SATB1 is Down-regulated in Clear Cell Renal Cell Carcinoma and Correlates with miR-21-5p Overexpression and Poor Prognosis

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Abstract. *Background: Altered expression of special AT-rich sequence binding protein 1 (SATB1) was reported in several types of human cancers. This study aimed to determine the expression levels of SATB1, as well as miR-21-5p -the post-transcriptional repressor of SATB1 expression- in clear cell renal cell carcinoma (ccRCC) and to investigate their association with the progression of ccRCC. Materials and Methods: Immunohistochemistry and quantitative polymerase chain reaction were used to assess the expression of SATB1 protein and mRNA as well as miR-21-5p in tumor and matched normal kidney tissues collected from 56 ccRCC patients. Results: Nuclear SATB1 immunoreactivity was elevated in ccRCC cells while its cytoplasmic expression was decreased. SATB1 mRNA level was down-regulated in ccRCC tissue and inversely correlated with the content of miR-21-5p. Down-regulation of SATB1 mRNA and up-regulation of miR-21-5p were associated with shorter patient survival. Conclusion: Decreased expression of SATB1 in ccRCC may result from over-expressed miR-21-5p. Our data suggest that SATB1 may have a potential value as a prognostic marker in ccRCC.*

Renal cell carcinoma (RCC) comprises of several histological subtypes that are characterized by different histopathological, genetic and clinicopathological features. Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype, accounting for 70-80% of all RCC cases (1). ccRCC originates from the proximal convoluted tubules epithelium (renal cortex) and presents a predominantly expansive growth pattern. Hematogenous metastases are relatively common in clear cell carcinomas and it has been shown that ccRCC carries a worse prognosis compared to the other histological variants (2). Many genes are involved in ccRCC development and progression (3, 4). Therefore, for decades, the genomic alterations and underlying molecular mechanisms have been investigated to test potential clinical usefulness for early diagnosis, treatment and surveillance of ccRCC patients.

Special AT-rich sequence-binding protein 1 (SATB1) is a cell type-specific nuclear matrix attachment region binding protein that functions as the global chromatin structure organizer and transcription factor. SATB1 forms a docking site for the chromatin-modifying enzymes, transcription activators or repressors and, as a potent epigenetic regulator, may affect the expression of numerous genes (5-8). Targeted silencing of *SATB1* has led to altered expression of more than 1,000 genes, including those implicated in the pathogenesis of human neoplasms (9). The reports indicate that *SATB1* is expressed in a tissue-typical manner and its prognostic value may be cancer-type specific. Overexpression of *SATB1* gene has been associated with the progression of several types of cancers such as breast (9, 10), gastric (11), ovarian (12), liver (13), prostate cancer (14), laryngeal squamous cell carcinoma (15), and cutaneous malignant melanoma (16). However, contradictory results have also been demonstrated. A study by Iorns *et al*. reported that SATB1 had no role in breast cancer pathogenesis (17). Moreover, in one study of lung cancer the loss of SATB1 expression correlated with poor survival (18),

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Key Words: SATB1 expression, miR-21-5p, clear cell renal cell carcinoma, clinicopathological parameters, survival, qPCR, IHC.

while in an another investigation of the non-small cell lung cancer the highest level of *SATB1* mRNA was observed in metastatic cancers (19). To date, two reports have described *SATB1* expression in ccRCC; however, their results were not consistent (20, 21). Cheng *et al*. (20) found that levels of *SATB1* mRNA and protein were dramatically increased in ccRCC tissues. Furthermore, they suggested that overexpression of SATB1 may play a critical role in the acquisition of an aggressive phenotype for RCC cells. On the contrary, a study by Guo *et al*. (21) revealed that SATB1 levels were significantly down-regulated in ccRCC tissues compared to those in the paired normal tissues. Moreover, the association between *SATB1* expression and patient survival so far, remains unknown. Therefore, the objective of our study was to analyze and compare *SATB1* gene expression in samples of the ccRCC tissue and normal kidney. To estimate the prognostic value of the *SATB1* expression level in ccRCC tissues, we analyzed correlations between the expression level of *SATB1* and clinicopathological features, as well as overall survival (OS) of the patients.

The expression of SATB1 can be regulated by microRNAs (miRs), including a validated post-transcriptional repressor of SATB1 expression, miR-21-5p (22). It was previously reported that miR-21-5p overexpression in ccRCC can be directly induced in response to hypoxic environment (23). The studies of The Cancer Genome Atlas Research Network carried out in 446 ccRCC patients reported that miR-21-5p overexpression in the tumor is caused by the hypomethylation of *MIR21* promoter (24). Moreover, the expression level of miR-21-5p significantly correlated with poor prognosis in ccRCC. On the basis of bioinformatics the hypomethylation of *MIR21* resulting in miR-21-5p overexpression was proposed as a key factor driving glycolytic shift in ccRCC (24). Therefore, we also evaluated the level of miR-21-5p in the studied tissues and its association with the expression of SATB1, its potential target in ccRCC.

