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Expression and Prognostic Significance of *EP300*, *TP53* and *BAX* in Clear Cell Renal Cell Carcinoma

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Abstract. *Background: Histone acetyltransferase E1A-binding protein p300 (EP300), tumor protein p53 (TP53) and B-cell lymphoma-2-associated X protein (BAX) contribute to the regulation of the cell cycle and apoptosis, cellular processes that are often impaired in cancer cells. The aim of this study was to determine the expression levels of EP300, TP53 and BAX genes and their respective proteins in clear cell renal cell carcinoma (ccRCC) and evaluate the value of these factors as prognostic factors. Materials and Methods: EP300, TP53 and BAX expression at the transcript and protein levels were determined by quantitative polymerase-chain reaction (QPCR) and immunohistochemistry (IHC) in paired tumor and kidney specimens from 31 patients with ccRCC. Results: Levels of EP300, TP53 and BAX transcripts were found increased in tumor tissues. Immunoreactivity for TP53 was elevated in cancer cells when compared to unchanged kidney, while EP300 and BAX immunoexpression in ccRCC did not differ from that of normal renal tissue. Immunoreactivity for TP53 was positively associated with larger tumor size. In contrast, stronger BAX immunoexpression correlated with smaller tumor diameters. The average immunoreactivity for BAX was higher in localized, kidney-confined tumor than in advanced/recurrent tumors. None of the analyzed transcripts or proteins correlated with the overall survival of patients. Conclusion. Although TP53 and BAX immunoreactivity levels were associated with some clinicopathological parameters of the patients, the expression of EP300, TP53 and BAX did not reveal any prognostic significance in ccRCC.*

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Renal cell carcinomas account for 2-3% of adult malignancies worldwide, with clear cell (ccRCC) being the most prevalent histological subtype (1). Patients with ccRCC are characterized by poor prognosis compared to non-clear cell subtypes of RCC (2). Despite a continuous improvement of imaging techniques and surgical procedures, one-third of RCC cases present with metastatic disease at diagnosis and a one-third of those undergoing nephrectomy of localized tumor will experience relapse (1). Most sporadic ccRCC cases are characterized by the loss of function of the von Hippel-Lindau (*VHL*) tumor-suppressor gene, while mutations in known cancer-related genes such as tumor protein p53 (*TP53*) or *RAS* remain infrequent (3, 4). The inactivation of *VHL* results in accumulation of hypoxia-inducible factors (HIFs) and overexpression of many genes, including those that promote angiogenesis and reprogramming of cellular metabolism. Significant progress in our understanding of the molecular etiopathogenesis of ccRCC has been made over the past decade (3, 4). Nevertheless, new discoveries have not resulted in validation of any prognostic or predictive molecular biomarker that can be routinely used in clinical practice (5, 6).

Histone acetyltransferase E1A-binding protein p300 (EP300) is a ubiquitously expressed pleiotropic multidomain coactivator that catalyzes acetylation of histone and non-histone proteins and functions as a scaffold protein mediating recruitment of transcription machinery to gene promoters (7, 8). EP300 interacts with multiple factors including tumor suppressors or oncogenes. *EP300* mutations contribute to unfavorable phenotype in a number of solid tumors and hematological malignancies and therefore EP300 is often considered as tumor suppressor. In RCC cells, EP300 forms a complex with stabilized HIF1α, which makes it

indispensible for the expression of genes encoding factors associated with the progression of ccRCC: vascular endothelial growth factors, carbonic anhydrase 9, and erythropoietin (9-11). EP300 recruitment is required for transactivation of target genes by both HIF1α and TP53, which compete for limited resources of histone acetyltransferases (12). *VHL* mutations were shown to determine the switch between activity of HIF1α- and TP53-related pathways in RCC cells (13). Inactivation of VHL promoted recruitment of EP300 protein to HIF1α, accompanied by the repression of TP53 pathway, down-regulation of its downstream target gene B-cell lymphoma-2-associated X protein (*BAX*) and low apoptotic activity in RCC cells (13).

Numerous types of cancers are characterized by accumulation of altered, nonfunctional TP53 protein. Therefore, a high level of TP53 immunoreactivity in cancer cells usually indicates the mutation of *TP53* gene and predicts shorter survival (14). However, the latter does not apply to ccRCC, since the whole-exome sequencing of ccRCC tumors showed that *TP53* is mutated only in 2.2% and remains wild-type in most ccRCC cases (4). Nevertheless, the TP53 pathway in RCC cells seems to be repressed and nonfunctional by a mechanism alternative to that of gene mutations or deletions (15). To date, there is still no consensus on either prognostic nor predictive significance of TP53 expression in ccRCC due to conflicting results of many studies (16, 17).

Recent data indicate that interactions between EP300 and TP53 may be of greater importance for ccRCC progression than previously assumed (13). Therefore, the objective of this study was to determine by quantitative polymerase chain reaction (QPCR) and immunohistochemistry (IHC) the expression levels of genes encoding histone acetyltransferase EP300, tumor suppressor TP53 and their downstream target, a programmed cell death regulator BAX, in paired samples of ccRCC tumor and of normal kidney tissue. To estimate the prognostic value of *EP300, TP53* and *BAX* in ccRCC, the associations between the expression data and clinicopathological parameters, as well as overall survival (OS), of the patients were analyzed.

In our previous study of the same group of patients with ccRCC, we found that the expression level of transcription factor PLAG1 like zinc finger 1 (PLAGL1) was associated with the development of metastatic disease and unfavorable prognosis (18). PLAGL1 protein acts as modulator of enzymatic activity and transactivating capacity of other factors, with particular focus on EP300 and TP53 (19). For this reason, we included results of *PLAGL1* expression in the analysis of associations between the expression levels of *EP300, TP53* and *BAX* in the tumor and normal kidney tissues of patients with ccRCC.

