde bulundu. Sitoplazmik β-katenin ekspresyonunun vasküler endotelyal büyüme faktörü ekspresyonu ile anlamlı olarak ilişkisi ortaya çıktı (p=0.011). Nukleer ve sitoplazmik β-katenin ile siklin D1 ekspresyonu arasında ilişki bulunmadı. β-katenin, vasküler endotelyal büyüme faktörü ve siklin D1 ekspresyonu ile bazı klinikopatolojik özellikler arasında ilişki görülmedi.* ***Sonuç:*** *Sonuçlarımız β-katenin ve vasküler endotelyal büyüme faktörü arasında fonksiyonel ilişkiye katkıda bulunabilir. Rektosigmoid kanserlerde siklin D1 ekspresyonunun sadece β-katenin düzenlemesine bağlı olmadığı, daha komplike olabileceğini düşünüyoruz. Wnt/β-katenin, hedef gen aktiviteleri ve protein ekspresyonlarını daha iyi anlamak ve kolorektal kanser patogenezindeki rollerini belirlemek için daha ileri çalışmalar gereklidir.*

Anahtar kelimeler: β-katenin, vasküler endotelyal büyüme faktörü, siklin D1, Wnt signaling, kolorektal kanser

INTRODUCTION

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in the survival of cancer cells, in local tumor growth and in the development of distant metastasis (1). The formation of tumor microvessels is stimulated by angiogenic factors such as vascular endothelial growth factor (VEGF, VEGF-A), a 45 kDa glycoprotein that is mitogenic for endothelial cells.

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VEGF binds to specific receptors on endothelial cells, where it induces endothelial proliferation and capillary tube formation, enhancing tumor neovascularization or angiogenesis (1, 2). VEGF is overexpressed by the vast majority of solid human cancers, including colorectal cancer (2, 3).

Cyclin D1 encodes a cell-regulatory protein that is expressed at a high level during the G1 phase of the cell cycle. Cyclin D1 binds to cyclin-dependent kinases and proliferating cell nuclear antigens. The formation of these complexes has been implicated in the control of cell proliferation. Cyclin D1 is an essential protein for cell cycle progression from the G1 to the S phase and has been studied in various malignancies, including colon cancer (4, 5).

β-catenin exists in three different subcellular forms: membrane-bound, cytoplasmic and nuclear. Free β-catenin is degraded after binding to a complex consisting of adenomatous polyposis coli (APC), Axin and glycogen synthase kinase-3β. Binding of Wnt leads to phosphorylation of the cytoplasmic protein, which then binds to Axin and causes dissociation of the APC/Axin/GSK complex (6, 7). This, in turn, means that β-catenin is unable to bind and free β-catenin accumulates. It translocates to the nucleus where it binds to T-cell factors and activates the transcription of a number of genes, including cyclin D1 (8), c-myc (9) and VEGF (10).

The aim of this study was to analyze the expression patterns of β-catenin, VEGF and cyclin D1, and to establish a possible link between loss of β-catenin and VEGF or cyclin D1 expression in rectosigmoid cancers. We also investigated the correlations of β-catenin, VEGF and cyclin D1 expression with some clinicopathological features.

MATERIALS AND METHODS

Archival cases of rectosigmoid cancer and adjacent normal mucosa were retrieved from the archives of the Department of Pathology. Stage was defined according to the American Joint Committee on Cancer criteria (11). Almost all of our cases were pT3 tumors. We selected 46 pT3 tumors classified as adenocarcinomas, and according to World Health Organization (WHO) tumor differentiation grade, 18 tumors were well differentiated (grade 1), 24 were moderately differentiated (grade 2), 3 were poorly differentiated (grade 3), and one was classified as undifferentiated (grade 4) (12). We excluded poorly differentiated and undifferentiated tumors from statistical analysis owing to their small number. None of these patients had received neo-adjuvant chemotherapy or radiotherapy before surgery.