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 (decision No. 22/2010), 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 2014. The study included 56 patients with ccRCC (aged 64.9±9.5 years, mean±SD). None of the patients had a history of other tumor or received preoperative treatment. Clinical and demographic data were obtained at the time of enrollment. The tumor grade was determined according to the Fuhrman grading system. Data on the OS were collected for all patients. Median follow-up time was 28.8 months.

Two types of samples from each patient subjected to radical nephrectomy were harvested immediately after surgical resection: (i) ccRCC tissue, and (ii) matched normal, macroscopically unchanged renal cortex tissue from a distant part of the resected kidney. Specimens for routine histological evaluation and immunohistochemistry were fixed in neutral 4% buffered-formaldehyde and further processed into paraffin blocks. Tissue samples for qPCR analysis were frozen in liquid nitrogen and stored at –80˚C.

Total RNA extraction, reverse transcription and real-time quantitative PCR. Total RNA was extracted from renal tissues and reverse transcribed as previously described (25). Quantification of genes expression was carried out using ABI 7500/7500 Fast Real-Time PCR System (Life Technologies – Applied Biosystems, Foster City, CA, USA). TATA box binding protein (*TBP*) and peptidylprolyl isomerase A (*PPIA*) genes were used as an internal control to normalize the transcript levels of *SATB1*. The levels of *SATB1, TBP* and *PPIA* cDNAs in collected isolates were determined using TaqMan®Fast Advanced Master Mix and a respective TaqMan® Gene Expression Assay (for *SATB1*: Hs00161515_m1, *TBP*: Hs00427620_m1 and *PPIA*: Hs99999904_m1; all: Life Technologies – Applied Biosystems) according to the manufacturer's instructions and using the following conditions: polymerase activation for 20 s at 95˚C, followed by 40 cycles of denaturation at 95˚C for 3 s and annealing/extension at 60˚C for 30 s.

To estimate the level of miR-21-5p expression, total RNA specifically enriched in small RNA was isolated from frozen tissues samples using the *mir*Vana™ Isolation Kit (Ambion, Cambridge, MA, USA), according to the provided protocol. RNU48 was used as an endogenous control to normalize miR-21-5p levels. Reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription Kit and *miRNA-21* or *RNU48* specific RT primers (all: Life Technologies – Applied Biosystems). The resulting cDNA was amplified with the use of TaqMan Universal PCR Master Mix and specific primers with probes for *miR-21* (TaqMan MicroRNA Assays ID: 000397) and *RNU48* (TaqMan MicroRNA Assays ID: 001006). Optimized amplification conditions were 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min.

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 qPCR reactions. Relative quantification of *SATB1* and miR-21-5p expression was evaluated using the ΔΔCt method (26). The fold change in the relative *SATB1* gene or miR-21-5p expression was determined by calculating the $2^{-\Delta\Delta Ct}$ value. Fold increase above 1 ($2^{-\Delta\Delta Ct}$ >1) indicated overexpression of target RNA in ccRCC tissue, and fold decrease under 1 ($2^{-\Delta\Delta Ct}$ <1) indicated its down-regulation.

Immunohistochemistry and staining analysis. SATB1 immunostaining in ccRCC and normal renal tissues was conducted according to previously described methods (27) on 4 μm-thick paraffin sections using the Autostainer Link48 (DakoCytomation, Glostrup, Denmark). Rabbit monoclonal primary antibody directed against SATB1 (1:100, EPR3951, GeneTex, Irvine, CA, USA) was applied, while the negative controls were performed by omitting the primary antibody.

The SATB1 immunostained sections were evaluated using Olympus BX53 light microscope (Olympus, Tokyo, Japan) by two independent pathologists in a blinded manner regarding the clinical data of the patients. In doubtful cases, re-evaluation was performed until a consensus was achieved. SATB1 immunoexpression was