Patients and Methods

Ethics statement. This study was carried out in accordance with the principles and procedures approved by the Bioethics Committee for Scientific Research at the University of Warmia and Mazury in Olsztyn, Poland (agreement no. 4/2010). Appropriate written informed consent regarding the use of tissue was obtained from each patient in the study.

Patients and the collection of tissue samples. Specimens were obtained from postoperative material of 31 patients (17 men and 14 women, aged 61.7±10.8, mean±SD, range=27-80 years) with ccRCC who were operated on at the Department of Oncological Surgery, Warmia and Mazury Oncological Center in Olsztyn in the period between March 2010 and August 2012. None of the patients had suffered from a second neoplastic disease or other serious disease. The clinical characteristics, recurrence of ccRCC (regarded as a new onset of metastatic disease) and OS data of the patients were collected. The median observation period was 37.7 months. During this time, tumor recurrence was reported in 6/26 (23.1%) of patients with initially localized disease and 13/31 (41.9%) patients included in the follow-up died.

Paired tissue fragments were obtained immediately after nephrectomy from primary ccRCC tumor tissue and unchanged kidney tissue of the same patient. Tumor and kidney tissue samples for RNA extraction were snap-frozen in liquid nitrogen and stored at −80˚C until further analysis. Tumor and kidney fragments for histological and IHC were placed in 4% buffered formaldehyde and processed into paraffin blocks. The tumor stage was characterized by pathologist according to the TNM system (American Joint Committee on Cancer) (20). Hematoxylin and eosin (H&E)-stained sections of collected tumor and matching kidney specimens were evaluated by a pathologist to confirm presence of cancer or cancer-free phenotype, respectively. None of the collected tumors contained a sarcomatoid component. The degree of tumor malignancy was determined using the Fuhrman nuclear grading system (21). Tumors were grouped by depth of invasion (T) as 'localized' (T1+T2) or 'locally invasive' (T3); by Fuhrman grade (G) as 'low nuclear grade' (G1+G2) or 'high nuclear grade' (G3); and by 'initial metastatic status' (M) as negative (M0) or positive (M1). Additionally, we combined the TNM system with the recurrence status of patients to group ccRCC tumors by their mode of growth and behavior as localized 'kidney-confined' lesions (T1 or T2, negative for metastasis both initially and during follow-up) or potentially harmful 'advanced/recurrent' tumors (meeting at least one of the following criteria: T3 or M1 or recurrence).

RNA extraction, reverse transcription and QPCR. Total RNA was isolated and reverse transcribed according to a modified method described elsewhere (22). The levels of *EP300, TP53* and *BAX* mRNAs in tissue homogenates were determined by QPCR using an ABI 7500 Fast Real-Time PCR System, TaqMan®Fast Advanced Master Mix and TaqMan® Gene Expression Assay: Hs00914223_m1 for *EP300*, Hs01034249_m1 for *TP53* and Hs00180269_m1 for *BAX* (all: Applied Biosystems, Foster City, CA, USA). Gene-expression data were normalized according to TATA box binding protein (*TBP*) mRNA (Hs00427620_m1; Applied Biosystems). The ΔΔCt method was used to determine the fold differences in expression between the paired samples of ccRCC tumor and unchanged kidney.

IHC and evaluation of p300, p53 and BAX immunoreactivity. Paraffin sections of the tumors and renal tissues were stained by the IHC technique using primary rabbit antibodies directed against p300 (polyclonal, diluted 1:400 in PBS; #ab61217; Abcam, Cambridge, UK) and BAX (monoclonal, 1:400 in PBS; #32503, Abcam) according to the modified procedure described previously (18). IHC determination of TP53 immunoreactivity was carried out using ST5010 Autostainer (Leica, Nussloch, Germany) and Monoclonal Mouse Anti-Human p53 Protein Clone DO-7 Ready-to-Use Antibodies for Autostainer Link (#IR616; Daco, Glostrup, Denmark) according to manufacturers' recommendations. All sections were counterstained with hematoxylin.

IHC reactions for EP300, TP53 and BAX in the cancer cells of tumor sections and epithelium of proximal convoluted tubules (PCTs) of unchanged renal tissue were evaluated by pathologist. The intensity of EP300 and BAX IHC reactions was scored as follows: 0 for negative, 1 for trace, 2 for weak, 3 for moderate and 4 for strong. The scoring system for nuclear TP53 immunoreactivity was based on the percentage of TP53-positive ccRCC cells or PCT epithelial cells: 0 for negative, 1 for up to 1%, 2 for up to 2%, 3 for up to 5% and 4 for more than 5% of TP53-positive cells.

To test the correlations between the clinical-pathological data and p300, p53 and BAX immunoreactivity in ccRCC tumors, the specimens were divided into two groups regarded as 'weak' reaction (IHC score less than or equal to the median value) and 'strong' reaction (IHC score above the median value).

PLAGL1 expression data. To calculate the correlation coefficients between EP300 immunoreactivity and *PLAGL1* mRNA and protein expression levels in the same specimens from patients with ccRCC we used PLAGL1 expression data presented in our earlier study (18).

Statistical analyses. These were carried out using Prism software (v. 6.04; Graphpad, La Jolla, CA, USA). Quantitative expression data are presented as means±SEMs. The differences in mRNA and immunoreactivity levels in the tumor and normal kidney tissues were examined by Wilcoxon matched-pairs test. Pearson's coefficients were calculated to measure the strength and direction of the relationships between the expression data of the analyzed genes. The correlation between the clinicopathological characteristics and gene-expression data was disclosed by Fisher's exact test followed by Mann-Whitney test or by Pearson's correlation. The log-rank test was used to evaluate the statistical significance of differences in OS between groups of patients. For all performed analyses, the results were considered statistically significant for values of $p<0.05$.

