Expression of Immunohistochemical Markers of Progression in Pre-cancerous and Cancerous Human Colon: Correlation with Serum Vitamin D Levels

ARGJIRA JUNIKU-SHKOLOLLI[1,2], SUZANA MANXHUKA-KERLIU[3], HALIL AHMETAJ[1], VINEETA KHARE[4] and SADIK ZEKAJ[2]

Departments of [1]Pathophysiology, [2]Gastrohepatology, and [3]Pathology, University Clinical Center of Kosovo, Prishtina, Republic of Kosovo; [4]Christian Doppler Laboratory for Molecular Cancer Chemoprevention, Medical University of Vienna, Vienna, Austria

Abstract. *Background/Aim: We aimed to evaluate vitamin D levels in blood, as well as the immunohistological expression of β-catenin, p21 activated kinase (PAK1), p53 and Ki67 in relation to histological type and grading of colonic tumors. Results were compared to the expression in normal and adenomatous colon. Materials and Methods: We analyzed colorectal specimens from 20 patients with colorectal tumors for expression of β-catenin, PAK1, p53 and Ki67. Associations between the expression of these markers and levels of vitamin D in serum were analyzed. Results: The average 25-hydroxyvitamin D (25OHD) level in a healthy population was 20.53 ng/ml, while that in patients with colorectal cancer was 5.99 ng/ml. The average vitamin D level in patients with positive nuclear β-catenin was 4.58 ng/ml, which was lower than that of patients with negative nuclear β-catenin expression. Patients with positive nuclear PAK1 also had low vitamin D levels in their blood (4.51 ng/ml). Patients with positive nuclear p53 had significantly lower vitamin D levels (4.18 ng/ml), compared to patients without nuclear p53 expression. In patients with Ki67 expression in at least 50% of cells, the average vitamin D level was 6.27 ng/ml, while in patients with Ki67 staining in fewer than 50% of cells, the average vitamin D levels in serum was double (13.42 ng/ml).*

Cancer remains the second leading cause of mortality after cardiovascular disease in industrialized societies. As a result of early detection of colonic polyps by screening and removal before they can develop into cancer, death rates have been dropping. In addition, screening and treatment for colorectal cancer at early stages has improved over the last decades, resulting in more than 1 million survivors of colorectal cancer in the United States.

Fearon and Vogelstein were the first to postulate a genetic model of colorectal tumorigenesis, the adenoma-carcinoma sequence. In this model, the primary event is aberrant activation of the adenomatous polyposis coli (APC)/β-catenin pathway, followed by Rat sarcoma (*RAS*) mutations and subsequently loss of function of the *p53* gene (1). Further research demonstrated that mutations in all three genes occur in only 7% of colorectal cancers, and diverse other pathways can be involved in the process of tumor formation. Observational studies have shown that the adenoma-carcinoma sequence lasts approximately 10 years.

Vitamin D is a secosteroid synthesized in the skin by sun (UV-B) irradiation. Although it is called a vitamin, it is rather recognized as a pro-hormone due to its synthesis and the multiple systemic actions of its metabolites (2). Many epidemiological studies have shown a negative association between colorectal cancer incidence and vitamin D levels (3, 4), as well as colorectal cancer risk and calcium intake (5, 6).The dihydroxylated form of vitamin D, 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) directly affects growth factor and cytokine synthesis and signaling in colonic epithelium and modulates the cell cycle, apoptosis and differentiation (7).

$1,25(OH)_2D_3$ exerts its biological effects by binding to the vitamin D receptor (VDR), thereby regulating gene expression. The active metabolite has prominent antiproiferative, anti-angiogenic, and pro-differentiating action in a wide range of tumor cells due to the VDR being expressed in almost all tissues. Several important cellular signaling pathways can, thus, be acted upon. However, for clinical

Correspondence to: Argjira Juniku-Shkololli, University Clinical Center of Kosovo, Department of Pathophysiology, Hospital Area, Prishtina 10 000, Republic of Kosovo. Tel: +377 44155282, e-mail: argjira.juniku@uni-pr.edu

KeyWords: Vitamin D, β-catenin, PAK1, p53, Ki67, malignant and benign colon tumors.

Table I. *Clinicopathological characteristics of patients with adenoma and patients with adenocarcinoma.*

Gender	Adenoma (n=5)	Carcinoma (n=15)
Male	3	8
Female	2	7
Age, years		
Median	49	64
Range	33-64	41-81
<65 years	5	7
>65 years	0	8
Localisation		
Proximal	0	4
Distal	5	8
Histologic classification		
Adenocarcinoma		11
Mucinous carcinoma		4
Tubular adenoma	3	
Tubulovillous adenoma	1	
Serrated adenoma	1	
Degree of dysplasia (polyps)		
Low	4	
High	1	
Differentiation grade (cancer)		
Well	3	
Moderate	10	
Poor	2	
Tumor staging (TNM-system)		
I (T1-T2,N0,M0)	4	
II (T3-T4,N0,M0)	4	
III (anyT,N1-N2,M0)	3	
IV (any T, any N,M1)	2	
X	2	
Lymph node status		
NX	4	
N0	7	
N1	4	
Metastatical status		
MX	6	
M0	8	
M1	1	