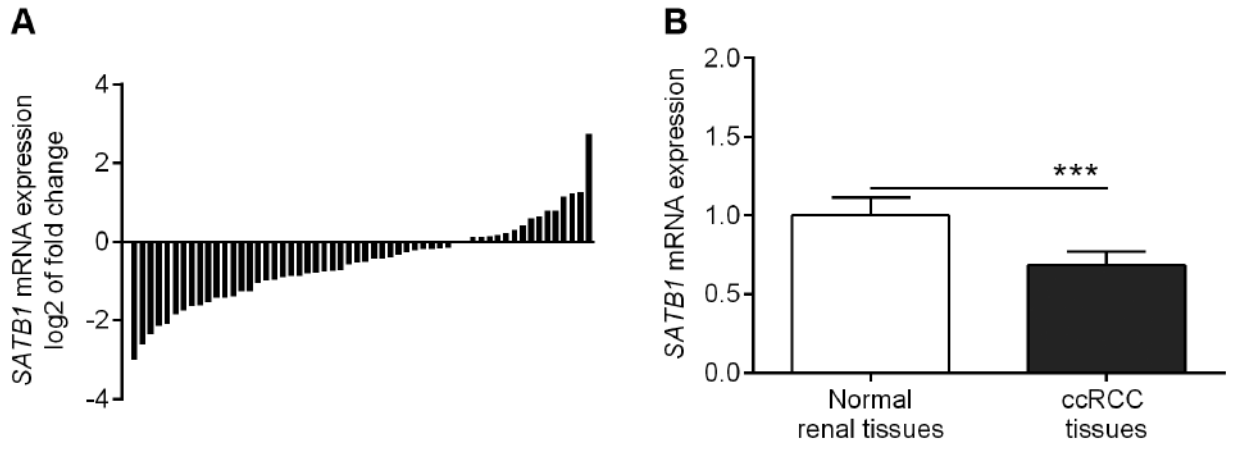

Figure 1. *mRNA levels of special AT-rich sequence binding protein 1 (SATB1) in the tumor and normal renal tissues of clear cell renal cell carcinoma (ccRCC) patients (N=56) as determined by quantitative polymerase chain reaction. (A) SATB1 mRNA levels in tumors of individual ccRCC patients are shown in relation to the SATB1 mRNA content in matched normal renal tissue. (B) The average expression of SATB1 mRNA (mean±SEM) in ccRCC tissues is shown in relation to the value obtained for normal kidney tissue (1.0). ***p<0.001.*

assessed in whole tissue sections in cancer cells and non-transformed, normal epithelial cells of the proximal tubules. The nuclear SATB1 expression was evaluated semi-quantitatively based on the percentage of cells presenting positive expression of SATB1 (% cells score), and encoded as follows: 0 - absence of staining, 1 point when 1-10% cells stained, 2 points when 11-50%, 3 when 51-80%, and 4 points when 81-100%. The cytoplasmic SATB1 expression was assessed according to the immunoreactive score (IRS) of Remmele and Stegner (28). The scale is based on the percentage of cells showing positive reaction (as previously described), as well as reaction intensity (0: no reaction, 1: low intensity reaction, 2: moderate intensity reaction, 3: intense reaction). The final score is the product of both parameters and ranges from 0 to 12 points. Based on median expression values, ccRCC cases which showed cytoplasmic SATB1 expression less than or equal to six IRS scores were regarded as having 'low' expression, whereas those scored more than six were regarded as 'high' SATB1 expression. In the case of nuclear SATB1 immunoreactivity, cases scoring less than two were regarded as having 'low' expression and those scoring equal to two or more were regarded as 'high' SATB1 expression.

Statistical analyses. Statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, USA) and STATISTICA v.10 (StatSoft, Tulsa, OK, USA) software. The differences in *SATB1* mRNA and protein levels between matched tumor and normal samples of ccRCC 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 levels of *SATB1* mRNA and miR-21-5p expression. 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, results were considered statistically significant when $p<0.05$.

Results

SATB1 mRNA expression in ccRCC tissues is down-regulated. All tumor and matched normal renal tissues samples of ccRCC patients expressed *SATB1* mRNA. Among the 56 cancer specimens tested, the relative *SATB1* mRNA level (ccRCC *vs*. matching normal renal tissues) was decreased in 40 (71.4%) tumors while it was increased in 16 (28.6%) specimens (Figure 1A; Table I). The expression of *SATB1* mRNA was significantly decreased in ccRCC compared to the normal renal tissues (0.68±0.09 *vs*. 1.00±0.11, respectively; $p=0.0001$; Figure 1B).

miR-21-5p expression in ccRCC tissues is up-regulated and inversely correlated with SATB1 mRNA expression. To evaluate the impact of miR-21-5p on *SATB1* expression, the level of miR-21-5p expression in tumor and normal renal tissues of ccRCC patients was determined and correlated with *SATB1* mRNA. miR-21-5p expression was detected in all studied tissues. Among the 56 cancer specimens tested, the relative miR-21-5p level (ccRCC *vs*. matching normal renal tissues) was up-regulated in 49 (87.5%) tumors while it was down-regulated in 7 (12.5%) specimens (Figure 2A). miR-21-5p expression was increased in ccRCC in comparison to the normal renal tissues (2.21±0.78 *vs*. 1.00±0.22, respectively; $p<0.0001$; Figure 2B). Significantly negative correlation was found between the levels of miR-21-5p and *SATB1* mRNA in ccRCC (r=–0.5101; $p<0.0001$; Figure 3A) and normal renal tissues (r=–0.4863; $p=0.0001$; Figure 3B).