Results

EP300 mRNA but not protein expression is elevated in ccRCC. EP300 mRNA expression was up-regulated in 25 and down-regulated in six out of 31 ccRCC tumors. The average level of *EP300* mRNA was significantly increased in the tumors as compared to corresponding normal kidney tissues of the patients with ccRCC ($p<0.0001$) (Figure 1A). EP300 immunoreactivity was found in the sections of 26/31 ccRCC and 23/31 normal kidney specimens and was localized mainly to the cell nuclei (Figure 2A and B). The difference between average level of EP300 immunoreactivity in the tumor cells and in PCT epithelial cells of kidney tissue was statistically not significant ($p=0.1073$) (Figure 1B). We did not find any correlation between *EP300* mRNA or EP300 immunoreactivity levels and clinicopathological parameters of patients with ccRCC (Table I).

TP53 mRNA is elevated in ccRCC and TP53 protein immunoreactivity correlates with primary tumor size. TP53 mRNA expression was up-regulated in 27 and down-regulated in 4 out of 31 ccRCC tumors. The average level of *TP53* mRNA in the tumors was significantly higher compared to corresponding normal renal tissues ($p<0.0001$) (Figure 1C). Immunoreactivity for TP53 protein, localized in cell nuclei (Figure 2C and D) was disclosed in 11/31 (35.5%) of ccRCC and only in 1/31 (3.2%) of normal kidney specimens. The average level of TP53 immunoreactivity in tumor cells was much higher than in PCT epithelial cells of renal tissue ($p=0.0020$) (Figure 1D). We did not find any correlations between the levels of *TP53* relative expression and clinicopathological data of the patients (Table II). However, the score for TP53 immunoreactivity of ccRCC cells positively correlated with tumor size (r=0.3807; $p=0.0346$) (Table II). TP53-positive immunoreactivity tended to occur with higher frequency in specimens obtained from men and in those with T3 tumors ($p=0.0570$ and $p=0.0665$, respectively) (Table II), but this correlation was not confirmed by the Mann–Whitney test. Moreover, the differences in TP53 expression with respect to gender (1.12±0.31 for men *vs*. 0.43±0.41 for women; $p=0.0506$) and T status (1.75±0.57 for T3 *vs*. 0.58±0.22 for T1+T2; $p=0.0560$) were close to the cutoff value for statistical significance.

The immunoexpression of BAX protein is negatively associated with tumor growth. BAX mRNA expression was up-regulated in 26 and down-regulated in 5 ccRCC tumors. The average level of *BAX* mRNA was significantly increased in the tumors compared to corresponding normal kidney tissues of the patients with ccRCC ($p<0.0001$) (Figure 1E). Moderate BAX immunoreactivity was observed in the cytoplasm of all studied ccRCC and normal kidney specimens. However, nuclear expression of BAX protein was less common, being noticeably present in cancer cells but only rarely in PCT epithelial cells. The average level of BAX immunoreactivity in tumors and unchanged kidney tissues were similar ($p=0.1210$) (Figure 1F). No relationships were found between *BAX* mRNA and qualitative clinicopathological parameters. BAX immuno-expression in cancer cells negatively correlated with the size of primary tumor (r=–0.3787, $p=0.0356$) (Table III). Moreover, BAX showed a tendency to be underexpressed in advanced/recurrent ccRCCs when compared to kidney-confined lesions ($p=0.0756$) (Table III). The latter observation was confirmed by Mann–Whitney test (2.13±0.19 for advanced/recurrent *vs*. 2.69±0.12 for kidney-confined tumors; $p=0.0295$).