Table II. *Expression of PAK1, β-catenin, p53 and Ki67 by immunohistochemistry in colorectal adenomatous polyps and carcinomas*

Marker	Adenoma (n=5)	Adenocarcinoma (n=15)	*p*-Value
PAK1			
Nuclear			0.011
Weak to moderate	0	2	
Strong	0	0	
Cytoplasmic			0.152
Weak to moderate	1	5	
Strong	0	5	
β-Catenin			
Nuclear			<0.0001
Weak to moderate	0	6	
Strong	0	0	
Membranous+cytoplasmic			0.101
Weak to moderate	4	1	
Strong	0	11	
p53			
Nuclear			0.034
Weak to moderate	1	4	
Strong	0	4	
Cytoplasmic			0.001
Weak to moderate	0	4	
Strong	0	1	
KI67			0.008
Weak to moderate	3	3	
Strong	0	10	

trials, the problem remains of how to administer side-effect-free doses of $1,25(OH)_2D_3$ (8).

The measured range for serum levels of the mono-hydroxylated form of vitamin D, 25OHD (which is the indicator of vitamin D status in the body) in adults is 10-50 ng/ml. Intestinal calcium absorption is optimized at levels above 30 ng/ml and there is an inverse relation between parathyroid hormone secretion and serum levels of 25OHD below 25 ng/ml, the latter indicating vitamin D insufficiency (9).

In kidney cells, 25OHD is converted by CYP27B1, the 1-alpha-hydroxylase into the active metabolite $1,25(OH)_2D_3$. However, other cell types, such as colonocytes, also express vitamin D hydroxylases (10), indicating an autocrine/paracrine function of the active metabolite. Low serum levels of the $25OHD_3$ precursor could result in colonic $1,25(OH)_2D_3$ production that is insufficient for maintenance of autocrine/paracrine regulation of cellular growth and function (11).

In this study, serum vitamin D levels were determined for the first time in the Republic of Kosovo. The Republic of Kosovo is situated at a latitude of 35˚N and has 287 sunny days per year, supposedly ample to provide healthy vitamin D levels.

Numerous investigations reported an increased risk for colorectal cancer in individuals with serum $25OHD_3$ levels below 12 ng/ml (12). It has been suggested that the antitumoral action of $1,25(OH)_2D_3$ in colorectal cancer relies on several mechanisms at the cellular level, such as inhibition of cell proliferation, sensitiveness to apoptosis, induction of epithelial differentiation, cell detoxification metabolism, inhibition of angiogenesis and cell-cell adhesion (2). This prompted us to evaluate immunohistochemical expression of β-catenin and p21 activated kinase (PAK1) (which are involved in Wingless related integration site (WNT)-β-catenin pathway), as well as expression of p53 and Ki67 (markers of apoptosis and cell proliferation, respectively) in colorectal polyps and cancer. To our knowledge, this is the first study of the relationship of histological type and grade of colorectal tumor with the expression of these markers, and a correlation of these results with serum vitamin D levels.

Patients and Methods

Patients. The patient group included nine women and 11 men whose tissue specimens were obtained with consent after colorectal surgery/endoscopy at the University Clinical Center of Kosovo, during the period of April 2012-December 2012. Of the cases initially identified, some had to be excluded due to insufficiency of tissue in the paraffin blocks for analysis. Consequently, a total of 20 cases were considered suitable for the study.

Patients' biochemical characteristics for being included in this study were: normal serum total bilirubin, serum aspartate-amino-transferase (AST) and alanin-amino-transferase (ALT) less or equal to 2.5×allowed limit (2-37 U/L for AST and 3-41 U/L for ALT), normal serum creatinine, serum albumin of3.0 mg/dl or more, and normal serum calcium levels.

The age range overall was from 33 to 81 years (33-64 years for patients with colorectal adenoma and 41-81 years for those with carcinoma). The median age was 60.25 years (49 years for those with adenoma and 64 years forpatients with carcinoma).

Specimen handling. The study group consisted of patients with clinically, endoscopically and microscopically confirmed colorectal cancer or adenoma. All specimens consisted of colonic resection or biopsy samples. The paraffin blocks from selected patients were reviewed by an experienced pathologist (S.M.) in order to choose a representative tumor block. A surrounding normal mucosal tissue block (the tumor margin) was obtained when available. The distribution of cases using histopathological classification was as follows: adenoma, n=5; adenocarcinoma, n=11; and mucinous carcinoma, n=4.

