Expression of the *EP300*, *TP53* and *BAX* genes in colorectal cancer: Correlations with clinicopathological parameters and survival

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Abstract. E1A binding protein P300 (*EP300*), tumor protein P53 (*TP53*) and BCL2 associated X, apoptosis regulator (*BAX*) genes encode proteins which cooperate to regulate important cellular processes. The present study aimed to determine the expression levels of *EP300*, *TP53* and *BAX* in colorectal cancer (CRC) and to investigate their prognostic value and association with the progression of CRC. Tumor and matched unchanged colorectal tissues were collected from 121 CRC patients. Quantitative polymerase chain reaction and immunohistochemistry were used to assess the mRNA and protein levels of the studied genes. Altered expression of the studied genes in CRC tissues was observed at both the mRNA and protein levels. The depth of invasion was associated with *TP53* mRNA levels and was correlated negatively with *BAX* mRNA expression. Moreover, a relationship between tumor location and *BAX* mRNA content was noted. BAX immunoreactivity was correlated positively with the intensity of p300 immunostaining and was associated with lymph node involvement and tumor-node-metastasis (TNM) disease stage. Univariate regression analysis revealed that overexpression of p53 and BAX in CRC tissues was associated with poor patient outcome. In conclusion, dysregulation of the expression of the studied genes was found to contribute to CRC pathogenesis. The association between p300 and BAX levels suggests the existence of an interdependent regulatory mechanism of their expression. Moreover, BAX expression may be regulated alternatively, in a p53-independent manner, since the lack of correlations between expression of these factors was observed.

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Introduction

Worldwide, colorectal cancer (CRC) is the third most common cancer in men and the second in women (1). The mortality rate can be reduced by the detection of early-stage disease, since the majority of cases are diagnosed at the late stage, and distant metastasis is the major cause of death in individuals suffering from CRC. Therefore, there is a high need for identification of predictive markers for early detection and more effective prevention and treatment of CRC.

Although many factors and biological mechanisms related to CRC development have been defined, the etiology of this disease is not completely known. The most important causes of CRC include molecular abnormalities, including alterations in gene expression that can be due to aberrations in the epigenetic regulation of chromatin structure and function (2,3). Chromatin activity is influenced by the modulation of nucleosomal histones including their acetylation (4). Histone acetylotransferase (HAT) p300, encoded by the E1A binding protein P300 (*EP300*) gene, mediates histone and non-histone protein acetylation and is involved in gene activation (5). Apart from HAT activity, p300 can also function as a bridge by connecting sequence-specific transcription factors to the transcription apparatus. Moreover, p300 acts as a protein scaffold for the assembly of multicomponent complexes that confer transcriptional activation (6). The p300 transcriptional coactivator protein plays a central role in coordinating and integrating multiple signal-dependent events with the transcription apparatus, allowing the appropriate level of gene activity to occur in response to various physiological cues that influence important cellular processes such as proliferation, differentiation, cell cycle regulation, DNA damage response and apoptosis (6,7). The *EP300* gene is altered in various human tumors. Somatic mutations in this gene have been observed in gastric, colorectal, breast and pancreatic cancers (8,9). Most *EP300* mutations may clearly lead to the loss of function,

supporting the suggestion that loss of p300 activity contributes to tumor development (10). Moreover, high expression of p300 was found to be associated with poor prognosis in breast, hepatocellular, nasopharyngeal, small and non-small cell lung cancers (11-15). The expression dynamics of p300 in CRC and its prognostic significance remain unclear. Huh *et al* reported that p300 overexpression was an indicator of good prognosis in patients with CRC (16), while in a study by Ishihama *et al*, the opposite relationship was revealed since p300 overexpression correlated with a poor prognosis (17). The role of p300 in tumorigenesis is debatable as various studies show that this protein is a tumor suppressor, while others indicate that p300 is a coactivator of several oncogenic transcription factors and promotes cell cycle progression and tumor metastasis (5,7). p300 is an important cofactor in the proper functioning of other tumor-suppressor proteins, including p53. The p53 pathway is modulated by p300 at multiple levels (7,18). p300 controls p53 stability by regulating its ubiquitination and degradation (19). Vleugel *et al* indicated that p300 is a cofactor highly associated with p53 accumulation in invasive breast cancer (20). After DNA damage, p53 is activated, *inter alia*, by acetylation at specific amino acid residues by p300. It has been suggested that p53 acetylation also increases the stability of the p53-DNA complex at target gene promoters. Moreover, p300 is required for p53-mediated transactivation of target genes through its coactivator function and acetylation of target gene histones (7,21). p53 is a transcriptional regulator of the BCL2 associated X, apoptosis regulator (*BAX*) gene, which encodes a downstream pro-apoptotic effector protein (22), and p300 knockdown has been shown to inhibit apoptosis, by disrupting the p53-mediated response to DNA damage (7,23).

Studies of the expression of *EP300*, tumor protein P53 (*TP53*) and *BAX* genes in CRC and their prognostic significance provide contradictory results (16,17,24-29). Furthermore, the association between the expression levels of these genes remains ambiguous. Therefore, the aim of the present study was to analyze and compare the expression levels of *EP300*, *TP53* and *BAX* genes in samples of tumor and unchanged colorectal tissues of CRC patients by quantitative real-time PCR (qPCR) and immunohistochemical (IHC) techniques. Moreover, we studied the relationships between the expression levels of analyzed factors in CRC tissues. To estimate the prognostic value of the studied gene expression levels, we investigated their correlations with clinicopathological parameters, as well as the overall survival (OS) of patients with CRC.