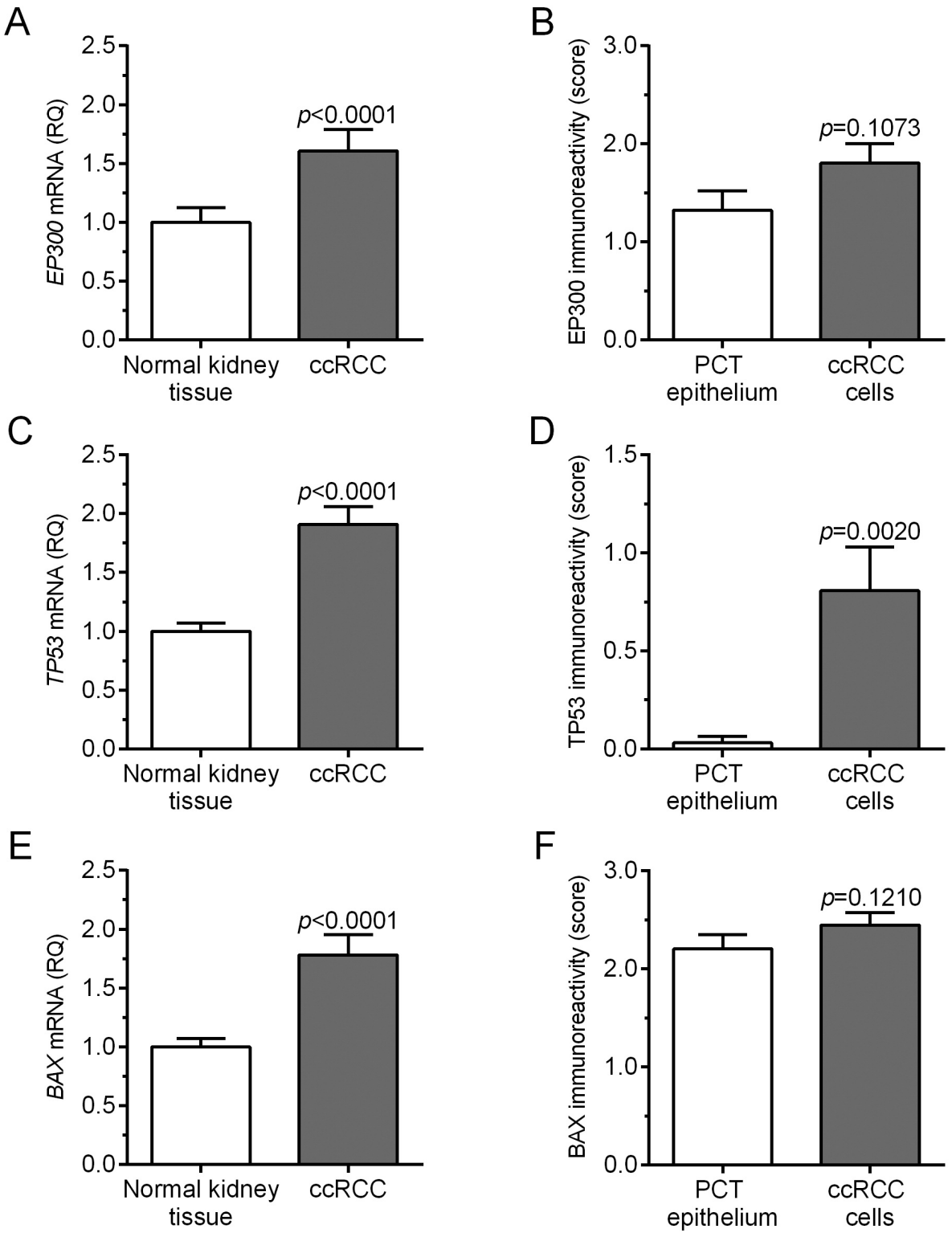

Figure 1. *E1A binding protein p300 (EP300) mRNA (A), EP300 protein (B), tumor protein p53 (TP53) mRNA (C), TP53 protein (D), B-cell lymphoma-2-associated X protein (BAX) mRNA (E) and BAX protein (F) expression levels in clear cell renal cell carcinoma (ccRCC) in relation to the corresponding normal kidney tissue determined by quantitative real-time polymerase chain reaction (A, C and E) or immunohistochemistry (B, D and F). Data are presented as the means±SEMs (N=31). p-Values were calculated using the Wilcoxon matched-pair test. RQ: Relative quantification; PCT: proximal convoluted tubule.*