Heterogenous nuclear and cytoplasmic SATB1 immunohisto-chemical staining in ccRCC tissue. Nuclear

Table I. *Associations between demographic and clinicopathological features of clear cell renal cell carcinoma (ccRCC) patients and the relative mRNA expression of special AT-rich sequence binding protein 1 (SATB1) in renal cancer tissues.*

Parameter	Patients' number n (%)	SATB1 mRNA levels in tumor tissue *vs.* normal renal tissue of ccRCC patients		*p*-Value
		Down (ratio <1) n (%)	Up (ratio >1) n (%)	
Total	56 (100.0)	40 (71.4)	16 (28.6)	
Gender				
Men	27 (48.2)	20 (74.1)	7 (25.9)	0.7712
Women	29 (51.8)	20 (69.0)	9 (31.0)	
Age (years)				
≤62	28 (50.0)	18 (64.3)	10 (35.7)	0.3753
>62	28 (50.0)	22 (78.6)	6 (21.4)	
Tumor size (cm)				
≤7	35 (62.5)	25 (71.4)	10 (28.6)	1.0000
>7	21 (37.5)	15 (71.4)	6 (28.6)	
Depth of invasion (pT status)				
T1	31 (55.4)	22 (71.0)	9 (29.0)	1.0000
T2+T3	25 (44.6)	18 (72.0)	7 (28.0)	
Fuhrman grade				
G2	42 (75.0)	28 (66.7)	14 (33.3)	0.3054
G3+G4[a]	14 (25.0)	12 (85.7)	2 (14.3)	
Distant metastases				
Absent	41 (73.2)	27 (65.9)	14 (34.1)	0.1194
Present	15 (26.8)	13 (86.7)	2 (13.3)	

[a]Two patients had Fuhrman grade 4 ccRCC.

and cytoplasmic SATB1 immunoreactivity was observed in epithelial cells of proximal convoluted tubules (Figure 4A) as well as cancer cells of the analyzed tissues (Figure 4B). Among 56 cancer tissue specimens tested, nuclear immunoreactivity of the SATB1 protein was low in 33 (58.9%) and high in 23 (41.1%) specimens (Table II). The average nuclear SATB1 immunostaining was significantly higher in ccRCC tissues compared to SATB1 immunoreactivity in epithelial cells of proximal convoluted tubules of the normal renal cortex (1.45±0.09 *vs.* 1.11±0.06, respectively; p=0.0017; Figure 5B).

Cytoplasmic SATB1 expression was low in 31/56 (55.4%) ccRCC specimens, whereas high immunoreactivity was observed in 25/56 (44.6%) cancer tissues (Table II). The average intensity of cytoplasmic SATB1 expression was significantly lower in cancer cells compared to its expression in the proximal tubules epithelium of the matched normal renal tissues (6.77±0.45 *vs.* 8.43±0.27, respectively; p=0.0021; Figure 5D).

The lack of correlation between SATB1 expression and demographic or clinicopathological features in ccRCC tissues. Possible correlations of *SATB1* expression with selected demographic and clinicopathological parameters were analyzed based on the results of qPCR and immunohistochemical analysis. *SATB1* expression, at both the mRNA and protein levels, did not correlate with any of the tested parameters, including gender, age, primary tumor size, depth of invasion, Fuhrman grade and the presence of metastases (p>0.05; Tables I and II).

OS of patients is associated with the SATB1 mRNA level. To estimate the significance of *SATB1* expression as a prognostic factor, *SATB1* expression levels in ccRCC patients were correlated with their OS. Patients were followed-up for 28.8 months. During this observation period, 15 (26.8%) patients died.

Univariate Cox regression model revealed that down-regulated expression of *SATB1* mRNA (p=0.0079) and increased level of miR-21-5p (p=0.019) are associated with worse prognosis in ccRCC patients (Table III). The intensity of nuclear and cytoplasmic SATB1 immunostaining did not significantly correlate (p>0.05) with patients' OS. Multivariate Cox regression analysis confirmed that the expression of *SATB1* mRNA in the tumor is an independent prognostic factor in ccRCC. Kaplan-Meier plots presenting the survival of ccRCC patients are shown in Figure 6.

Discussion

Under physiological conditions SATB1 has been shown to play an important role in T-cell development (29), early erythroid differentiation (30), cellular homeostasis and response to various stimuli (6, 7). SATB1 seems to be of particular importance for cells that adapt to a changing internal and external environment (9, 31). This protein provides a nuclear platform for docking of chromatin-remodeling enzymes, and through this mechanism, controls expression of numerous genes (5, 7-9). Post-translational modifications of SATB1 such as acetylation and phosphorylation act as molecular switches regulating its transcriptional activity as either activator or repressor (6). A number of studies in the past years demonstrated the role of SATB1 in cancer. Genes regulated by SATB1 include those associated with metastasis, proliferation, cell adhesion and apoptosis (9). Altered expression of *SATB1* has been observed in various human neoplasms (9-21, 27), attracting attention as a putative diagnostic and prognostic biomarker.

