

Low Expression of Single-stranded DNA Binding Protein 2 (SSBP2) Predicts Unfavourable Postoperative Outcomes in Patients With Clear Cell Renal Cell Carcinoma

HYUNSUNG KIM, YESEUL KIM, SEONGSIK BANG, SEONGEON PARK, SEUNGYUN JEE,
JONGMIN SIM, SU-JIN SHIN, SEUNG SAM PAIK and KISEOK JANG

Department of Pathology, College of Medicine, Hanyang University, Seoul, Republic of Korea

Abstract. *Background:* Single-stranded DNA binding protein 2 (SSBP2) is a subunit of a single-stranded DNA binding complex, which is involved in the maintenance of hematopoietic stem cells and stress responses. Numerous studies have suggested that SSBP2 functions as a tumor suppressor and is silenced through a pathway mediated by promoter hypermethylation. However, the role of SSBP2 in human renal cell carcinoma has not been reported, to date. *Herein, we investigated the clinicopathological significance of SSBP2 expression in clear cell renal cell carcinoma (ccRCC). Materials and Methods:* We constructed tissue microarrays consisting of 173 ccRCC tissues, and SSBP2 expression was evaluated semi-quantitatively based on the staining intensity and the proportion of stained cells. *Regarding statistical analysis, the tissues were divided into two groups according to SSBP2 expression, and correlation of SSBP2 expression with various clinicopathological characteristics and patient outcomes was evaluated. Results:* Low SSBP2 expression was observed in 114 of 175 (65.9%) of ccRCC cases, and low SSBP2 expression was significantly correlated with larger tumor size ($p=0.005$, Chi-square test), higher WHO/ISUP histological grade ($p<0.001$, Chi-square test), tumor necrosis ($p=0.008$, Chi-square test), sarcomatoid change ($p=0.021$, Chi-square test), and higher pT AJCC stage ($p=0.002$, Chi-square test). Kaplan-Meier survival curves revealed that patients with low SSBP2 expression had worse recurrence-free survival ($p=0.041$, log-rank test).

Conclusion: ccRCC with low SSBP2 expression was associated with adverse clinicopathological characteristics and poor patient outcomes.

Renal cell carcinoma (RCC) is the most common malignancy in the kidney and is the ninth most common cancer in both men and women in Korea (1). Clear cell renal cell carcinoma (ccRCC) is a histological subtype of RCC that accounts for about 80% of all RCC cases (2). Although nephrectomy can cure most localized ccRCCs, distant or local recurrence occurs in 20-30% of patients within 5 years after curative surgery (3, 4). Recent proteomic analyses revealed numerous dysregulated proteins and cancer-related signalling pathways in RCC, which are potential diagnostic and prognostic biomarkers and molecular targets for treatment (5, 6). However, due to the molecular phenotype heterogeneity of RCC, there is no well-established molecular biomarker for prognosis (7, 8). Therefore, prognostic biomarkers for ccRCC are greatly needed.

Single-stranded DNA binding protein 2 (SSBP2) was isolated as a tumor suppressor of myeloid leukemia and is located in a critical region of loss in chromosome 5q14.1 (9). SSBP2 is a subunit of a ssDNA-binding complex that is involved in the maintenance of hematopoietic stem cells and stress responses (10). SSBPs interact with the transcriptional adaptor protein Lim domain-binding protein 1 (LDB1) through a highly conserved amino terminal motif (11); LDB1 binds to the LIM domains of LIM only proteins (LMO) and LIM homeodomain proteins (LHX) through a carboxy-terminal LIM interacting domain (12). Although precise levels of LMO, LHX, and LIM-binding proteins are known to be critical for many developmental programs, accumulating evidence suggests that these complexes are also key molecules in various human cancers (13). The role of SSBP2 in human malignancies is an area of active investigation, and many studies have suggested that SSBP2 functions as a tumour suppressor and is silenced through a pathway mediated by promoter hypermethylation (14-18). However, among

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Correspondence to: Kiseok Jang, MD, Ph.D., Department of Pathology, College of Medicine, Hanyang University, 222 Wangsimni-ro, Seongdong-gu, Seoul 04763, Republic of Korea. Tel: +82 222908960, Fax: +82 222967502, e-mail: medartisan@hanyang.ac.kr

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glioblastoma patients, a SSBP2 variant was associated with poor survival (19), and the role of SSBP2 in human RCC has not yet been reported (20).