Immunostaining was performed using the standard streptavidin-biotin peroxidase technique (LabVision, Anti-polyvalent HRP; CA, USA). Briefly, sections were deparaffinized in xylene and then hydrolyzed in ethanol. Antigen retrieval was performed using a citric acid solution (pH 6.0), which was heated with a microwave (10 min at 650 W). Endogenous peroxidase activity was blocked with a hydrogen peroxide solution (LabVision, CA, USA). Polyclonal catenin-beta Ab-1 (Neomarkers, CA, USA) and monoclonal antibodies (Neomarkers, LabVision, CA, USA), VEGF (clone VG1, dilution 1:50), and cyclin D1 (Clone SP4, dilution 1:100) were used. Sections were incubated with the primary antibody for 30 min at room temperature. The peroxidase reaction was visualized with AEC (3-amino-9-ethylcarbozole). Sections were then counterstained with hematoxylin, dehydrated and mounted. All series included positive and negative staining controls.

β-Catenin expression was evaluated using two different categories as membranous or cytoplasmic expression and nuclear expression. The membranous or cytoplasmic expression of β-catenin and cytoplasmic expression of VEGF were assessed semiquantitatively. Intensity of stained cells was compared with normal colonic epithelium adjacent to the tumor. Scoring of the immunohistochemistry results was performed on the basis of both the distribution of immunopositive cells and the immuno-intensity, as previously described with slight modification (13, 14). The percentage of β-catenin- and VEGF-positive cells was graded from 0 to 4 (0 = less than 5% of positive cells; 1 = 5%-25%; 2 = 26%-50%; 3 = 51%-75%; 4 = >75%). The intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final score was calculated by adding the scores for the percentage and intensity. An overall immunohistochemistry score of 0-1 was recorded as 0, 2-4 as + (grade 1), and 5,6,7 as ++ (grade 2). We determined, by counting, the percentage of nuclear staining of β-catenin and cyclin D1 among 1,000 tumor cells at 400-fold magnification. The results were decoded and statistically analyzed. We performed the Kruskal-Wallis test, Mann-Whitney U test and Spearman's rank test as correlation analysis for statistical analysis

using the SPSS software (SPSS Standard Software 9.05). A value of p <0.05 was considered statistically significant. The histopathologic characteristics of the patients were compared and assessed using the chi-square test. For these antibodies, tumors were classified as positive or negative for statistical analysis using the chi-square test.

RESULTS

The mean age of patients (21 women, 21 men) at operation was 64.71± 10 years (range: 32–82 years). Tumors ranged in size from 2 to 12 cm in diameter, with a mean size of 5.7 ± 2.61 cm). Twenty-one of the 42 pT3 patients (50.0%) were node-positive and 13 patients (31.0%) had liver metastasis. Thirteen patients were assigned to stage III (31.0%) and 16 to stage II (38.1%). The relationship between immunohistochemical results and clinicopathologic features is summarized in Table 1 and Table 2.

Normal colorectal tissue did not exhibit nuclear staining, showing a membranous localization of β-catenin. Membranous or cytoplasmic immunoreactivity to β-catenin was seen in 39 (92.8%) tumors; of these, 28 (71.8%) cases showed grade 2 staining (Figure 1a). Nuclear β-catenin was seen in 11 (26.1%) carcinomas (Figure 1b). Statistical analysis indicated no significant correlation between the percentage of cells with membranous or cytoplasmic β-catenin immunostaining and that with nuclear expression of the antigen (r=0.107, p>0.05).