Aberrant *SATB1* expression was also noted in ccRCC; however, results of previous reports are not consistent (20, 21). Similarly to our findings, Guo *et al.* demonstrated that *SATB1* mRNA levels were significantly down-regulated in ccRCC

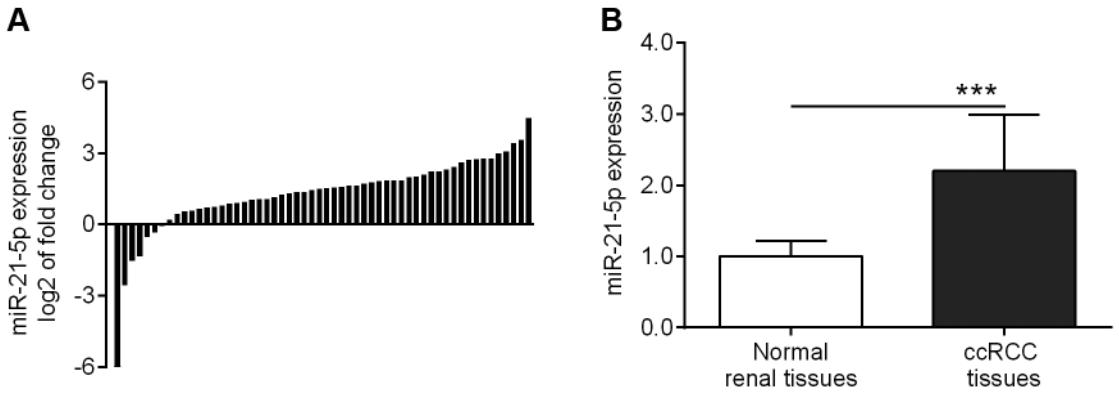

Figure 2. *miR-21-5p levels in the tumor and normal renal tissues of clear cell renal cell carcinoma (ccRCC) patients (N=56) as determined by quantitative polymerase chain reaction. (A) miR-21-5p levels in tumors of individual ccRCC patients are shown in relation to the miR-21-5p content in matched normal renal tissue. (B) The average expression of miR-21-5p (mean±SEM) in ccRCC tissues is shown in relation to the value obtained for normal kidney tissue (1.0). ***p<0.001.*

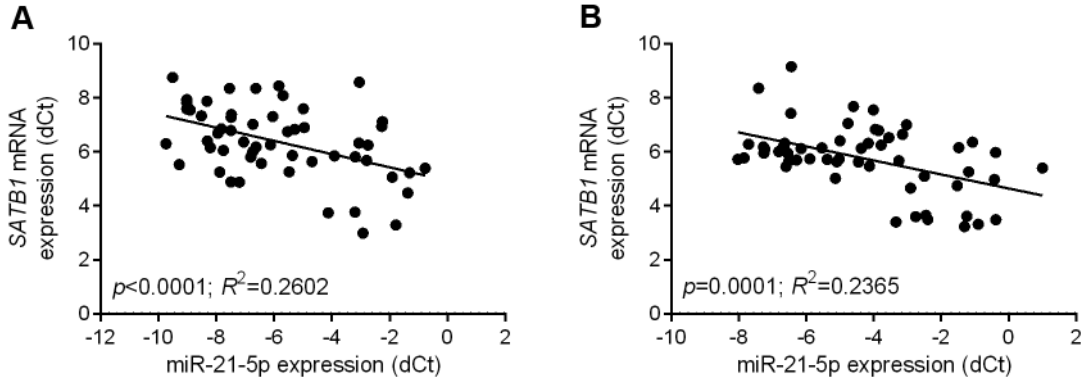

Figure 3. *Negative correlations between mRNA of special AT-rich sequence binding protein 1 (SATB1) and miR-21-5p expression levels in clear cell renal cell carcinoma (A) and normal renal tissues (B). The solid line indicates the correlation trend.*

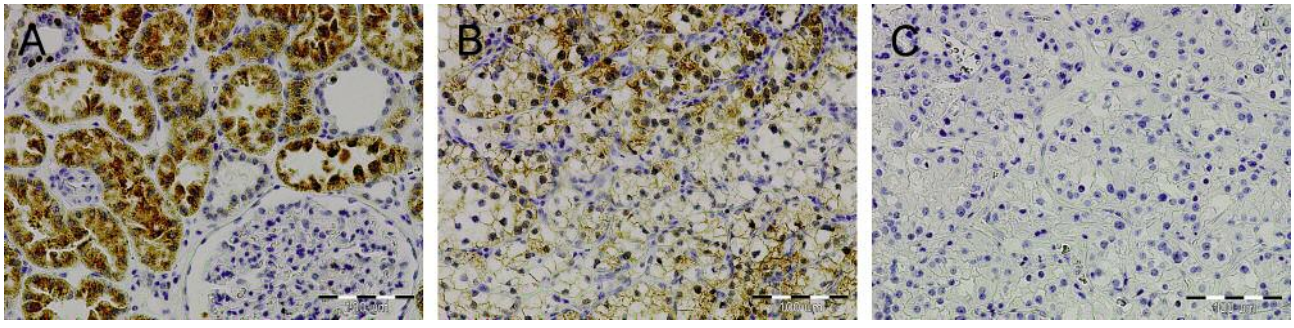

Figure 4. *Expression of special AT-rich sequence binding protein 1 (SATB1) in clear cell renal cell carcinoma (ccRCC) and normal renal tissues assessed by immunohistochemistry. Immunohistochemical staining of SATB1 protein in representative sections of normal (A) and cancer (B) tissues of ccRCC patients. Negative controls were performed by omitting the primary antibody (C). Magnification ×200.*

tissues compared with those in paired normal tissues (21). The results of our study prompted us to assume that down-regulation of *SATB1* expression in ccRCC may be caused by microRNAs since we found a negative correlation between the levels of *SATB1* mRNA and miR-21-5p expression. miRs are capable of regulating target mRNAs post-transcriptionally by