Materials and methods

Patients and the collection of tissue samples. The present study was performed in accordance with the ethical standards and was approved by the Bioethics Committee of the University of Warmia and Mazury in Olsztyn, Poland (decision nos. 3/2010 and 34/2010), and written informed consent regarding the participation in the study and use of tissue was obtained from each patient.

The specimens were collected at the Hospital of the Ministry of Internal Affairs and Administration in Olsztyn (Poland) from 2010 to 2013. The study included 121 patients with CRC (mean age ± SD, 67.91±10.57 years; range, 33-91 years). None of the CRC patients had a second neoplastic disease or suffered from inflammatory bowel disease. None of the patients had previously undergone chemotherapy or radiotherapy. Clinical and demographic data were obtained at the time of enrollment. Data concerning OS were collected for all patients.

Two types of matched samples were obtained within 20 min after the partial surgical resection of the large intestine: i) tumor tissue and ii) macroscopically unchanged mucosa from a distant part of the resected large intestine. Specimens were immediately frozen in liquid nitrogen, and stored at -80˚C for qPCR analysis, whereas for routine histological evaluation and immunohistochemistry, the samples were fixed in 10% neutral buffered formalin and further processed into paraffin blocks.

Total RNA extraction, reverse transcription and real-time quantitative PCR. Total RNA was extracted from all studied tissues and reverse transcribed as previously described (30). Quantification of genes expression was carried out using ABI 7500/7500 Fast Real-Time PCR System (Life Technologies, Applied Biosystems, Foster City, CA, USA). Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) gene was used as an internal control to normalize the transcript levels of *EP300*, *TP53* and *BAX*. The levels of *EP300*, *TP53*, *BAX* and *HPRT1* cDNAs in collected samples were determined using TaqMan® Fast Advanced Master Mix and a respective TaqMan® Gene Expression Assay (for *EP300*, Hs00914223_m1; *TP53*, Hs01034249_m1; *BAX*, Hs00180269_m1; and *HPRT1*, Hs02800695_m1; all from Life Technologies, Applied Biosystems) according to the manufacturer's instructions, and using the following conditions: polymerase activation for 20 sec at 95˚C, followed by 40 cycles of denaturation at 95˚C for 3 sec and annealing/extension at 60˚C for 30 sec. All samples were prepared in duplicates. No template control reactions were performed for each qPCR run. Standard curves consisting of serial dilutions of the appropriate cDNA were used to control the efficiency of the qPCR reactions. Relative quantification of *EP300*, *TP53* and *BAX* expression was evaluated using the ΔΔCt method (31). The fold-change in the relative gene expression was determined by calculating the $2^{-\Delta\Delta Ct}$ value. Fold increase >1 ($2^{-\Delta\Delta Ct}$ >1) indicated overexpression of target RNA in CRC tissue, and fold decrease <1 ($2^{-\Delta\Delta Ct}$ <1) indicated its downregulation.

Immunohistochemistry and staining analysis. Immunoreactivity of the studied proteins was analyzed in sections of 49 tumor and 37 unchanged colorectal tissues of CRC patients. Immunohistochemistry was performed according to previously described methods (30), using rabbit primary antibodies directed against p300 [diluted 1:400 in phosphate-buffered saline (PBS); #ab61217] or BAX (1:400; #ab32503) (both from Abcam, Cambridge, UK). The p53 protein immunostaining was conducted using the Leica ST5010 Autostainer (Leica, Wetzlar, Germany) and ready-to-use antibodies (#IR616; Dako, Glostrup, Denmark) according to the manufacturer's instructions. The negative controls were performed by omitting the primary antibody.

The p300, p53 or BAX immunostained sections were evaluated using Olympus BX41 light microscope (Olympus, Tokyo, Japan) by a pathologist who was blinded to the patient clinical data. Immunoreactivity of p300 and BAX