Table 1. Association between β-catenin, VEGF, cyclin D1 and some clinicopathological features in 42 rectosigmoid carcinomas

Category	n	B-catenin positive (nuclear) N (%)	p value	B-catenin positive (cytoplasmic) N (%) Grade 1	Grade 2	P value	Cyclin D1 positive N (%)	P value	VEGF positive N (%) Grade 1	Grade 2	P value
Age, yrs			.281			.340		.462			.756
< 50	4	0		1(9.1)	2(7.1)		3(12.0)		1(6.3)	2(10.0)	
≥ 50	38	11(100.0)		10(90.9)	26(92.9)		22(88.0)		15(93.8)	18(90.0)	
Sex			.500			.809		.104			.160
Male	21	6(54.5)		6(54.5)	14(50.0)		10(40.0)		10(47.6)	10(47.6)	
Female	21	5(45.5)		5(45.5)	14(50.0)		15(60.0)		6(28.6)	10(47.6)	
Grade			.443			.410		.555			.662
I	18	4(36.4)		3(27.3)	14(50.0)		11(44.0)		6(37.5)	10(50.0)	
II	24	7(63.6)		8(72.7)	14(50.0)		14(56.0)		10(62.5)	10(50.0)	
Tumor size,			.500			**.022***		.104			.331
cm < 5	16	6(54.5)		8(72,7)	7(25,0)		10(40.0)		8(50.0)	7(35.0)	
≥ 5	26	5(45.5)		3(27.3)	21(75.0)		15(60.0)		8(50.0)	13(65.0)	
N			.500			.809		.265			.177
N0	21	6(54.5)		5(45.5)	14(50.0)		11(44.0)		8(50.0)	8(40.0)	
N1-2	21	5(45.5)		6(54.5)	14(50.0)		14(56.0)		8(50.0)	12(60.0)	
M			.538			.286		.115			.695
M0	29	8(72.7)		6(54.5)	20(71.4)		15(60.0)		11(68.8)	13(65.0)	
M1	13	3(27.3)		5(45.5)	8(28.6)		10(40.0)		5(31.3)	7(35.0)	
Stage			.843			.594		.191			.621
II	16	5(45.5)		3(27.3)	11(39.3)		7(28.0)		6(37.5)	6(30.0)	
III	13	3(27.3)		3(27.3)	9(32.1)		8(32.0)		5(31.3)	7(35.0)	
IV	13	3(27.3)		5(45.5)	8(28.6)		10(40.0)		5(31.3)	7(35.0)	

Ps were calculated by the chi-square test. * significant association (p<0.05).

Table 2. Association between nuclear, cytoplasmic β-catenin, and VEGF, cyclin D1 and some clinicopathological features in rectosigmoid carcinomas (the correlation coefficiency (r) and P value were calculated by Spearman's rank analysis)

	VEGF p(r)	Cyclin D1 p(r)	Age p(r)	Sex p(r)	Tumor size p(r)	Grade p(r)	N p(r)	M p(r)	Stage p(r)	β-catenin (cytoplasmic) p(r)
β-catenin (cytoplasmic)	**0.011* (.389)**	.786 (-.043)	.577 (.089)	.835 (-.033)	.835 (-.033)	.214 (-.196)	.835 (-.033)	.923 (-.015)	.845 (.031)	1.000
β-catenin (nuclear)	.677 (-.066)	.703 (.061)	.190 (.227)	.975 (-.005)	.975 (-.005)	.698 (.062)	.975 (-.005)	1.000 (.000)	.927 (-.015)	.499 (.107)

N: Lymph node metastasis. M: Distant metastasis. * Significant association (p<0.05).