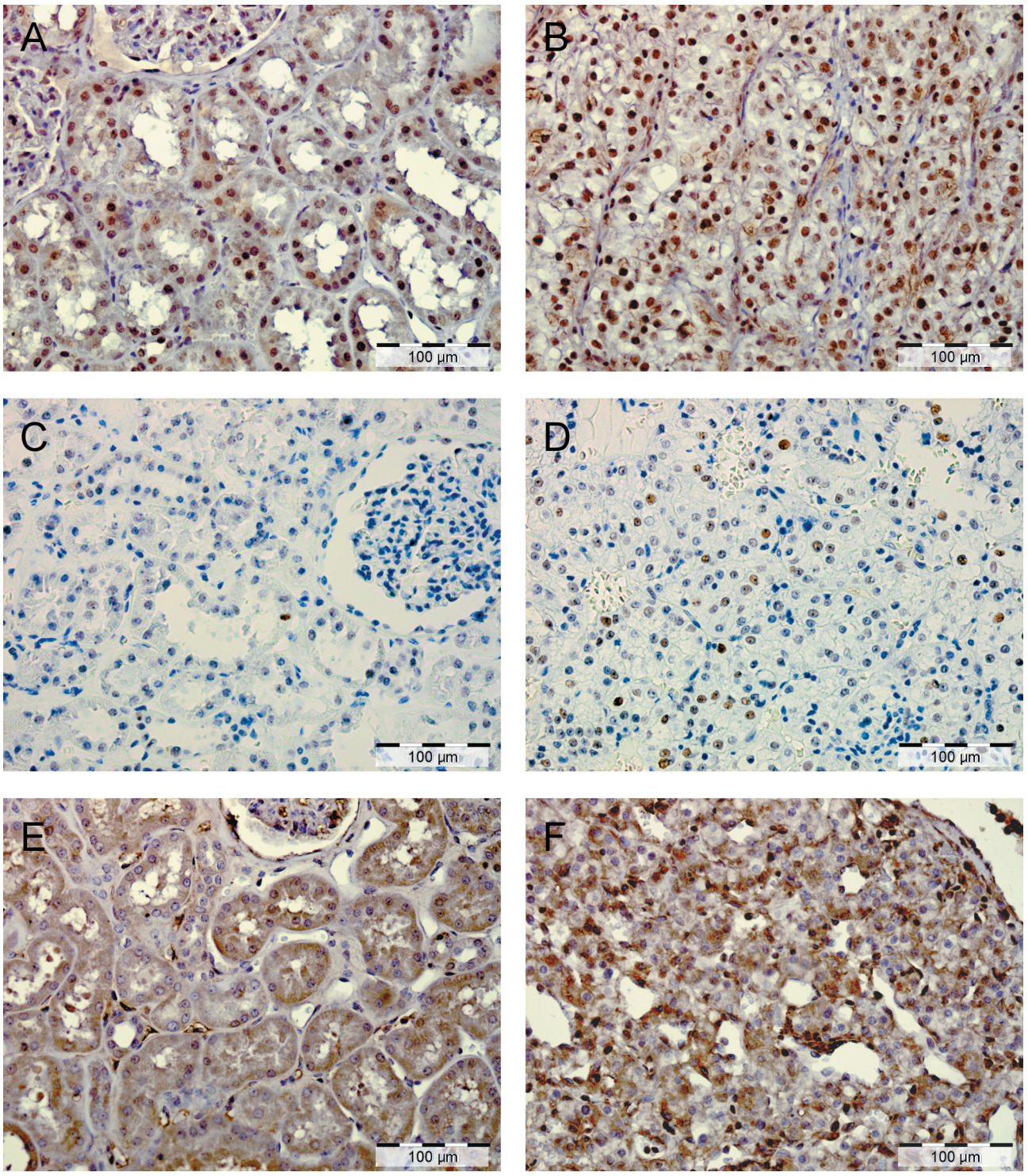

Figure 2. *Immunoreactivity of the studied proteins in sections of normal kidney tissue and clear cell renal cell carcinoma (ccRCC). E1A binding protein p300 (EP300) immunoreactivity was localized mainly in cell nuclei and revealed relatively moderate to strong reactions in epithelial cells from proximal convoluted tubules (PCTs) (A) and cancer cells (B). Nuclear tumor protein p53 (TP53) immunoreactivity was present in normal renal tissue of only one patient in a few cells of PCT epithelium (C), while in the TP53-positive ccRCC tumors,the percentage of imunoreactive cancer cells was considerably higher (D). Similarly, moderate to strong B-cell lymphoma-2-associated X protein (BAX) immunoreactivity was present in the cytoplasm of PCT epithelium (E) and cancer cells (F). In addition to the cytoplasmic BAX expression, some cancer cells revealed nuclear BAX immunoreactivity. Immunohistochemistry was performed as described in the Materials and Methods. Total magnification ×200.*

Table I. *Correlations between clinicopathological characteristics of patients with clear cell renal cell carcinoma (ccRCC) and relative quantification (RQ) of E1A-binding protein p300 (EP300) mRNA in homogenates of the tumors and renal tissues by quantitative real-time polymerase chain reaction and EP300 immunoreactivity in cancer cells by immunohistochemistry.*

Qualitative parameter	Number of patients	*EP300* mRNA in tumors *vs.* renal tissues			EP300 immunoreactivity in cancer cells		
		RQ≤1, n (%)	RQ>1, n (%)	*p*-Value[a]	Score≤2, n (%)	Score>2, n (%)	*p*-Value[a]
Total	31	6 (19.4)	25 (80.6)		22 (71.0)	9 (29.0)	
Men	17	3 (17.6)	14 (82.4)	1.0000	13 (76.5)	4 (23.5)	0.6927
Women	14	3 (21.4)	11 (78.6)		9 (64.3)	5 (35.7)	
Depth of invasion							
T1+T2	24	5 (20.8)	19 (79.2)	1.0000	17 (70.8)	7 (29.2)	1.0000
T3	7	1 (14.3)	6 (85.7)		5 (71.4)	2 (28.6)	
Fuhrman grade							
G1+G2	24	4 (16.7)	20 (83.3)	0.5959	17 (70.8)	7 (29.2)	1.0000
G3	7	2 (29.0)	5 (71.4)		5 (71.4)	2 (28.6)	
Distant metastasis							
M0	26	6 (23.1)	20 (76.9)	0.5533	18 (69.2)	8 (30.8)	1.0000
M1	5	0 (0)	5 (100.0)		4 (80.0)	1 (20.0)	
Tumor growth							
Kidney-confined	16	4 (25.0)	12 (75.0)	0.6539	13 (81.3)	3 (18.8)	0.2524
Advanced/recurrent	15	2 (13.3)	13 (86.7)		9 (60.0)	6 (40.0)	
Quantitative parameters		r		*p*-Value[b]	r		*p*-Value[b]
Age		0.2210		0.2322	–0.1011		0.5884
Tumor size		0.1250		0.5028	0.1663		0.3713

[a]Fisher's exact test; [b]Pearson's correlation.

Altered EP300 immunoreactivity is associated with the expression of TP53 mRNA, PLAGL1 mRNA and PLAGL1 protein. Relative EP300 immunoreactivity, expressed as the fold change between tumor specimens and normal kidney tissues of patients with ccRCC, significantly correlated with relative expression of *TP53* mRNA (r=0.4097, p=0.0221), *PLAGL1* mRNA (r=0.3975, p=0.0268) and PLAGL1 protein (r=0.5488, p=0.0014) (Figure 3).

Expression levels of EP300, TP53 and BAX do not correlate with OS of patients with ccRCC. The levels of *EP300, TP53* and *BAX* mRNA and immunoreactivity did not correlate with OS of patients with ccRCC (Table IV). Higher Fuhrman grade (hazard ratio (HR)=3.76, p=0.0098), presence of distant metastases (HR=3.31, p=0.0331) and invasive or metastatic status of the disease (HR=4.531, p=0.0108) were found to correlate with unfavorable prognosis (Table IV).

Discussion

The present study provides new insights into the expression of *EP300, TP53* and *BAX* in ccRCC and the relationship of their mRNA and protein levels with the progression of this disease. We found that immunoreactivity of TP53 and BAX in tumor cells was associated with clinicopathological data of the patients, including tumor size and progression of the disease. Nonetheless, none of the analyzed molecular factors had prognostic significance in a cohort of patients with ccRCC followed-up for OS. In addition, this study provides the first evidence that in ccRCC, the expression of *PLAGL1* gene at the mRNA and protein levels is associated with immunoreactivity of EP300 protein.