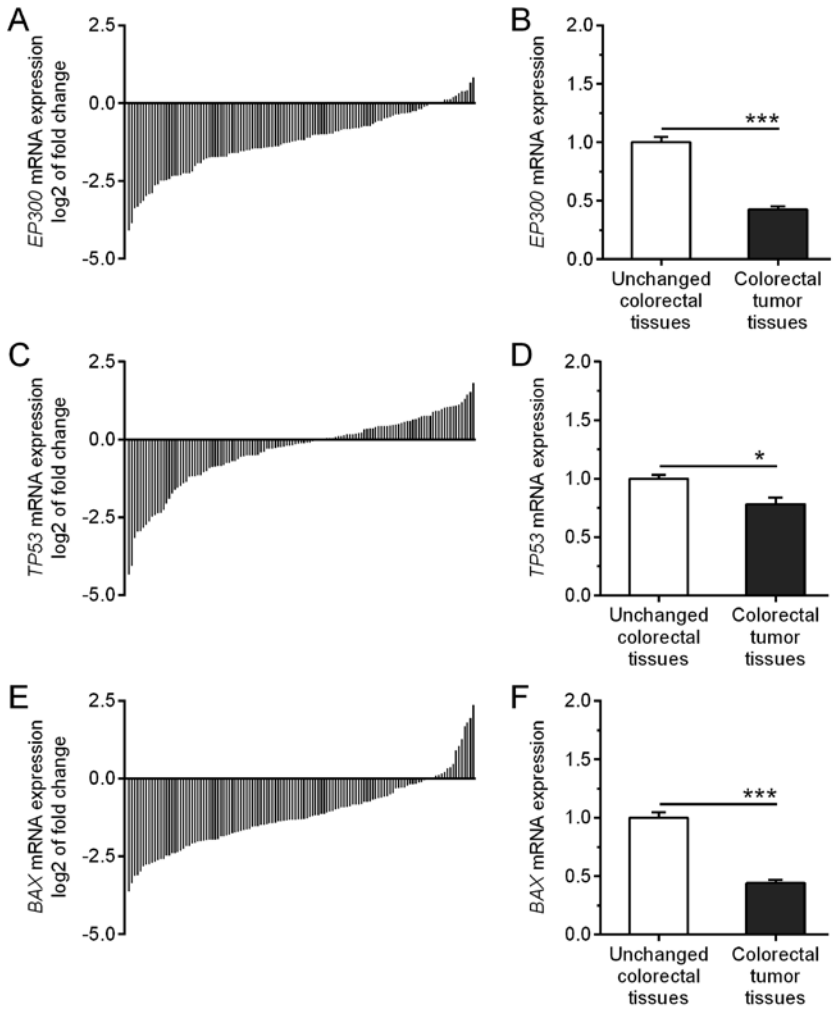

Figure 1. mRNA levels of *EP300*, *TP53* and *BAX* genes in the tumor and unchanged colorectal tissues of the colorectal cancer (CRC) patients (n=121) as determined by quantitative polymerase chain reaction. (A) *EP300*, (C) *TP53* and (E) *BAX* mRNA levels in tumors of individual CRC patients are shown in relation to the *EP300*, *TP53* and *BAX* mRNA content, respectively, in matched unchanged colorectal tissue. The average expression of (B) *EP300*, (D) *TP53*, and (F) *BAX* mRNA (mean ± SEM) in CRC tissues is shown in relation to the value obtained for the unchanged colorectal tissue (1.0); *P<0.05, ***P<0.001.

was assessed in enterocytes or cancer cells of the studied sections using a scale based on the reaction intensity (0, no reaction; 10, up to 10%; 30, 11-30%; 60, 31-60%; 80, 61-80%; and 100, >80%), while immunostaining of p53 was evaluated using a scale based on the percentage of cells showing positive reaction (0, absence of staining; 10, when 1-10% cells were stained; 30, 11-30%; 60, 31-60%; 80, 61-80%; and 100, >80%). Based on median expression values, CRC cases which showed expression scores <30 were regarded as having 'low' expression, whereas scores ≥30 were regarded as 'high' p300, p53 or BAX expression.

Statistical analyses. Statistical analyses were performed using Prism 6 (GraphPad Software, La Jolla, CA, USA) and STATISTICA v.10 (StatSoft, Tulsa, OK, USA) software. The differences in mRNA and protein expression between matched tumor and unchanged colorectal tissue samples of CRC patients were examined by the Wilcoxon matched-pairs test. The correlations between the demographic, clinicopathological and molecular parameters were analyzed by Fisher's exact and Chi-square tests. Pearson's correlation coefficient was used to determine the relationship between the expression levels of the studied factors. The univariate and multivariate survival associations were analyzed using the Cox proportional hazards regression model. The survival curves were plotted according to the Kaplan-Meier method. In all the analyses, the results were considered statistically significant at P<0.05.

Results

Altered expression of EP300, TP53 and BAX mRNAs in CRC tissues. Among the 121 tumor specimens tested, the relative *EP300* mRNA level (tumor tissue vs. matching unchanged mucosa of CRC patients) was decreased in 106 (87.6%) tumors while it was increased in 15 (12.4%) cases (Fig. 1A). The average expression of *EP300* mRNA was significantly decreased in the tumor tissues when compared to that noted in the unchanged tissue of the CRC patients (0.43±0.02 vs. 1.00±0.05; P<0.0001; Fig. 1B). The relative expression of *TP53* mRNA was down-regulated in 67 (55.4%) tumors whereas it was upregulated in 54 (44.6%) specimens (Fig. 1C; Table I). The average expression of *TP53* mRNA was lower in the tumor tissues in comparison to that noted in the non-cancerous colorectal tissues (0.78±0.06 vs. 1.00±0.03; P=0.0207; Fig. 1D). The level of *BAX* mRNA

Table I. Associations between demographic and clinicopathological features of the CRC patients and the relative mRNA expression of the *TP53* gene in CRC tissues.

Parameters	Patients n (%)	*TP53* mRNA levels in tumor vs. unchanged tissues of CRC patients		P-value
		Down (ratio <1) n (%)	Up (ratio >1) n (%)	
Total	121 (100.0)	67 (55.4)	54 (44.6)	
Sex				0.8543
Male	67 (55.4)	38 (56.7)	29 (43.3)	
Female	54 (44.6)	29 (53.7)	25 (46.3)	
Age (years)				0.1005
≤67	60 (49.6)	38 (63.3)	22 (36.7)	
>67	61 (50.4)	29 (47.5)	32 (52.5)	
Location				0.2501
Right	44 (36.4)	20 (45.5)	24 (54.5)	
Left	29 (24.0)	18 (62.1)	11 (37.9)	
Rectum	48 (39.7)	29 (60.4)	19 (39.6)	
Malignancy grade				0.7528
G2	110 (90.9)	60 (54.5)	50 (45.5)	
G3	11 (9.1)	7 (63.6)	4 (36.4)	
Depth of invasion (pT status)				0.0249[a]
T1+T2	20 (16.5)	16 (80.0)	4 (20.0)	
T3+T4	101 (83.5)	51 (50.5)	50 (49.5)	
Lymph nodes (pN status)				0.8550
N0	63 (52.1)	34 (54.0)	29 (46.0)	
N1+N2	58 (47.9)	33 (56.9)	25 (43.1)	
Distant metastases (pM status)				0.2688
M0	106 (87.6)	61 (57.5)	45 (42.5)	
M1	15 (12.4)	6 (40.0)	9 (60.0)	
TNM stage				1.0000
I+II	59 (48.8)	33 (55.9)	26 (44.1)	
III+IV	62 (51.2)	34 (54.8)	28 (45.2)	