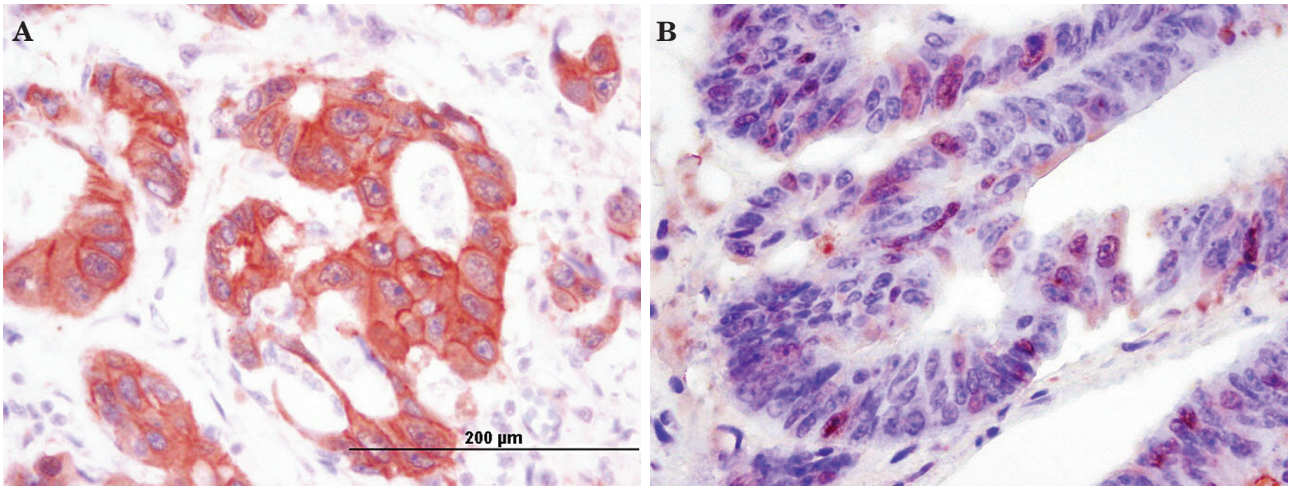

Figure 1. An example of cytoplasmic/membranous (A) and nuclear (B) immunopositivity for β-catenin.

The pattern of VEGF expression in carcinomas was granular cytoplasmic. Adjacent "normal" peritumoral colonic epithelium was only occasionally positive for VEGF. In these cases, its expression was more prominent in the superficial luminal cells. On the contrary, tumors presented a high number of cells stained at the cytoplasmic level (Figure 2a). Thus, **36/42 (85.7%)** were positive and **20 (55.5%)** were positive grade 2. Correlation analysis showed a significant, positive association between cytoplasmic or membranous β-catenin and VEGF expression (r=0.389, p=0.011). No association was found between nuclear β-catenin or VEGF expression and grade or stage of the tumor.

VEGF staining was also observed in lymphocytes, macrophages and fibroblasts of surrounding epithelium and in the tumor. Inflammatory cell positivity was observed in 32 carcinomas. Some tumor vessels also showed VEGF reactivity, which was detected in 31/42 carcinomas. There was no association between VEGF positive inflammatory cells or vessels and clinicopathologic features.

Peritumoral colonic epithelium exhibited no staining of cyclin D1. Twenty-four carcinomas (57.1%) exhibited nuclear cyclin D1 expression (Figure 2b).

Employing Spearman's correlation test, we found that the expression of cytoplasmic β-catenin and VEGF was correlated positively (r=0.389, p=0.011). Interestingly, cyclin D1 expression in tumors was not associated with nuclear and cytoplasmic β-catenin expression (p=0.703, p=0.786, respectively). Correlation analysis showed no significant association between cyclin D1 positivity and some clinicopathologic features such as stage.