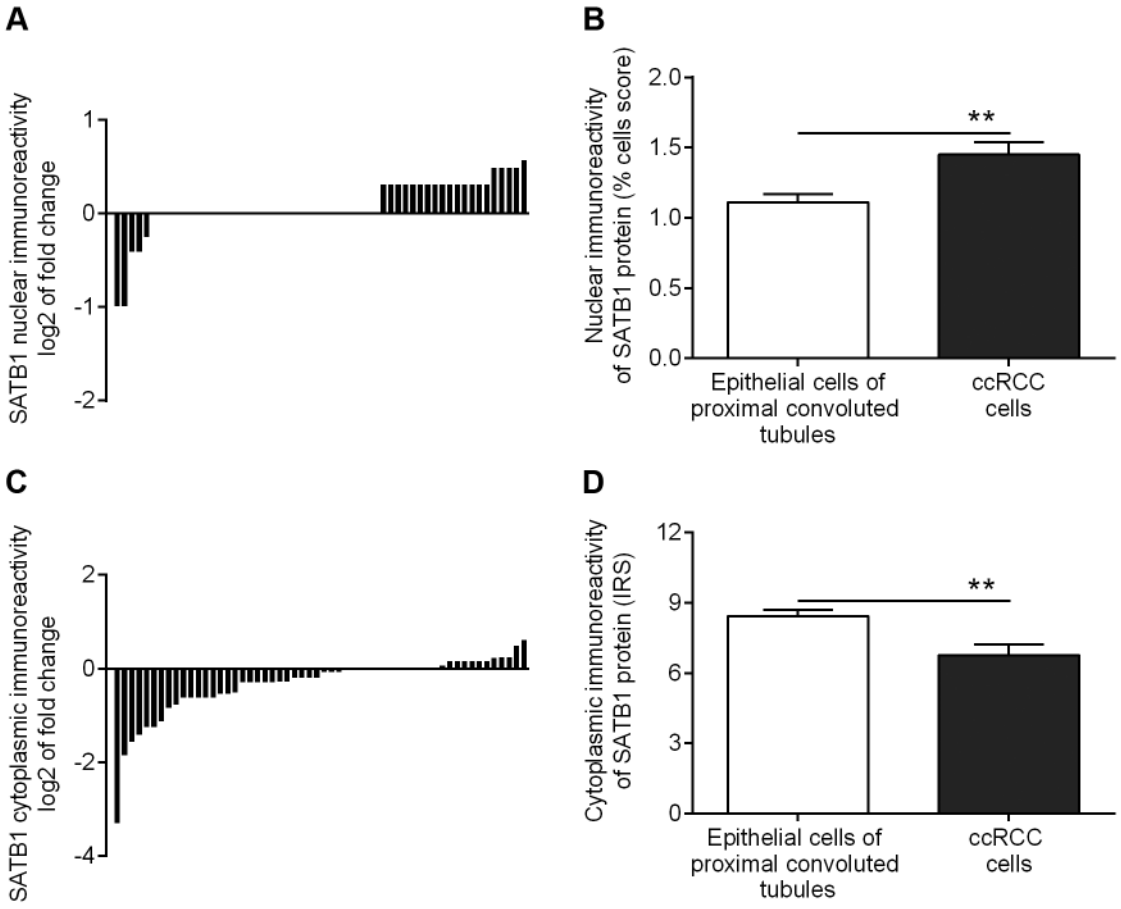

Figure 5. *Evaluation of special AT-rich sequence binding protein 1 (SATB1) expression in the tumor and normal renal tissues by immunohistochemistry. The level of nuclear (A) and cytoplasmic (C) SATB1 immunoreactivity in tumors of individual ccRCC patients is shown in relation to the level of SATB1 immunostaining in matched normal renal tissue. The average nuclear (B) and cytoplasmic (D) immunoreactivity of the SATB1 protein in epithelial cells of proximal convoluted tubules and clear cell renal cell carcinoma (ccRCC) cells. Bars represent mean±SEM; IRS, immunoreactive score of Remmele and Stegner (28);**p<0.01.*

inducing their cleavage or translational repression (32). Lopes-Ramos *et al*. indicated that *SATB1* is a target gene for miR-21-5p (22). After transfection of colorectal cancer cells with miR-21-5p, they observed a reduction of *SATB1* mRNA expression, while knock-down of miR-21-5p resulted in an increase in *SATB1* mRNA levels (22). Our finding of significantly up-regulated miR-21 expression in ccRCC compared with normal kidney is in line with the results of other authors (23, 33-35). Moreover, in accordance with previous studies (33-35), we observed that higher miR-21-5p levels were associated with poor OS. Furthermore, in the present study, we demonstrated relationship between shorter patient OS and decreased level of *SATB1* mRNA in ccRCC tissue and suggest that the *SATB1* mRNA level may serve as an independent prognostic factor; however, we failed to find any correlations of *SATB1* mRNA content with patient's and disease characteristics. So far, associations between clinicopathological parameters, ccRCC patients' survival and *SATB1* mRNA levels have not been investigated.