CRC, colorectal cancer; TNM, tumor-node-metastasis. [a]Significant P-value (<0.05).

Table II. Associations between demographic and clinicopathological features of the CRC patients and the relative mRNA expression of *BAX* gene in CRC tissues.

Parameters	Patients n (%)	*BAX* mRNA levels in tumor vs. unchanged tissues of CRC patients		P-value
		Down (ratio <1) n (%)	Up (ratio >1) n (%)	
Total	121 (100.0)	104 (86.0)	17 (14.0)	
Sex				0.5999
Male	67 (55.4)	59 (88.1)	8 (11.9)	
Female	54 (44.6)	45 (83.3)	9 (16.7)	
Age (years)				
≤67	60 (49.6)	52 (86.7)	8 (13.3)	1.0000
>67	61 (50.4)	52 (85.2)	9 (14.8)	
Location				
Right	44 (36.4)	43 (97.7)	1 (2.3)	0.0074[a]
Left	29 (24.0)	25 (86.2)	4 (13.8)	
Rectum	48 (39.7)	36 (75.0)	12 (25.0)	
Malignancy grade				
G2	110 (90.9)	93 (84.5)	17 (15.5)	0.3598
G3	11 (9.1)	11 (100.0)	0 (0.0)	
Depth of invasion (pT status)				
T1+T2	20 (16.5)	13 (65.0)	7 (35.0)	0.0081[a]
T3+T4	101 (83.5)	91 (90.1)	10 (9.9)	
Lymph nodes (pN status)				
N0	63 (52.1)	53 (84.1)	10 (15.9)	0.6080
N1+N2	58 (47.9)	51 (87.9)	7 (12.1)	
Distant metastases (pM status)				
M0	106 (87.6)	91 (85.8)	15 (14.2)	1.0000
M1	15 (12.4)	13 (86.7)	2 (13.3)	
TNM stage				
I+II	59 (48.8)	49 (83.1)	10 (16.9)	0.4381
III+IV	62 (51.2)	55 (88.7)	7 (11.3)	

CRC, colorectal cancer; TNM, tumor-node-metastasis. [a]Significant P-value (<0.05).

expression was decreased in 104 (86.0%) tumors while it was increased in 17 (14.0%) cases (Fig. 1E; Table II). The average expression of *BAX* mRNA was significantly decreased in the tumor tissues when compared to the unchanged tissues of the CRC patients (0.44±0.02 vs. 1.00±0.05; P<0.0001; Fig. 1F).

Correlations between the mRNA expression of the studied genes in CRC tissues and clinicopathological features. Possible associations of *EP300*, *TP53* and *BAX* expression with selected demographic and clinicopathological parameters were analyzed based on the results of the qPCR analysis. The depth of