Immunohistochemistry (IHC). Staining was performed by the avidin-biotin method with a monoclonal antibody as primary and diamino-benzidine (DAB) as the chromogen, according to the manufacturers' instructions. From formalin-fixed paraffin-embedded tissue, sections of 3 μm were cut, dewaxed and rehydrated in xylene and ethanol. Endogenous peroxidase was blocked with 7% solution of hydrogen peroxide (15%) in methanol. Antigen retrieval was performed by placing the slides in a citrate buffer and heating in a microwave at 600 W and cooking for 10 minutes, (replacing evaporated water after 5 min). The primary antibodies used were the following: PAK1 (Cell Signaling Techonolgy. Inc, Danvers, Massachusetts, USA; 1:100 dilution), β-catenin (BD Biosciences, Becton, Dickinson and Company, New Jersey, USA; 1:1000 dilution), p53 (Santa Cruz Biotechnology Inc., Heidelberg, Germany; 1:200 dilution), and Ki67 (Abcam Inc., Cambridge, UK; 1:500 dilution). The slides were incubated with the primary antibody overnight at 4˚C, in a humidified chamber, followed by incubation with biotinylated secondary antibody (anti-mouse for β-catenin and anti-rabbit for p53, Ki67 and PAK1, Vector Laboratories, Orton Southgate, Peterborough, UK) and avidin-biotin-horseradish peroxidase complex (Vectastain ABC Kit, PK-6100; Vector Laboratories, Orton Southgate, Peterborough, UK). Staining was visualized with DAB and nuclear counterstaining was performed using hematoxylin. Slides were dehydrated and embedded in Histofluid (Marienfeld, Lauda-Koenigshofen, Germany).

All IHC slides were evaluated by two pathologists and a consensus was reached for each sample. Photography was carried out on an Olympus BX41 microscope (Olympus Corporation, Tokio, Japan). Morphological classification of the colorectal lesions conformed to the recommendations of the WHO and staging was according to the TNM system (13).

For evaluation, positive cells were counted and expressed as a percentage of the total number of neoplastic cells. If staining was present in fewer than 10% of cells, the slide was scored as negative, and if more than 10% of nuclei/cytoplasm were stained, the slide was scored as positive. Staining in positive slides was defined as weak to moderate if 10-50% of cells were stained, and strong if 50-90% of cells were stained. Nuclear Ki67staining ranged from 10% to 70% positivity.

Measurement of serum vitamin D. Serum vitamin D and calcium levels were measured once, during the period April-October. prior to therapy. Serum calcium was normal (not shown). Only in four patients measurement was obtained in late fall, but results fitted into the range obtained between April and October. Serum $25OHD_3$ level was determined by electrochemiluminescence immunoassay in 20 patients (Roche Diagnostics GmBH Mannheim, Germany; limits of detection: 4-100 ng/ml. For comparison, serum vitamin D levels were evaluated in a normal (non cancer) population, consisting of subjects on routine examinations (n=121).

Statistical analysis. ANOVA was used to evaluate the differences in expression of PAK1, β-catenin and p53, between the three groups of patients established by histopathological diagnosis. ANOVA was also used for determining differences between these groups of patients with respect to serum vitamin D levels. A *t*-test was used for Ki67 evaluation. SPSS for Windows software was used to analyze the data (IBM Company, Copyright 1989, 2010 SPSS Inc, New York, USA)

Results

In our study, there were 15 patients with adenocarcinoma and five with adenoma.Three adenomas were tubular, one tubular-villous and one serrated. Only one adenoma exhibited high-grade dysplasia, four had only low-grade dysplasia (Table I).

Among the patients with adenocarcinoma, 11 had non-mucinous adenocarcinomas and four had mucinous adeno-carcinomas (Table I).

Serum vitamin D analysis. While in the normal population the average $25OHD_3$ level was 20.53 ng/ml (range=4-76.6 ng/ml), in patients with colorectal cancer the average $25OHD_3$ level was only 5.99 ng/ml (range=3-23.04 ng/ml); (p=0.0001). Interestingly, the vitamin D level was much higher in patients with adenoma (21.4 ng/ml, ranging from 11.3-30.6 ng/m). When compared to measurements from patients with colorectal cancer, the difference was statistically highly significant again (p=0.0001), according to ANOVA.

Immunohistochemical analysis. PAK1: We evaluated the nuclear and cytoplasmic expression of PAK1. Nuclear PAK1 was expressed in tumors from three out of 15 patients with adenocarcinoma. None of the adenoma specimens exhibited nuclear PAK1 staining (Table II); the difference in extent of staining between adenomas and carcinomas was significant (p=0.011).

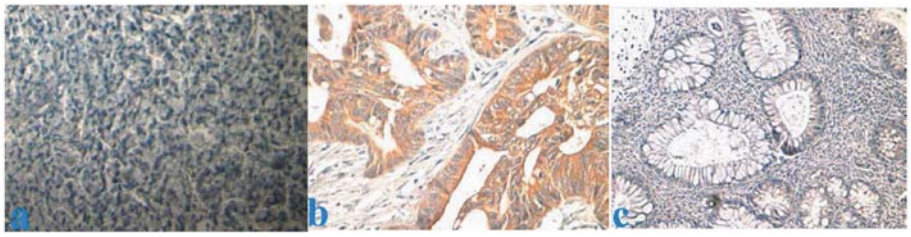

Figure 1. *PAK1 expression in a) mucinous colorectal adenocarcinoma (nuclear expression); b) non-mucinous colorectal adenocarcinoma (cytoplasmic expression); and c) colorectal polyp (no expression).*