Contrary to our and Guo *et al*. (21) results, Cheng *et al*. found that *SATB1* mRNA expression was dramatically increased in human ccRCC tissues (20). This discrepancy may be due to the selection of different reference genes to normalize qPCR measurements since, in accordance with published recommendations (36), we used *TBP* and *PPIA* genes as normalizers, while Cheng *et al*. (20) chose *GAPDH* gene. Jung *et al*. (36) examined commonly used housekeeping genes, including those mentioned above, with regard to their usefulness as reference genes for relative gene expression quantification in gene profiling studies of ccRCC. Their study demonstrated the suitability of the two housekeeping genes *PPIA* and *TBP* as endogenous reference genes when comparing malignant tissue samples with adjacent normal kidney samples of ccRCC patients. All other examined genes,

Table II. *Associations between demographic and clinicopathological features of clear cell renal cell carcinoma (ccRCC) patients and nuclear and cytoplasmic immunoreactivity of special AT-rich sequence binding protein 1 (SATB1) in tumor cells.*

Parameter n (%)	Patients' number n (%)	SATB1 nuclear immunoreactivity in tumor cells		*p*-Value n (%)	SATB1 cytoplasmic immunoreactivity in tumor cells		*p*-Value
		Score 0-1 n (%)	Score 2-3		IRS ≤6 n (%)	IRS >6	
Total	56 (100.0)	33 (58.9)	23 (41.1)		31 (55.4)	25 (44.6)	
Gender							
Men	27 (48.2)	14 (51.9)	13 (48.1)	0.4157	16 (59.3)	11 (40.7)	0.6016
Women	29 (51.8)	19 (65.5)	10 (34.5)		15 (51.7)	14 (48.3)	
Age (years)							
≤61	28 (50.0)	16 (57.1)	12 (42.9)	1.0000	14 (50.0)	14 (50.0)	0.5913
>61	28 (50.0)	17 (60.7)	11 (39.3)		17 (60.7)	11 (39.3)	
Tumor size (cm)							
≤7	35 (62.5)	21 (60.0)	14 (40.0)	1.0000	20 (57.1)	15 (42.9)	0.7859
>7	21 (37.5)	12 (57.1)	9 (42.9)		11 (52.4)	10 (47.6)	
Depth of invasion (pT status)							
pT1	31 (55.4)	20 (64.5)	11 (35.5)	0.4175	18 (58.1)	13 (41.9)	0.7880
pT2+pT3	25 (44.6)	13 (52.0)	12 (48.0)		13 (52.0)	12 (48.0)	
Fuhrman grade							
G2	42 (75.0)	25 (59.5)	17 (40.5)	1.0000	25 (59.5)	17 (40.5)	0.3570
G3+G4a	14 (25.0)	8 (57.1)	6 (42.9)		6 (42.9)	8 (57.1)	
Distant metastases							
Absent	41 (73.2)	22 (53.7)	19 (46.3)	0.2305	23 (56.1)	18 (43.9)	1.0000
Present	15 (26.8)	11 (73.3)	4 (26.7)		8 (53.3)	7 (46.7)	

[a]Two patients had Fuhrman grade 4 ccRCC. IRS: immunoreactive score of Remmele and Stegner (28).

Table III. *Univariate and Multivariate Cox proportional hazard regression of clear cell renal cell carcinoma (ccRCC) patients overall survival.*

Covariates	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Gender (men *vs*. women)	0.90	0.32-2.49	0.8372			
Age (years)	1.01	0.96-1.07	0.6927			
Size of primary tumor (cm)	1.16	1.01-1.34	0.0372*	1.03	0.79-1.33	0.8506
Depth of invasion (T1 *vs*. T2+T3)	0.17	0.05-0.61	0.0066*	0.33	0.05-2.07	0.2339
Fuhrman grade (G2 *vs*. G3+G4)	0.17	0.06-0.50	0.0014*	0.62	0.16-2.38	0.4833
Distant metastasis (absent *vs*. present)	0.24	0.09-0.68	0.0072*	0.57	0.16-2.02	0.3877
SATB1 mRNA relative expression (RQ)	0.08	0.01-0.52	0.0079*	0.12	0.01-0.99	0.0484*
SATB1 nuclear immunoreactivity in ccRCC cells (score)	0.61	0.26-1.43	0.2558			
SATB1 cytoplasmic immunoreactivity in ccRCC cells (score)	0.87	0.73-1.04	0.1311			
miR-21-5p relative expression (RQ)	1.11	1.02-1.21	0.0190*	1.02	0.92-1.13	0.7237

Medium follow-up time: 28.8 months. *Significant *p*-values (<0.05); HR: hazard ratio; CI: confidence interval; RQ: relative quantification.

including *GAPDH*, showed significant differences in expression between malignant and non-malignant pairs. Another possible explanation for the discrepancy may be the presence of distinct subclasses of ccRCC that differ in clinical behavior as demonstrated by the analysis of gene expression profiles of ccRCC tumors obtained from patients with diverse clinical outcomes by using 21,632 cDNA microarrays (3). These findings may explain our data in which down-regulation of *SATB1* mRNA levels was observed in majority, however, not all ccRCC samples.