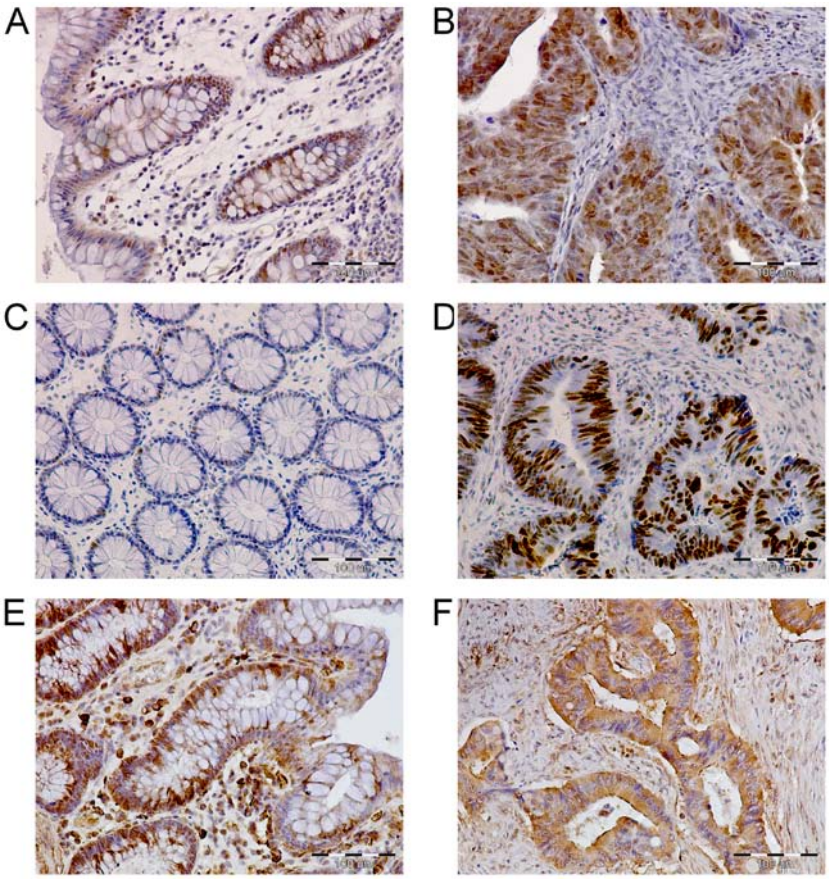

Figure 2. Expression of p300, p53 and BAX proteins in colorectal cancer (CRC) and unchanged colon mucosa as assessed by immunohistochemistry. (A, C and E) Representative sections of unchanged colon mucosa and (B, D and F) CRC show the immunoreactivity for (A and B) p300; (C and D) p53 and (E and F) BAX. Magnification, x200.

invasion was correlated positively with the relative *TP53* mRNA level in CRC tissues (T1+T2 vs. T3+T4, P=0.0249; Table I) and negatively with *BAX* mRNA expression (P=0.0081; Table II). The *BAX* mRNA content was also associated with tumor location (P=0.0074; Table II), demonstrating the lowest levels in right-sided CRC. The *EP300* mRNA level did not correlate with any of the tested parameters, including sex, age, tumor location, malignancy grade, tumor-node-metastasis (TNM) disease stage, depth of invasion, lymph node involvement, or the presence of metastases (P>0.05).

Elevated p300 and p53 immunoreactivity in CRC tissues. p300 immunoreactivity was observed mainly in the nuclei of enterocytes (Fig. 2A) and cancer cells of the analyzed tissues (Fig. 2B). The average intensity of p300 immunostaining was significantly higher in CRC cells as compared to p300 immunoreactivity in enterocytes of the matched unchanged intestinal mucosa (40.54±4.33 vs. 28.38±3.06, respectively; P=0.0219; Fig. 3A). Among the 49 tumor tissue specimens tested, the intensity of p300 staining was high in 35 (71.4%) and low in 14 (28.6%) specimens. p53 immunoreactivity was observed in the nuclei of a few enterocytes of unchanged intestinal mucosa (Fig. 2C) and numerous cancer cells (Fig. 2D). The average p53 immunoexpression was significantly higher in CRC when compared to that in the matched large intestine tissues (46.22±5.58 vs. 0.81±0.45, respectively; P<0.001; Fig. 3B). p53 immunoreactivity was high in 34/49 (69.4%) CRC specimens, whereas low immunostaining was observed in 15/49 (30.6%) cancer tissues. BAX immunoreactivity was found in the cytoplasm of enterocytes (Fig. 2E) as well as cancer cells of the analyzed tissues (Fig. 2F). The average intensity of BAX immunostaining did not significantly differ between the tumor and unchanged tissues of the CRC patients (25.95±3.52 vs. 28.11±3.95, respectively; P>0.05; Fig. 3C). The studied proteins were not detected in the control immunonegative samples, in which immunostaining was performed with the omission of the primary antibodies.

The intensity of p300 immunostaining in CRC tissues was correlated positively with BAX immunoreactivity (r=0.2903; P=0.043), but not with the *BAX* mRNA level. There were no other significant associations between immunoreactivity of the studied proteins, as well as relationships with transcripts levels of the respective genes (P>0.05).

Correlations between the immunoreactivity of the studied proteins in CRC tissues and clinicopathological characteristics. Possible correlations of the expression levels of the studied proteins in CRC tissues with selected demographic and clinicopathological parameters were analyzed based on the results obtained by immunohistochemical analysis. The intensity of BAX immunostaining was higher in tumor specimens derived from patients diagnosed with: i) lymph

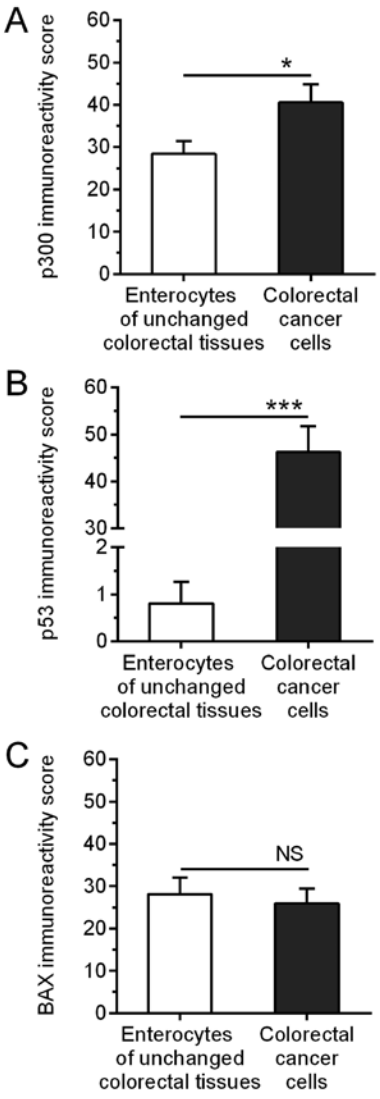

Figure 3. Evaluation of p300, p53 and BAX expression in the tumor and unchanged colorectal tissues by immunohistochemistry. The average immunoreactivity of the (A) p300, (B) p53 and (C) BAX proteins in enterocytes and colorectal cancer (CRC) cells. Bars represent mean ± SEM; *P<0.05, ***P<0.001. NS, not significant.

node involvement (N0 vs. N1+N2; P=0.0448; Table III); and ii) a higher TNM disease stage (I+II vs. III+IV; P=0.0421; Table III). The levels of p300 and p53 immunoreactivity did not correlate with any of the tested parameters, including sex, age, tumor location, malignancy grade, TNM disease stage, depth of invasion, lymph node involvement, or the presence of metastases (P>0.05).