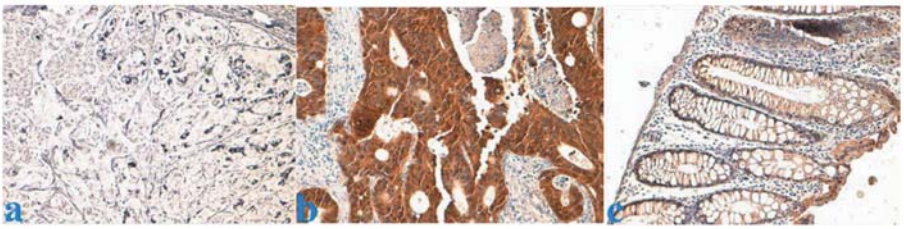

Figure 2. *β-Catenin expression in: a) mucinous adenocarcinoma (nuclear expression); b) non-mucinous colorectal adenocarcinoma (cytoplasmic expression); and c) adenomatous polyp (membranous expression).*

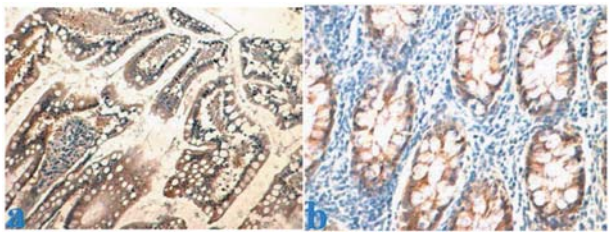

Figure 3. *Membranous expression of β-catenin in the margins of a) mucinous, and b) non-mucinous colorectal adenocarcinoma.*

Most of the adenocarcinomas, both mucinous and non-mucinous, exhibited cytoplasmic PAK1 expression and in 50% of positive specimens, the expression was high (Table II). In the adenoma group, only one patient had cytoplasmic PAK1 expression.

Figure 1 illustrates PAK1 expression. In mucinous adenocarcinomas we found nuclear expression (Figure 1a) in two out of four cases. In non-mucinous adenocarcinomas, there was nuclear expression in only one out of 11 patients, while cytoplasmic expression was found in nine out of 11 cases (strong in five cases and weak to moderate in four) (Figure 1b). In adenomas, we did not observe PAK1 expression, except for weak cytoplasmic expression in one case (Figure 1c).

PAK1 expression also correlated with Dukes' staging: overall PAK1 immunohistochemical staining was positive in 1 out of 3 of Dukes' stage I tumors, in 2 out of 3 of stage II tumors, whereas PAK1 staining was positive in 3 out of 3 stage III tumors and in one single IV stage tumor. All 4 patients with lymph node metastases had positive PAK1 staining, in 3 of which it was cytoplasmic and in the one remaining it was nuclear staining.

Interestingly, the three patients with adenocarcinoma with nuclear PAK1 expression had lower serum vitamin D levels (average=4.51 ng/ml), in comparison to patients with adenocarcinoma (n=12) with no nuclear PAK1 (average= 6.36 ng/ml). However, this difference did not reach statistical significance, probably due to the small number of patients with nuclear PAK1-positive tumor.

β-Catenin. As a dual-function protein, β-catenin regulates cell adhesion at the membrane and gene transcription in the nucleus. β-Catenin expression was evaluated as nuclear *versus* cytoplasmic and membranous staining. Nuclear β-catenin was expressed in six out of 15 adenocarcinomas, whereas in adenoma specimens, nuclear β-catenin staining was negative in all five cases. The difference was statistically significant ($p<0.0001$) (Table II).

Nuclear β-catenin expression was related to grading: grade 3 adenocarcinomas expressed nuclear β-catenin staining, whereas in grade 1 and grade 2 adenocarcinomas, nuclear β-catenin was expressed in only 4 patients out of a total of 13 cases. No adenomatous polyp stained positively for nuclear β-catenin.

Nuclear β-catenin expression increased with advancing tumor stage: in stage I tumors there was none, while in stage II and stage III tumors, expression was markedly present in 4 patients out of 6.

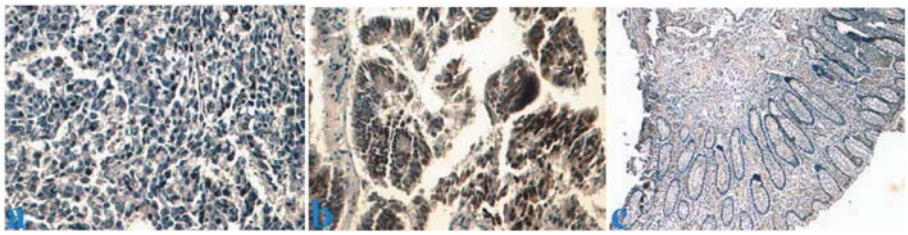

Figure 4. *p53 expression in a) mucinous colorectal adenocarcinoma (nuclear expression);, b) non-mucinous adenocarcinoma (nuclear and cytoplasmic expression); and c) adenomatous polyp (no expression).*