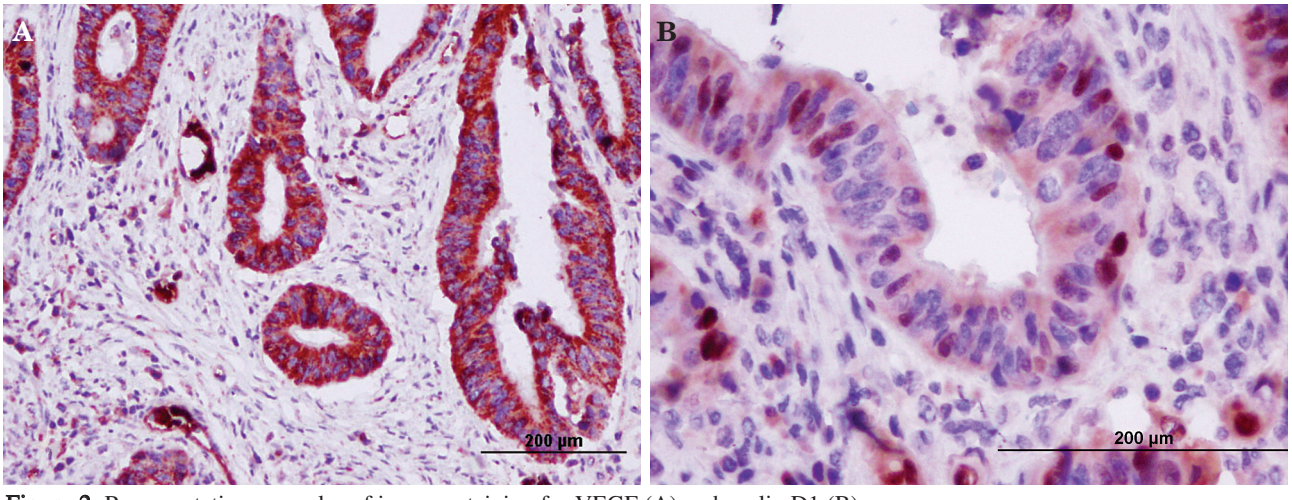

Figure 2. Representative examples of immunostaining for VEGF (A) and cyclin D1 (B).

There were no apparent differences in β-catenin, VEGF and cyclin D1 expression between the tumor periphery and more central areas.

DISCUSSION

Previous immunohistochemical studies of β-catenin in colorectal cancer have shown contradictory results with respect to the distribution of nuclear, cytoplasmic and membranous staining and clinical outcome. Loss of membranous and increased nuclear β-catenin expression in colorectal tumors has been shown to be associated with disease stage and short survival in several but not all studies (4, 14-18). A tissue microarray-based study has shown in a large series of colorectal carcinoma that the majority of cancers retained some degree of β-catenin membranous staining, whereas cytoplasmic or nuclear expression was seen in 42.5% and 20.4% of specimens, respectively (15). In addition, the authors suggested that increased expression of nuclear β-catenin was associated with higher T and N stage. In this study, we found that membranous or nuclear β-catenin expression was not associated with grade or stage in pT3 rectosigmoid cancer. In the tumors that we investigated, positive nuclear staining was seen in only 26%. Maruyama et al. (16) demonstrated 68% cytoplasmic and 66% nuclear accumulation in 96 patients, but only cytoplasmic localization correlated with a significantly worse metastasis-free survival.

VEGF expression in colorectal tumors has been investigated by multiple methods. Data on the prognostic value of VEGF expression in colorectal cancer derive primarily from immunohistochemical studies (19). The expression rate and prognostic value of VEGF expression in colorectal cancer remains unclear, as shown by the discordant results still reported in the literature (20-24). Several studies have indicated VEGF expression in tumor cells as a factor in predicting patient prognosis (21, 23, 24), while others reported no such association (20, 22). The percentage of VEGF-positive colorectal carcinomas has varied from 34% to 100% in studies (20, 21). Among those studies, some reported that VEGF expression was 70%, 67.3% and 66% (22-24). We identified VEGF expression in 36 cases (85.7%), and observed no significant correlations between the levels of VEGF staining intensity and clinicopathological variables such as age, gender, tumor size, tumor differentiation, and stage.

A few studies have investigated the expression of β-catenin and VEGF in colon carcinomas. Zhang et al. (25) showed that VEGF-A promoter activity could be stimulated by oncogenic β-catenin in HeLa cells and suggested that VEGF is the target of the Wnt pathway in early colonic neoplasia. Easwaran et al. (10) indicated that in primary human colon tumors, increased VEGF-A expression correlated with mutations in the APC tumor suppressor gene that activates the β-catenin signal. The authors suggested that there is a close link between β-catenin signaling and the regulation of VEGF expression in colon cancer. We found that cytoplasmic but not nuclear staining of β-catenin was correlated with VEGF expression immunohistochemically.