Levels of p53 and BAX immunoreactivity in CRC tissues are associated with patient OS. To estimate the prognostic significance of the studied genes, the levels of their expression in CRC tissues were correlated with patient OS. Median follow-up time of the 49 patients whose tissues were used in both analyses, qPCR and immunohistochemistry, was 47.2 months. During this observation period, 20 (40.8%) patients died.

Univariate Cox regression model revealed that higher levels of p53 and BAX immunoreactivity in CRC tissues were associated with worse patient prognosis (P=0.0499 and P=0.0127, respectively; Table IV). The intensity of p300 immunostaining, as well as expression of the studied genes at the mRNA level were not significantly correlated with patient OS (P>0.05; Table IV). The expression level of any of the studied genes was not an independent prognostic factor in CRC as was revealed by multivariate Cox regression analysis. Kaplan-Meier plots presenting the survival of CRC patients are shown in Fig. 4.

Table III. Associations between demographic and clinicopathological features of the CRC patients and the immunoreactivity of BAX protein in the tumor cells.

		BAX immunoreactivity in CRC cells		
Parameters	Patients n (%)	Score <30 n (%)	Score ≥30 n (%)	P-value
Total	49 (100.0)	20 (40.8)	29 (59.2)	
Sex				0.7733
Male	25 (51.0)	11 (44.0)	14 (56.0)	
Female	24 (49.0)	9 (37.5)	15 (62.5)	
Age (years)				0.0903
≤67	22 (44.9)	12 (54.5)	10 (45.5)	
>67	27 (55.1)	8 (29.6)	19 (70.4)	
Localization				0.9733
Right	14 (28.6)	6 (42.9)	8 (57.1)	
Left	13 (26.5)	5 (38.5)	8 (61.5)	
Rectum	22 (44.9)	9 (40.9)	13 (59.1)	
Malignancy grade				0.6359
G2	45 (91.8)	19 (42.2)	26 (57.8)	
G3	4 (8.2)	1 (25.0)	3 (75.0)	
Depth of invasion (pT status)				0.2096
T1+T2	6 (12.2)	4 (66.7)	2 (33.3)	
T3+T4	43 (87.8)	16 (37.2)	27 (62.8)	
Lymph nodes (pN status)				0.0448[a]
N0	28 (57.1)	15 (53.6)	13 (46.4)	
N1+N2	21 (42.9)	5 (23.8)	16 (76.2)	
Distant metastases (pM status)				0.4446
M0	41 (83.7)	18 (43.9)	23 (56.1)	
M1	8 (16.3)	2 (25.0)	6 (75.0)	
TNM stage				0.0421[a]
I+II	25 (51.0)	14 (56.0)	11 (44.0)	
III+IV	24 (49.0)	6 (25.0)	18 (75.0)	

CRC, colorectal cancer; TNM, tumor-node-metastasis. [a]Significant; P-value (<0.05).

Table IV. Univariate and multivariate Cox proportional hazard regression of overall survival of the colorectal cancer patients.

Covariates	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Sex (men vs. women)	1.82	0.74-4.47	0.1886			
Age (years)	1.05	1.01-1.10	0.0116[a]	1.03	0.96-1.10	0.4010
Location (cecum, ascending and transverse colon vs. rectum)	0.24	0.05-1.05	0.0487[a]	0.19	0.03-1.21	0.0616
Location (descending and sigmoid colon vs. rectum)	1.07	0.40-2.86	0.1568			
Depth of invasion (T1+T2 vs. T3+T4)	NA[b]	NA[b]	0.9925			
Lymph node metastasis (N1 vs. N0)	1.59	0.55-4.58	0.4211			
Lymph node metastasis (N2 vs. N0)	5.56	1.88-16.4	0.0031[a]	2.78	0.33-23.2	0.1415
Distant metastasis (present vs. absent)	6.87	2.62-18.0	0.0001[a]	7.46	1.92-28.9	0.0036[a]
TNM stage (I+II vs. III+IV)	4.22	1.52-11.7	0.0056[a]	1.09	0.08-14.8	0.9458
EP300 mRNA relative expression (RQ)	0.32	0.06-1.73	0.1881			
p300 immunoreactivity (score)	1.00	0.98-1.01	0.8418			
TP53 mRNA relative expression (RQ)	1.14	0.67-1.95	0.6288			
p53 immunoreactivity (score)	1.01	1.00-1.03	0.0499[a]	1.00	0.99-1.02	0.7323
BAX mRNA relative expression (RQ)	1.01	0.49-2.09	0.9735			
BAX immunoreactivity (score)	1.03	1.01-1.05	0.0127[a]	1.03	1.00-1.06	0.0504

Medium follow-up time, 47.2 months. [a]Significant P-value (<0.05). [b]Lack of completed observations in one of the groups. HR, hazard ratio; CI, confidence interval; RQ, relative quantification; NA, not applicable.