To date, altered expression of EP300 protein or transcript has been reported only in few other cancer types, including esophageal (23), hepatocellular (24) and nasopharyngeal (25) carcinomas, and the overexpression of EP300 was reported to be associated with unfavorable prognosis in these studies. In our cohort of patients with ccRCC, the expression of *EP300* mRNA was up-regulated in the tumors but EP300 immunoreactivity levels in cancer cells were similar to those in tumor-uninvolved renal tissue. Recent bioinformatic assessment of genomic and mRNA expression data in ccRCC revealed that the level of *EP300* transcript was up-regulated in 4% and down-regulated in 1% of cases (26). It is of particular interest that in the cited study, an alteration of *EP300* mRNA was a part of the gene signature associated with better survival of patients with ccRCC (26). The expression levels of *EP300* mRNA and protein determined in our study did not reveal any association of *EP300* with patient outcome, although we did not analyze the contribution of EP300 to the multivariate prognostic signatures.

Table II. *Correlations between clinicopathological characteristics of the patients with clear cell renal cell carcinoma and relative quantification (RQ) of tumor protein p53 (TP53) mRNA in homogenates of tumors and renal tissues by quantitative real-time polymerase chain reaction and TP53 immunoreactivity in cancer cells by immunohistochemistry.*

Qualitative parameter	Number of patients	*TP53* mRNA in tumors *vs*. renal tissues			TP53 immunoreactivity in cancer cells		
		RQ≤1, n (%)	RQ>1, n (%)	*p*-Value[a]	Score=0, n (%)	Score≥1, n (%)	*p*-Value[a]
Total	31	4 (12.9)	27 (87.1)		20 (64.5)	11 (35.5)	
Men	17	1 (5.9)	16 (94.1)	0.3041	8 (47.1)	9 (52.9)	0.0570
Women	14	3 (21.4)	11 (78.6)		12 (85.7)	2 (14.3)	
Depth of invasion							
T1+T2	24	3 (12.5)	21 (87.5)	1.0000	18 (75.0)	6 (25.0)	0.0665
T3	7	1 (14.3)	6 (85.7)		2 (28.6)	5 (71.4)	
Fuhrman grade							
G1+G2	24	3 (12.5)	21 (87.5)	1.0000	14 (58.3)	10 (41.7)	0.3717
G3	7	1 (14.3)	6 (85.7)		6 (85.7)	1 (14.3)	
Distant metastasis							
M0	26	4 (15.4)	49 (84.6)	1.0000	16 (61.5)	10 (38.5)	0.6310
M1	5	0 (0)	6 (100.0)		4 (80.0)	1 (20.0)	
Tumor growth							
Kidney-confined	16	3 (18.8)	13 (81.3)	0.5996	12 (75.0)	4 (25.0)	0.2734
Advanced/recurrent	15	1 (6.7)	14 (93.3)		8 (53.3)	7 (46.7)	

Quantitative parameters	r	*p*-Value[b]	r	*p*-Value[b]
Age	0.1991	0.2829	0.0195	0.9169
Tumor size	0.1106	0.5535	0.3807	**0.0346**

[a]Fisher's exact test; [b]Pearson's correlation. Significant *p*-values (<0.05) are given in bold.

In the present study, we identified a significant up-regulation of *TP53* mRNA in ccRCC. TP53 immunoreactivity was revealed in the cell nuclei of 35.5% of analyzed tumors, while the majority of tumor and all, except for one, unchanged renal tissues were TP53-negative. The expression of TP53 assessed by IHC positively correlated with tumor size and tended to correlate with greater depth of invasion (T3 *vs*. T1+T2). These findings are supported by earlier observation reporting a correlation between higher TP53 expression and advanced T-stage (T3+T4 *vs*. T1+T2) in RCC (27). It was also demonstrated that in patients with RCC with delayed treatment, TP53 immunoreactivity positively correlated with the growth rate of RCC (28). Despite these correlations, TP53 expression level failed to prove useful in the risk assessment for our cohort of patients with ccRCC and this finding is also consistent with some earlier studies (17, 29). However, a prognostic significance of TP53 in ccRCC is still being discussed since others reported that TP53 immunoreactivity together with tumor grade and T-stage were independent prognostic factors of metastasis-free survival in ccRCC (30). It was also demonstrated that TP53 immunoreactivity was a prognostic factor for disease-specific survival in ccRCC and was useful for the construction of prognostic models with high discriminative power (31). Similarly, TP53 overexpression in tumor tissues was significantly associated with shorter survival in two groups of patients presenting different histological types of RCC, with a prevalence of ccRCC cases (27, 32). Although the above-cited studies differ according to the protocols and evaluation criteria for IHC reactions, these differences do not seem to be sufficient to explain the observed discrepancies. We suggest that in certain cohorts of ccRCC cases, TP53 immunoreactivity may be associated with patient outcome, however, the results of our present study do not support the significance of TP53 as a prognostic factor in ccRCC.

We found that *BAX* mRNA was up-regulated in ccRCC specimens, while BAX protein immunoreactivity was similar in cancer cells and PCT epithelium of normal kidney tissue. BAX immunoreactivity in cancer cells correlated negatively with the size of primary tumor and was down-regulated in advanced or recurrent ccRCCs when compared to localized, kidney-confined tumors. Nevertheless, the immunoreactivity for BAX did not reveal prognostic significance in the analyzed cohort of patients with ccRCC. Earlier studies reported negative to weak immunoreactivity for BAX in ccRCC tumors, that was similar to that in PCT epithelium of normal renal tissues (33). BAX protein was prevalently localized to the cytoplasm, but several cancer cells exhibited

Table III. *Correlations between clinicopathological characteristics of the patients with clear cell renal cell carcinoma and relative quantification (RQ) of B-cell lymphoma-2-associated X protein (BAX) mRNA in homogenates of tumors and renal tissues by quantitative real-time polymerase chain reaction and BAX immunoreactivity in cancer cells by immunohistochemistry.*