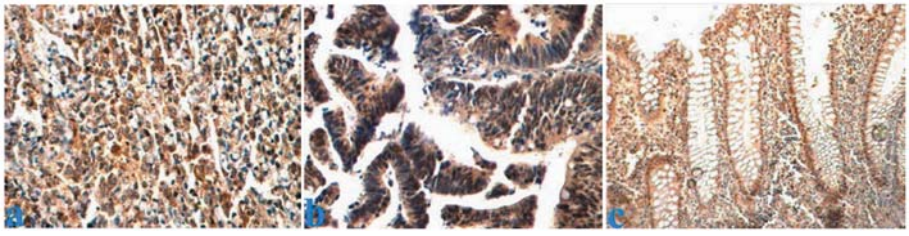

Figure 5. *Ki67 expression in a) mucinous colorectal adenocarcinoma (25% positivity); b) non-mucinous colorectal adenocarcinoma (70% positivity); and (c) adenomatous polyp (10% positivity).*

There was positivity for cytoplasmic and membranous β-catenin expression in 12 out of 15 adenocarcinomas, as well as in four out of five adenomas (Table II).

With respect to tumor grade, cytoplasmic β-catenin staining was strong in 3 out of 3 G1 adenocarcinomas, as well as in 8 out of 10 G2 adenocarcinomas, whereas there was no cytoplasmic staining in G3 adenocarcinomas (for β-catenin staining see Figure 2). Membranous expression was also present in the margins (normal mucosa adjacent to the tumor) of colorectal adenocarcinomas, both mucinous (Figure 3a) and non-mucinous (Figure 3b).

Interestingly, serum vitamin D levels were particularly low in patients with adenocarcinoma with nuclear β-catenin staining; the average serumvitamin D level was 4.58 ng/ml (n=6) compared to patients without nuclear β-catenin expression; 6.93 ng/ml(n=9). The difference almost reached significance (p=0.069).

p53. p53 is frequently mutated in cancer and its nuclear accumulation is associated with further mutations. In our study, nuclear p53 expression in adenocarcinomas (8 of 15) compared to that in adenomas (1 of 5) reached a significant difference (p=0.034) (Figure 4a).

Nuclear p53 expression was found in both G3 adenocarcinomas, in 5 out of 10 G2 adenocarcinomas, and in 1 out of 3 G1 adenocarcinomas, indicating an inverse trend of nuclear p53 expression with increasing differentiation.

5 of 15 patients with adenocarcinomas expressed cytoplasmic p53 (Figure 4b). No polyp specimen exhibited cytoplasmic p53 staining (Figure 4c) and the difference from expression in malignant tumors was highly significant (p=0.001).

Patients with nuclear p53 expression had a lower average serum vitamin D level (4.18 ng/ml; n=8) compared to patients with no nuclear p53 expression (8.05 ng/ml; n=7) and the difference reached significance (p=0.049).

Ki67. Ki67 is a nuclear non-histone protein that is present at low levels in quiescent cells but whose expression is increased in proliferating cells. The median value of Ki67 expression in all colorectal adenocarcinomas was 47%. Ki67 expression was positive (when present in >10% of cells) in 13 out of 15 adenocarcinomas, and in three out of five patients with colorectal adenomas. The staining difference in benign tumors compared to malignant tumors was statistically significant (p=0.008) (Table II).

With respect to tumor grade, we observed a positive Ki67 expression of 48% in well-differentiated carcinomas (G1), 53% in moderately differentiated carcinomas (G2), and 17% in poorly-differentiated carcinomas (G3).

In patients with non-mucinous adenocarcinomas, Ki67 expression was highest (55%), in patients with mucinous adenocarcinoma Ki67 average expression was 26%; and in patients with polyps, Ki67 expression was 16% (p=0.016) (Figure 5).

Regarding normal colonic mucosa, we obtained tissue from margins of colorectal adenocarcinoma from six patients. In all of them, Ki67 expression was very low: less

than 10% (deemed negative) in four cases and less than 15% in the remaining two patients.

In patients with Ki67 expression of ≥50%, serum vitamin D levels were less than half of patients with Ki67 staining in fewer than 50% of cells (average serum´vitamin D was 6.27 ng/ml *versus* 13.42 ng/ml; p=0.0014).

Discussion

Our study summarizes changes in the expression of certain immunohistochemical markers during tumor progression. We investigated markers responsible for apoptosis (PAK1 and p53), cell adhesion (β-catenin), differentiation (p53) and proliferation (Ki67), as well as correlation of their expression with serum vitamin D levels.

Our results support the important concept of vitamin D adequacy in the defense against colorectal tumorigenesis. To the best of our knowledge, no previous study has compared IHC of such markers with serum $25OHD_3$ levels.

In our study, the average serum vitamin D level in patients with colorectal cancer was much lower than that in patients with colorectal adenoma (p=0.0001). Interestingly, among our patients with adenocarcinoma, one had a serum vitamin D level (23.04 ng/ml) within the normal range, while only one patient with adenoma approached vitamin D deficiency (11.3 ng/ml).