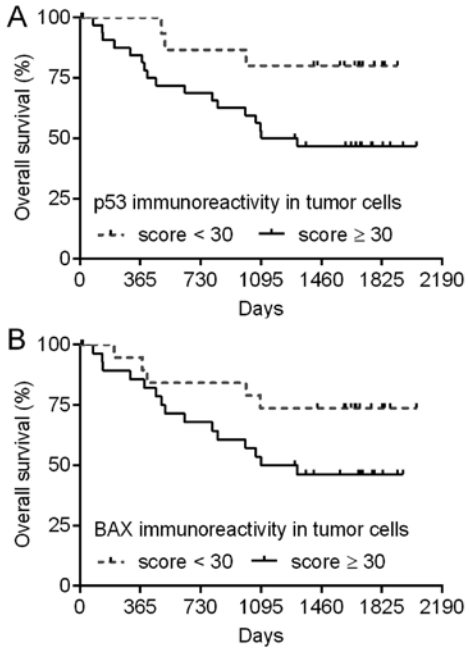

Figure 4. Kaplan-Meier survival curves of 49 colorectal cancer patients regarding immunoreactivity of (A) p53 and (B) BAX proteins.

Discussion

Altered expression of genes that encode important transcriptional coactivators acting with other factors to regulate gene expression can disrupt key cellular processes and lead to carcinogenesis. A better understanding of the mechanisms underlying colorectal cancer (CRC) development and progression may allow improvement in the diagnostic, prognostic and anti-CRC therapeutic approaches. Results of previous studies suggest that p300 protein is a pleiotropic coactivator involved in a number of different pathways, which affect apoptosis, cell cycle control, differentiation and proliferation (32). Somatic mutations in the *EP300* gene have been found in various human malignancies, including CRC (8,9), supporting an idea that this is a tumor-suppressor gene and its dysfunction contributes to tumor formation. Bhandaru *et al* reported that patients with low nuclear p300 expression in melanoma samples have worse 5-year survival (33). Moreover, a study by Ionov *et al* indicated that expression of *EP300* in colon cancer cell lines was associated with slower growth and a higher level of acetylated p53 (34). Furthermore, Krubasik *et al* found that absence of p300 in HCT116 CRC cells induced cellular phenotypic changes characteristic of epithelial to mesenchymal transition (EMT) (35), supporting the finding by Peña *et al* that p300 levels are important in the control of the expression of genes crucial for EMT, and therefore, for tumor progression in human colon cancer (36). However, apart from participating in various tumor-suppressor pathways, p300 is also essential for the activity of many oncogenes (37). It has been found that p300 binds to and acetylates metastasis-associated protein MTA2 to promote CRC cell growth (38). In prostate cancer, p300 was shown to be involved in cell proliferation and progression of this type of cancer (39). Previous investigations revealed that high expression of p300 is associated with aggressive

features and/or poor prognosis in breast, hepatocellular, nasopharyngeal, small and non-small cell lung cancers (11-15). Aberrant expression of the *EP300* gene was also indicated in CRC; however, studies present contradictory results (16,17). It was demonstrated that both mRNA and protein levels were increased in CRC and overexpression of p300 was found to be correlated with poor prognosis (17), while Huh *et al* found that p300 overexpression is an indicator of good prognosis in CRC patients (16). Similarly to Ishihama *et al* (17), we noted high immunoreactivity of p300 in CRC cells. However, we observed the opposite results at the mRNA level finding decreased levels of the *EP300* mRNA in the majority of the tested CRC samples. A low degree of correlation between mRNA levels and actual protein concentrations has also been reported by other authors (40-42). The present study, in contrast to the above mentioned findings, failed to reveal any relationships between the *EP300* expression level and patient survival, and this may result from a shorter follow-up time. Huh *et al* (16) demonstrated the association between p300 expression and lymph node involvement, which was not confirmed in our research. Discrepancies in the results of different studies may be due to the methodological aspects, e.g. choice of different antibodies, method of staining intensity estimation, and number of samples. Another explanation of these discrepancies may be the pleiotropic character of p300 and its function as a coactivator of oncoproteins and tumor-suppressor proteins. p300 appears to be capable of contributing to diametrically opposed cellular processes, and it has been suggested that whether p300 promotes apoptosis or cell proliferation appears to be highly context-dependent (37).