Qualitative parameter	Number of patients	*BAX* mRNA in renal tissues *vs*. tumors			BAX immunoreactivity in cancer cells		
		RQ≤1, n (%)	RQ>1, n (%)	*p*-Value[a]	Score≤2, n (%)	Score>2, n (%)	*p*-Value[a]
Total	31	5 (16.1)	26 (83.9)		15 (48.4)	16 (51.6)	
Men	17	1 (5.9)	16 (94.1)	0.1484	8 (47.1)	9 (53.9)	1.0000
Women	14	4 (28.6)	10 (71.4)		7 (50.0)	7 (50.0)	
Depth of invasion							
T1+T2	24	3 (12.5)	21 (87.5)	0.5622	10 (41.7)	14 (58.3)	0.2200
T3	7	2 (28.6)	5 (71.4)		5 (71.4)	2 (28.6)	
Fuhrman grade							
G1+G2	24	4 (16.7)	20 (83.3)	1.0000	11 (45.8)	13 (54.2)	0.6851
G3	7	1 (14.3)	6 (58.7)		4 (57.1)	3 (72.9)	
Distant metastases							
M0	26	5 (19.2)	21 (80.8)	0.5301	11 (42.3)	15 (57.7)	0.1719
M1	5	0 (0)	5 (100.0)		4 (80.0)	1 (20.0)	
Tumor growth							
Kidney-confined	16	3 (18.8)	13 (81.3)	1.0000	5 (31.3)	11 (68.7)	0.0756
Advanced/recurrent	15	2 (13.3)	13 (86.7)		10 (66.7)	5 (33.3)	
Quantitative parameters		r		*p*-Value[b]	r		*p*-Value[b]
Age		0.1810		0.3300	0.1412		0.4485
Tumor size		–0.1398		0.4531	–0.3787		**0.0356**

[a]Fisher's exact test; [b]Pearson's correlation. Significant *p*-values (<0.05) are given in bold.

Table IV. *Univariate analysis (log-rank test) of overall survival of patients with clear cell renal cell carcinoma.*

Parameter	Log-rank test	
	HR (95% CI)	*p*-Value
EP300 mRNA expression (RQ>1 *vs*. RQ≤1)	0.56 (0.10-2.29)	0.3691
EP300 immunoreactivity in ccRCC cells (score>2 *vs*. score≤2)	1.01 (0.31-3.32)	0.9764
TP53 mRNA expression (RQ>1 *vs*. RQ<1)	0.54 (0.07-3.02)	0.4173
p53 immunoreactivity in ccRCC cells (score>0 *vs*. score=0)	0.92 (0.30-2.77)	0.8781
BAX mRNA expression (RQ>1 *vs*. RQ<1)	0.93 (0.20-4.36)	0.9289
BAX immunoreactivity in ccRCC cells (score>2 *vs*. score≤2)	0.80 (0.27-2.36)	0.6867
Gender (women *vs*. men)	0.98 (0.33-2.93)	0.9775
Age (>61 *vs*. ≤61 years old)	0.42 (0.14-1.27)	0.1291
Depth of invasion (T3 *vs*. T1+T2)	2.54 (0.88-13.50)	0.0826
Fuhrman grade (G3 *vs*. G1+G2)	3.76 (1.59-25.95)	**0.0098**
Distant metastasis (M1 *vs*. M0)	3.31 (1.17-34.03)	**0.0331**
Tumor growth (kidney-confined *vs*. advanced/recurrent)	4.53 (1.41-12.76)	**0.0108**

EP300: Histone acetyltransferase E1A-binding protein p300; TP53: tumor protein p53; BAX: B-cell lymphoma-2-associated X protein; CI: confidence interval; HR: hazard ratio; RQ: relative quantification. Significant *p*-values (<0.05) are given in bold.

nuclear expression of this protein (33) and our findings correspond well with these observations. However, the prognostic significance of BAX was not evaluated (33). In another study, down-regulation of immunoexpression of BAX in ccRCC correlated with lymph node metastasis and higher tumor stage and grade, but BAX failed to be a prognostic factor for patient outcome (34). In contrast, two IHC studies carried out on specimens derived from different

A

Pearson's correlation of:	*vs.* EP300 immunoreactivity [b]	
	r	*p*-Value
TP53 mRNA[a]	0.4097	**0.0221**
TP53 immunoreactivity [b]	0.1563	0.4010
BAX mRNA[a]	0.1385	0.4573
BAX immunoreactivity [b]	- 0.0171	0.9274
PLAGL1 mRNA[a†]	0.3975	**0.0268**
PLAGL1 immunoreactivity [b†]	0.5488	**0.0014**

[a] fold change ccRCC tumors *vs.* renal tissues; [b] fold change ccRCC cells *vs.* proximal convoluted tubule epithelium; [†] Determined in our previous study (18); ccRCC – clear cell renal cell carcinoma; Significant *p*-values (<0.05) are given in bold.