Numerous studies have focused on the clinical relevance of nuclear β-catenin accumulation during colorectal pathogenesis, demonstrating its diagnostic as well as prognostic significance (14). A high density of β-catenin nuclear accumulation was associated with higher mortality in selected groups of patients with colorectal cancer (15). Under normal circumstances, β-catenin is part of a complex of proteins that constitute adherens junctions necessary for the creation and maintenance of epithelial cell layers. However, the gene that codes for β-catenin can also function as an oncogene. When β-catenin binds to the product of the mutated *APC* gene, free cytoplasmic β-catenin is destabilized. This leads to the accumulation of nuclear β-catenin, which functions as a transcriptional activator of genes specific for tumor formation (16). Aberrant activation of the WNt/β-catenin signaling pathway due to mutation of *APC* or of β-catenin is the most common and initial alteration in sporadic colorectal tumors (17). It is significant that reduction of transcriptional activity of β-catenin is known to be mediated by $1,25(OH)_2D_3$ and is accompanied by the export of nuclear β-catenin and its relocalization to the plasma membrane(18).

Our study showed a significant increase in nuclear β-catenin expression during progression from adenoma to non-mucinous adenocarcinoma, as well as from non-mucinous adenocarcinoma to mucinous adenocarcinoma. Normal colorectal mucosa (tumor margins) expressed membranous β-catenin and was used as internal positive control. In contrast to other results (19), we found β-catenin over-expression to be most pronounced in mucinous adenocarcinomas. Nuclear β-catenin expression correlated with both tumor grade and stage. Cytoplasmic β-catenin expression was present in most polyps and in all non-mucinous adenocarcinomas, whereas staining was positive in only 25% of mucinous adenocarcinomas. Cytoplasmic β-catenin expression was associated with better tumor differentiation. This observation correlates with results from other authors. The presence of β-catenin expression in the membrane and cytoplasm at an early tumor stage, and nuclear expression at advancing stages, illustrates the sequence of genetic mutations in normal epithelium developing into colorectal tumors.

The PAKs are a family of serine/threonine protein kinases with six isoforms (PAK1-6), which play important roles in cytoskeletal dynamics, cell survival and proliferation (20). Although PAKs are not mutated in cancerogenesis, they are overexpressed, hyperactivated or amplified in several human tumor types. PAK1 has been reported to be overexpressed in colorectal cancer, but its role remains unclear (21). Some recent studies have implicated a role for PAKs in activation of WNT-β-catenin signaling through direct interaction and phosphorylation of β-catenin (see *e.g.* 22). In colorectal cancer, nuclear PAK1 is associated with advanced tumor stage.

In adenocarcinomas, overall (nuclear and cytoplasmic) staining for PAK1 was found in 12 out of 15 cases. Nuclear PAK1 expression was negative in all adenomas, as well as in grade I adenocarcinomas. In grade II adenocarcinomas, nuclear PAK1 was expressed in 2 out of 10 patients, while in grade III adenocarcinomas it was expressed in 1 out of 2 cases.

Our results of PAK1 expression were similar to those of Ye and Field (20), and Zhu *et al.* (23), who found PAK1 expression in 70% of cells. Correlations with tumor grade and stage, as well as with the nodal status (24) indicate PAK1 to be a very important marker during colorectal tumor progression.

p53 is a nuclear protein that induces cell-cycle arrest or apoptosis in response to DNA damage. Its mutations are frequently associated with colorectal oncogenesis. The wild-type p53 gene product has a short half-life and is not detectable by IHC. In contrast, mutant p53 protein has a much longer half-life, accumulates in the nucleus, and creates a stable target for IHC detection (25).

Frequency of p53 expression in our study correlates well with the frequency of p53 mutations found when using sequencing techniques for identification of p53 mutations in sporadic colorectal cancer (26, 27).

We detected nuclear p53 expression in 8 out of 15 patients with adenocarcinoma, while cytoplasmic expression was present in 5 of them. In other studies, the frequency of p53 staining ranges from 45%-60% (28).

Nuclear p53 expression increased with increasing tumor grade: in grade III adenocarcinoma, p53 was expressed in all cases; in grade II adenocarcinomas in half of cases, and in grade I adenocarcinomas, nuclear p53 was expressed in one-third of cases. In the group with colorectal adenoma, only one patient had a tumor with nuclear p53 expression.

Ki67 is a nuclear non-histone protein that is present at low levels in quiescent cells, but is increased in proliferating cells, especially during G_2, M, and the latter half of the S phase. Thus, Ki67 reactivity, defined as the percentage of tumor cells staining positively on IHC staining, is a specific nuclear marker for cell proliferation. While the growth of malignant tumors is highly variable (although it might reflect their clinical course), proliferation still is a key feature of tumor progression.

In our study, the mean Ki67 expression in all colorectal adenocarcinomas was 47%. This is similar to that found by Georgescu *et al*. (48%) (29) and Oshima *et al*. (44%) (30). The proliferative activity as measured by Ki67 antibody was related to histological type and grade: Ki67 expression was higher in non-mucinous adenocarcinomas, compared with mucinous adenocarcinomas (p=0.0164). Ki67 expression was high in well-(G1) and moderately (G2) differentiated adenocarcinomas (52%), compared to poorly-differentiated (G3) adenocarcinomas (17%) (p=0.0314). In colorectal adenomas, Ki67 expression was very low (16%). This indicates a low level of proliferative activity in these lesions. In contrast Georgescu *et al*. observed that Ki67 expression was higher in poorly-differentiated (57%), than in moderately differentiated (34%) and well-differentiated (20%) adenocarcinomas (29).