p300 can both positively and negatively regulate p53 transactivation, as well as p53 protein turnover depending on cellular context and environmental stimuli (18). Previous studies have demonstrated that p300 is involved in controlling the stability of the p53 protein by facilitating both mdm2-dependent and -independent ubiquitination, leading to p53 degradation in unstressed cells (19,43). Moreover, it was shown that p300 siRNA increased steady-state p53 abundance and p53 half-life in human osteosarcoma U2OS cells (44). However, it has also been proposed that acetylation plays a positive role in the accumulation of p53 protein in stress response, since inhibition of deacetylation increased the half-life of p53 and promoted its stability (45). In a study by Vleugel *et al,* p300 staining intensity was correlated positively with p53 accumulation in invasive breast cancer (20). Our finding of high levels of p53 protein in CRC and only slight immunoreactivity of this protein in unchanged colorectal mucosa corresponds to the demonstration of p53 overexpression in the majority of CRC tissues (46). However, the present study did not reveal any relationship between expression of p300 and p53 proteins. Although we did not screen our cohort of patients for p53 gene mutations, we hypothesized that the overexpression of p53 protein could be due to genetic mutations that are thought to increase the protein half-life and occur in approximately half of all CRC cases (47). Similarly to other authors (24,25), we found no correlation between p53 levels and clinicopathological features. However, several previous studies demonstrated the relationship between p53 expression and lymph node involvement (48), tumor location, as well as disease stage (49) and T status (46). The present study indicated a correlation between the expression of *TP53* and depth of tumor invasion, but only at the mRNA level. The majority of published findings focusing on *TP53* gene expression and prognosis in CRC have been based exclusively on IHC analyses. The variables related to the staining protocols and scoring system hinder the comparison of the results of different studies. Although p53 protein is one of the most intensively studied, there is no consensus concerning the prognostic value of its expression. Some investigators revealed the lack of correlations between the p53 level in CRC and survival (24,28); however this could not be confirmed by us or by other authors (25,26,50,51). In line with previous studies (26,50), we demonstrated an association between p53 overexpression and worse survival, while in another studies of the same cancer type the opposite relationship was proposed (25,51). These discrepant results may be explained by findings indicating that the prognostic significance of p53 expression may depend on the ethnic group, site of tumor origin in the colon and stage of disease (47). Moreover, Morikawa *et al* demonstrated that p53 positivity was a significant independent predictor of shorter survival among non-obese CRC patients, but not among obese patients (49).

Stabilized p53 protein transactivates downstream targets that mediate apoptosis or cell cycle arrest. Protein levels of these p53-downstream effectors determine cell fate (43). p53 is a transcriptional regulator of the *BAX* gene that is known for its pro-apoptotic activity. It has been shown that the p53-p300-JMY (junction mediating and regulatory protein) complex is enriched in cells exposed to stress and upregulates a variety of p53-dependent target genes, including *BAX* (6). Our finding of the correlation between the expression of p300 and BAX proteins confirms the regulatory link between these proteins. The observed lack of relationship between expression of p300 protein and the level of the *BAX* transcript may result from different methodologies, since immunohistochemical analysis allows assessment of protein expression in specific cell types, while the estimation of the relative levels of mRNA in cancer tissue may be related not only to tumor cells but also to other non-cancerous cells. In contrast, using western blot assay, Iyer *et al* revealed that p300 absence in the HCT116 cell line appeared to have no effect on the pro-apoptotic BAX protein level in cells subjected to UV-induced damage (43). Moreover, it was shown that p300 co-transactivation was not required for BAX regulation (52), whereas an *in vivo* study suggested that p53 was not a major determinant for BAX expression in colorectal carcinomas (53). According to the analysis of BAX expression in breast cancer cells, probably additional regulators, apart from p53, are involved in the regulation of BAX protein expression (54). The lack of correlation between the p53 level and expression of the *BAX* gene in CRC found by us and other authors (27,28), supports this suggestion. Notably, Wincewicz *et al* demonstrated that expression of p53 in CRC was associated with BAX exclusively in younger patients (55). Thus, estimation of the interdependence among p300, p53 and BAX proteins requires further detailed functional studies.

Contrary to findings of enhanced expression of BAX in CRC compared to normal colorectal mucosa (56,57), results of our and Krajewska *et al* (58) studies did not reveal differences in the average Bax immunoreactivity between CRC and unchanged colorectal tissues. However, similarly to the breast cancer study (59), we observed a lower level of *BAX* mRNA

in CRC compared to that found in the matched tissue. Paul-Samojedny *et al* indicated that the ratio of the *BAX* mRNA expression in CRC in relation to that in normal tissue differs depending on the Dukes' stage (60). Previous studies provided contradictory results regarding the BAX expression level in CRC and correlations with clinicopathological parameters. Our findings, but only at the mRNA level, are consistent with the results of Ogura *et al* who found that BAX expression was significantly correlated with reduced depth of tumor invasion (61). In contrast to our observations, various studies revealed negative (29) or no associations (62) between BAX expression and lymph node status. We indicated that high BAX levels correlated with advanced TNM stage, however, the lack of any relationships with tumor stage has also been reported (62). Although studies have revealed that BAX expression is not associated with tumor location (56,62), our finding that the frequency of decreased *BAX* mRNA levels was the highest in right-sided tumors corresponds to a study by Nehls *et al* who observed a correlation between high BAX immunostaining and left-sided tumors (63). Similarly, the evaluation of the prognostic significance of BAX expression provided ambiguous findings since the absence of BAX or its reduced expression was related to poor prognosis (28,29,61) or lack of association was found by other authors (64). Our surprising observation that higher expression of the pro-apoptotic BAX protein in CRC tissues was associated with worse prognosis, reported also by Giatromanolaki *et al* (27), may be partially explained by the involvement of BAX in additional processes, apart from its role in the control of apoptosis, such as the regulation of cell proliferation, since the correlation between BAX expression and proliferative activity was revealed (65).

In summary, to the best of our knowledge, this is the first comprehensive study to analyze the expression of *EP300*, *TP53* and *BAX* genes at the mRNA and protein levels in a cohort of CRC patients as well as the relationships between their expression, clinicopathological parameters and OS of patients. The present study indicated that dysregulation of the studied gene expression may contribute to CRC pathogenesis. The association between p300 and BAX levels suggests the existence of an interdependent regulatory mechanism of their expression. Moreover, *BAX* expression may be regulated alternatively, in a p53-independent manner, since the lack of correlations between expression of these factors was observed. However, further studies are warranted to fully evaluate the mechanisms controlling the expression of the studied genes.

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