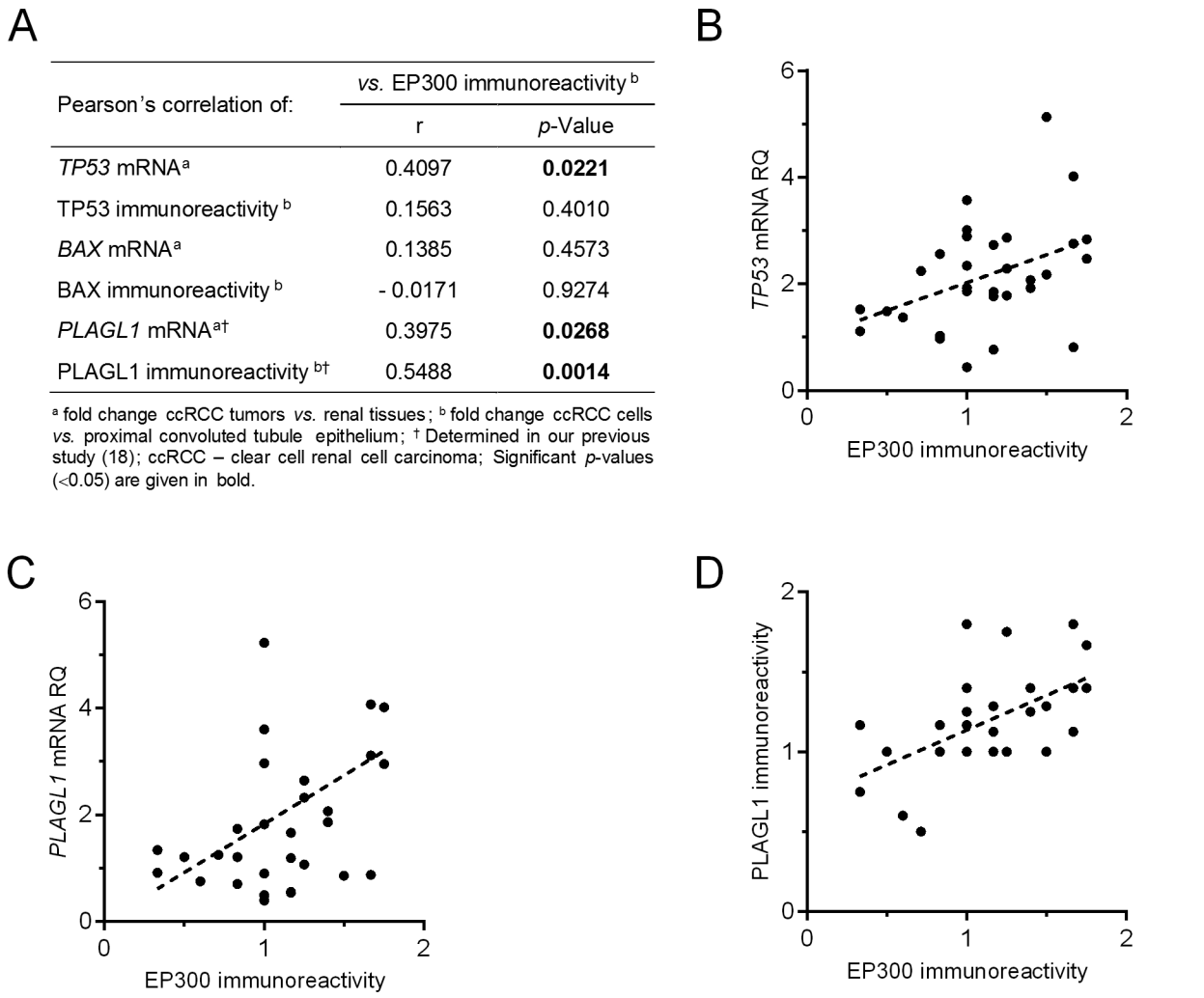

Figure 3. *Pearson's correlations between the fold change of E1A binding protein p300 (EP300) immunoreactivity and the relative fold change expression of tumor protein p53 (TP53) mRNA, TP53 protein, B-cell lymphoma-2-associated X protein (BAX) mRNA, BAX protein, PLAG1 like zinc finger 1 (PLAGL1) mRNA and PLAGL1 protein in tumor vs. normal kidney tissue of 31 patients with clear cell renal cell carcinoma (ccRCC). Correlation coefficients (r) and respective p-values are presented in the Table (inset) (A). Statistically significant correlations were identified between EP300 immunoreactivity and relative quantification (RQ) of TP53 mRNA (B), EP300 immunoreactivity and PLAGL1 mRNA RQ (C) and EP300 immunoreactivity and PLAGL1 immunoreactivity (D). Solid lines in (B-D) indicate the correlation trend.*

histological types of RCC (with prevalence of clear cell tumors) provided opposing results, demonstrating that higher levels of BAX immunoreactivity may be correlated with progression of disease and worse patient outcome, respectively (35, 36). Thus, to date, the prognostic significance of BAX expression in ccRCC remains questionable.

In our previous study, performed on the same group of patients with ccRCC, PLAGL1, a transcription factor and co-regulator of EP300 and TP53 (37, 38), was proposed as a potential factor correlated with tumor progression and worse patient outcome (18). In the present study, we showed that *PLAGL1* mRNA and protein expression positively correlated with EP300 immunoreactivity. Interestingly, promoters that are predicted to recruit EP300 protein contain CpG islands and their expression is significantly regulated by methylation and histone acetylation processes (39), and the P1 promoter of the *PLAGL1* gene fits this characteristic well (19, 40). Since the transcription of *PLAGL1* depends on H3 and H4 acetylation in the promoter region (40), the possibility of direct interaction of EP300 and PLAGL1 in ccRCC may justify further research aimed to determine the significance of the observed associations between these factors in the pathogenesis of ccRCC.

In conclusion, the results of our study indicate that the expression levels of EP300, TP53 and BAX proteins do not correlate with patient survival in ccRCC and cannot be used to discriminate between low-risk and high-risk patients. High Fuhrman grade, advanced/recurrent tumor growth and presence of distant metastases were the most reliable prognostic factors in the analyzed cohort of patients with ccRCC. To date the prognostic significance of TP53 protein and TP53-related factors in ccRCC remains controversial and the TP53 pathway does not seem to be of primary importance in this type of cancer. However, further analysis of the molecular background and interactions of histone acetyltransferase EP300 and PLAGL1 transcription factor could provide new insights into the pathogenesis of ccRCC.

Conflicts of Interest

None of Authors declare the conflict of interest.

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