The frequency of cyclin D1 positivity revealed in colon carcinoma in our cases was 57.1%, similar to the results described by McKay et al. (26) and higher than that reported by some other authors (27, 28). It has been shown that cyclin D1 gene amplification or enhanced expression was correlated with higher histological grade of tumor, lymphatic or hematogenous metastasis and poor prognosis (26, 29). A controversial report, however, exists (5, 27, 30). We have also reported lack of correlation between cyclin D1 positivity and clinicopathological tumor characteristics.

Some investigators thought cyclin D1 was the target gene of β-catenin in colon carcinoma (8, 31). Few studies have investigated the simultaneous expression of these two proteins in these tumors. Utsunomiya et al. (32) indicated that β-catenin overexpression in the cytoplasm may promote malignant transformation by triggering cyclin D1 expression in colorectal cancers. Although a good correlation between the expression of β-catenin and cyclin D1 was found, some cases showed inconsistent expression in their study. Another study showed an immunohistochemical correlation of β-catenin stabilization (cytoplasm/nucleus localization) with cyclin D1 expression in colon carcinomas (33). Our results have not confirmed this finding.

There are many reasons why the immunohistochemical results differ from each other and from ours, for example: sample size, patient population, stage of disease, site of tumor, intrinsic tumor heterogeneity, type of antibody, type of fixative, fixation time, storage time of tumor sections, staining protocol, and the scoring system. Use of different antibodies and their different clones in studies may

be an important factor in these different outcomes. The antibodies used in this study were totally different from those referred to in the previously published data. It is also possible that low levels of protein in various subcellular compartments cannot be distinguished by immunohistochemical studies. The number of samples investigated in our study was low and comprised only pT3 tumors. Studies dealing with different pT groups should be performed in the future.

A great deal of research interest is focused on different areas of the Wnt pathway and its role in carcinogenesis. Colorectal cancers (over 90%) have a mutation that activates this pathway (7,34). Many alterations to the pathway have been described, but β-catenin would appear to be one of the most important proteins associated with oncogenesis. Many interactions may regulate β-catenin's accumulation, intracellular distribution and regulatory effects (6, 35, 36). As cytosolic levels of β-catenin increase, the protein is accumulated in nuclei. Nuclear β-catenin can interact with members of T cell factor (TCF) family transcription factors, ultimately altering the expression of Wnt target genes such as VEGF and cyclin D1 (8, 10). Nuclear β-catenin is therefore the hallmark of the active Wnt pathway. However, LEF/TCF proteins are not the only nuclear proteins that interact with β-catenin; a subset of the large family of SOX-type transcription factors bind β-catenin and can antagonize β-catenin /TCF-mediated gene expression events through a number of mechanisms (6, 37). β-catenin binding to LEF/TCF, cadherins and APC appears to be mutually exclusive—as demonstrated by the ability of the cytoplasmic domain of cadherins to inhibit the nuclear import of β-catenin and the ability of LEF1 to inhibit the APC-mediated export of nuclear β-catenin (35, 36, 38). Another player in this regulatory network is ICAT (inhibitor of β-catenin and T cell factor), which interacts with β-catenin via the same region as do cadherin, LEF/TCF and APC (35, 36, 39).

The mechanisms by which alterations in this pathway are linked to carcinogenesis are not always clear. In addition, other factors can interact with members of the pathway and regulate the expression and effects of β-catenin.

We have demonstrated a significant association between cytoplasmic expression of β-catenin and VEGF in pT3 rectosigmoid cancers. We presume that VEGF expression may be related to β-catenin-mediated signaling. However, we found no correlation between nuclearly translocated β-catenin and VEGF expression. This lack of correlation suggests that there may be other mechanisms than translocated β-catenin that activate VEGF. The present study has not demonstrated a significant association between cytoplasmic or nuclear expression of β-catenin and cyclin D1 expression. We suggest that the overexpression of cyclin D1 in rectosigmoid cancers may be more complicated than purely upregulation by β-catenin. Additional larger studies will be needed to determine the different mechanisms of β-catenin-mediated signaling and changing target gene expression and to define their roles in colorectal carcinogenesis.

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