Correlation of Ki67 expression with histological type of adenocarcinoma resulted in 26% in mucinous *vs*. 55% in non-mucinous colorectal adenocarcinomas. This suggests that proliferative activity in mucinous adenocarcinomas is lower than that in non-mucinous adenocarcinomas.

Taken together, our data show that expression of these biomarkers increased with progression through adenoma to carcinoma sequence. Accumulation of PAK1, β-catenin and p53 in the nucleus revealed correlation with advanced tumor stage. Ki67 expression however, was higher in well-differentiated than in poorly differentiated carcinomas.

Serum vitamin D levels in patients with positive nuclear PAK1 and β-catenin expression had a negative trend, while patients with positive nuclear p53 expression had significantly lower vitamin D levels.

Vitamin D levels were lowest in patients with mucinous adenocarcinoma and correlated with nuclear accumulation of p53, nuclear β-catenin expression and higher expression of Ki67. Considering the importance of an adequate $25OHD_3$ supply for synthesis of the active metabolite $1,25(OH)_2D_3$ in colonic mucosal cells and the relevance of the latter for regulation of the WNT-β-catenin pathway, we suggest that serum $25OHD_3$ levels could be considered as an an indicator of WNT β-catenin activity and increased cell proliferation. This further emphasizes the chemopreventive role of vitamin D in CRC.

As we previously mentioned, the Republic of Kosovo is an area with plenty of incident sunshine and the population should, therefore, not have problems accumulating adequate serum levels of $25OHD_3$. Lifestyle, however, such as clothing and living and working indoors, could influence this. Since a causal relationship between low vitamin D levels and colorectal cancer incidence (with other contributing factors) is becoming increasingly convincing, our data suggest the importance of screening for serum $25OHD_3$ levels in the Kosovo population, with an option to supplement vitamin D against deficiency.

Acknowledgements

Special thanks are due to Professor Dr. Heide S. Cross for critically reviewing the draft and for her professional support and guidance in this research.

We are grateful to Professor Christoph Gasche for the opportunity to carry out part of this research at the Christian Doppler Laboratory for Molecular Chemoprevention at the Medical University of Vienna. We appreciate the help of Michaela Lang MSc. with immunohistochemistry.

This study was supported by Higher KOS Project (Promoting Institutional Development in Higher Education and Research in Kosovo), funded by the Austrian Development Cooperation and Ministry of Education, Science and Technology-Republic of Kosovo, implemented by WUS-Austria, ZSI and OEAD.

Dr.Vesa Kotori helped in evaluating the slides and taking microscopy pictures.

Statistical analysis was performed with the professional assistance of Professor Mytaher Haskuka.

Conflicts of Interest

The Authors declare no conflicts of interest.

References

1 Smith G, Carey FA, Beattie J, Wilkie MJV, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ and Wolf CR: Mutations in *APC*, Kirsten-ras, and p53-alternative genetic pathways to colorectal cancer. PNAS *99(14)*: 9433-9438, 2002.

2 Pereira F, Larriba MJ and Munoz A: Vitamin D and Colon Cancer. Endocrine-Related Cancer *19*: R51-R71, 2012.

3 Tangrea J, Helzlouer K, Pietinen P, Taylor P, Hollis B, Virtamo J and Albanes D: Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men. Cancer Causes Control *8*: 615-625, 1997.

4 Garland CF, Comstock GW, Garland FC, Helsing KJ, Shaw EK and Gorham ED: Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. Lancet *2*: 1176-1178, 1989.

5 Wu K, Willett WC, Fuchs CS, Colditz GA and Giovannucci E: Calcium intake and risk of colon cancer in women and men. J Natl Cancer Inst *94*: 437-446, 2000