Previous studies of *SATB1* expression at the protein level in ccRCC tissues also provided inconsistent results. In contrast to Cheng *et al*. (20) who noted higher immunoreactivity of SATB1 protein in majority of ccRCC samples compared to the

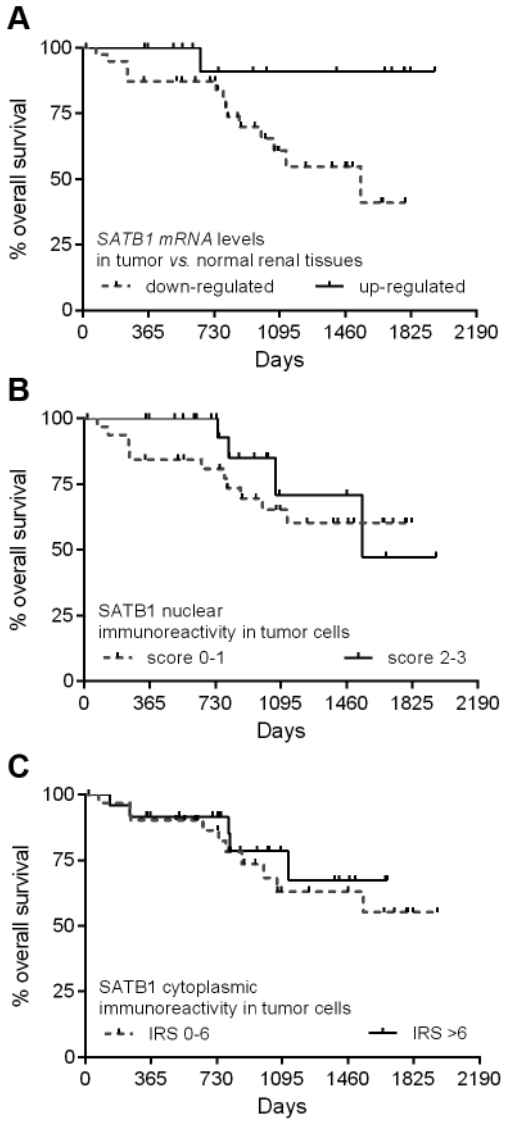

Figure 6. *Kaplan-Meier survival curves of 56 clear cell renal cell carcinoma patients regarding the expression levels of special AT-rich sequence binding protein 1 (SATB1) mRNA (A), as well as nuclear (B) and cytoplasmic (C) immunoreactivity of SATB1 protein. IRS, immunoreactive score of Remmele and Stegner (28).*

corresponding normal tissues, Guo *et al*. (21) demonstrated down-regulated SATB1 protein expression in ccRCC. We found that the SATB1 protein immunoreactivity in ccRCC cells is dependent on the cellular compartment demonstrating higher levels in the nuclei and lower in the cytoplasm compared to normal epithelial cells of proximal convoluted tubules. Our findings of high SATB1 nuclear and cytoplasmic immunoreactivity in 40.4% and 43.9% of ccRCC samples, respectively, are in line with results of Cheng *et al*. (20) who found high SATB1 expression in 46.1% of ccRCC cases. Similarly to these authors (20), we did not find correlations between SATB1 protein expression in ccRCC samples and demographic and clinicopathological parameters such as gender, age, tumor size, distant metastasis and Fuhrman grade. However, in contrast to our results, Cheng and co-workers (20) reported an association between increased SATB1 protein expression and depth of tumor invasion. Our study failed to reveal any relationships between the level of SATB1 protein immunoreactivity and patient survival contrary to the findings at the transcript level. This is probably due to the post-transcriptional mechanisms regulating SATB1 expression, or differences in methodologies. Immunohistochemical analysis allows for assessment of protein expression in specific cell types and its subcellular location, while studying mRNA content in cancer tissue may include not only tumor cells.

In conclusion, we provided evidence for the first time that the *SATB1* mRNA level in ccRCC tissue may serve as an independent prognostic factor. However, the discrepancies in prognostic significance of *SATB1* expression at the transcript and protein levels imply the need for further investigation using a longer period of follow-up. Moreover, the results of our experiments suggest, for the first time, that altered *SATB1* expression in ccRCC may be caused by miR-21-5p known for its oncogenic role in ccRCC.

Acknowledgements

This study was supported by statutory grant of University of Warmia and Mazury in Olsztyn, Poland and grant No. 2012-05/B/NZ4/01832 of the National Science Centre, Poland. The authors wish to thank Dr. Aleksandra Piotrowska from the Department of Histology and Embryology, Wroclaw Medical University for technical support.

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Received January 26, 2016
Revised March 22, 2016
Accepted March 23, 2016