6 Nittke T, Kallay E, Manhardt T and Cross HS: Parallel elevation of colonic 1,25-dihydroxyvitamin D3 levels and apoptosis in female mice on a calcium-deficient diet. Anticancer Research *29*: 372-3732, 2009.
7 Cross HS and Kallay E: Regulation of the Colonic Vitamin D System for prevention of tumor progression: an update. Future Oncol. *5(4)*: 493-507, 2009.
8 Ma Y, Trump DL and Johnson CS: Vitamin D in combination cancer treatment. J Cancer *1*: 101-107, 2010.
9 Lips P, Duong T, Oleksik A, Black D, Cummings S, Cox D and Nickelsen T: A global study of vitamin D status and parathyroid function in postmenopausal women with osteoporosis: baseline data from the multiple outcomes of raloxifene evaluation clinical trial. J Clin Endocrinol Metab *86*: 1212-1221, 2001.
10 Bises G, Kallay E, Weiland T, Wrba F, Wenzi E, Bonner E, Kriwanek S, Obrist P, Cross HS: 25-Hydroxyvitamin D3-1-alpha-hydroxylase expression in normal and malignant human colon. J Histochem Cytochem. *52(7)*: 985-9, 2004.
11 Cross HS: Vitamin D Metabolism and Colon Cancer Pathogenesis. CML-Colorectal Cancer *3(4)*: 71-9, 2010.
12 Peterlik M and Cross HS: Vitamin D and Calcium Deficits Predispose for Multiple Chronic Diseases. Eur. J. Clin. Invest. *35(5)*: 290-304, 2005.
13 Stanley R. Hamilton and Lauri A. Aaltonen: TNM staging of tumors of the colon and rectum. In: WHO Organization Classification of Tumours. Pathology & Genetics. Tumors of the Digestive System. IARC Press, Lyon, France, pp. *104*: 2000.
14 Wanitsuwan W, Kanngurn S, Boonpipattanapong T, Sangthong R and Sangkhathat S: Overall Expression of β-catenin Outperforms its Nuclear Accumulation in Predicting Outcomes of Colorectal Cancers. World J Gastroenterol. *14(39)*: 6052-6059, 2008.
15 Wong SCC, Lo ESF, Chan AKC, Lee KC and Hsiao WL: Nuclear β-catenin as a Potential Prognostic and Diagnostic Marker in Patients with Colorectal Cancer from Hong Kong. Mol Pathol. *56*: 347-352, 2003.
16 Behrens J: The Role of Wnt Signaling Pathway in Colorectal Tumorigenesis. Biochem Soc T. *33(4)*: 672-675, 2005.
17 Pendas-Franco N, Aguilera O, Gonzalez-Sancho JM and Munoz A: Vitamin D and Wnt/β-catenin Pathway in Colon Cancer: Role and Regulation of DICKKOPF Genes. Anticancer Research *28*: 2613-2624, 2008.
18 Larriba MJ, Valle N, Palmer HG, Ordonez-Moran P, Alvarez-Diaz S, Becker K-F, Gamallo C, Garcia de Herreros A, Gonzalez-Sancho JM and Munoz A: The Inhibition of Wnt/β-catenin Signalling by 1alpha,25-dihydroxyvitamin D3 is Abrogated by Snail 1 in Human Colon Cancer Cells. Endocr Relat Cancer *14*: 141-151, 2007.
19 Jamieson C, Sharma M and Henderson BR: Targeting the β-catenin nuclear transport pathway in cancer.Semin Cancer Biol. *27*: 20-9. 2014
20 Ye DZ and Field J : PAK Signaling in Cancer. Cellular Logist. *2*: 105-116, 2012.
21 Li LH, Zheng MH, Luo Q, Ye Q, Feng B, Lu AG, Wang ML, Chen XH, Su LP and Liu BY: P21-activated Protein Kinase 1 Induces Colorectal Cancer Metastasis Involving ERK Activation and Phosphorylation of FAK at Ser-910. Int J Oncol. *37(4)*: 951-62, 2010.
22 He H, Huynh N, Liu KH, Malcontenty-Wilson C, Zhu J, Christophi C, Shulkes A and Baldwin GS: P-21 Activated Kinase I Knockdown Inhibits β-catenin Signalling and Blocks Colorectal Cancer Growth. Cancer Lett. *317(1)*: 65-71, 2012.
23 Zhu G, Wang Y, Huang B, Liang J, Ding Y, Xu A and Wu W: A Rac/PAK1 Cascade Controls β-catenin Activation in Colon Cancer Cells. Oncogene *31(8)*: 1001-12, 2012.
24 Ong CC Jubb AM, Haverty PM, Zhou W, Tran V, Truong T, Turley H, O'Brien T, Vucic D, Harris AL, Belvin M, Friedman LS, Blackwood EM, Koeppen H and Hoeflich KP: Targeting p21-activated kinase 1 (PAK1) to induce apoptosis of tumor cells. PNAS *108*: 7177-7182, 2011.
25 Kraiss S, Spiess S, Reihsaus E and Montenarh M: Correlation of Metabolic Stability and Altered Quaternary Structure of Oncoprotein p53 with Cell Transformation. Exp Cell Res *192*: 157-164, 1991.
26 Takayama T, Miyanishi K, Hayashi T, Sato Y and Niitsu Y: Colorectal cancer: Genetics of Development and Metastasis. J Gastroenterol. *41*: 185-92, 2006.
27 Molaei M, Mansoori BK, Ghiasi S, Nemati F, Almasi S, Fatemi SR, Motlagh AG and Zali MR: Cancerogenesis in Colorectal Neoplasms: Evidence From Early Onset Colorectal Cancer. Clin Cancer Investig J. *1*: 57-64, 2012.
28 Poller DN, Baxter KJ and Sepherd NA: p53 and Rb1 protein Expression: Are they prognostically Useful in Colorectal Cancer? Br J Cancer *75*: 87-93, 1997.
29 Georgescu CV, Saftoiu A, Georgescu CC, Ciurea R and Ciurea T: Correlations of Proliferation markers, p53 Expression and Histological Findings in Colorectal Carcinoma. J Gastrointestin Liver Dis. *16*: 133-139, 2007.
30 Oshima TC, Iriva K and Forones NM: Ki-67 as a Prognostic Marker in Colorectal Cancer but not in Gastric Cancer. Neoplasm *5*: 420-4, 2005.

Received October 5, 2014
Revised December 19, 2014
Accepted December 23, 2014