To determine the clinical role of SSBP2 in human ccRCC, we investigated the expression of SSBP2 in ccRCC tissues by immunohistochemistry. The association of SSBP2 expression with various clinicopathological characteristics was assessed as well as whether SSBP2 is a prognostic factor for patient survival.

Materials and Methods

Patients and tumour samples. We enrolled a consecutive series of 252 patients with RCC in this study. All cases were diagnosed and underwent surgery at Hanyang University Hospital (Seoul, Korea) between 2006 and 2015. Patients who were diagnosed with non-ccRCC or had incomplete clinical follow-up data or unavailable paraffin blocks were excluded, leaving 173 patients. The median follow-up period was 80 months (range, 6-141 months). Patients were divided into Histologic grades 1 to 4 according to the World Health Organization/International Society of Urological Pathology (WHO/ISUP) grading system. Pathologic stage was determined according to the 8th edition of the American Joint Committee on Cancer (AJCC) staging system. We reviewed all hematoxylin and eosin (H&E)-stained slides, pathology reports, and other medical records to confirm the diagnoses. The clinicopathologic parameters assessed were tumour size, histologic grade (WHO/ISUP grading system), lymphovascular invasion (renal vein tumour thrombus was included), sinus fat invasion, perirenal soft tissue involvement, tumour necrosis, sarcomatoid change, and pT AJCC stage. This study was approved by the Institutional Review Board of Hanyang University Hospital (HYUH 2018-05-005), and the requirement for informed consent was waived.

Tissue microarray construction. We used a manual tissue microarrayer (Unitma, Seoul, Korea) to construct the tissue microarray (TMA) from archival formalin-fixed, paraffin-embedded tissue blocks. Non-necrotic tissues that were most representative of the centre of the carcinoma, spanning 0.5 cm or larger, were selected from the H&E-stained sections under light microscopy. Tissue cylinders (2-mm in diameter) were punched from a previously marked lesion of each donor block and transferred to the recipient block (Unitma). Each TMA was comprised of 5×10 samples.

Immunohistochemical staining. The immunohistochemical staining for SSBP2 was performed with 4- μ m-thick sections from the TMA blocks. The sections were deparaffinized in xylene and then rehydrated through graded ethanol. For antigen retrieval, the sections were heated in sodium citrate buffer (pH 6.0) in an autoclave at 100°C for 20 min. Endogenous peroxidase activity was blocked with peroxidase blocking solution (S2023; Dako, Glostrup, Denmark). The TMA slides were incubated with primary antibodies at 4°C overnight and then incubated with a labelled polymer (EnVision/HRP, K5007; Dako) for 30 min at room temperature. The primary antibody was a rabbit monoclonal anti-SSBP2 antibody (EPR11520, 1:100 dilution). The antigen detected by the monoclonal SSBP2 antibody (ab177944, Abcam, Cambridge, MA, USA) was reported to be a synthetic peptide 300 aa from the C-terminus

Table I. Baseline characteristics of studied cases (n=173).

Factors	Value (%)
Number of patients	173 (100%)
Median age at surgery (years)	58 (\pm 12.18)
Mean tumor size	3.63 (\pm 2.31)
Gender	
Male	123 (71.1%)
Female	50 (28.9%)
WHO/ISUP grade	
Grade 1	22 (12.7%)
Grade 2	95 (54.9%)
Grade 3	46 (26.6%)
Grade 4	10 (5.8%)
pT stages	
pT1a	108 (62.4%)
pT1b	25 (14.5%)
pT2a	6 (3.5%)
pT2b	0 (0.0%)
pT3	34 (19.6%)

(Cysteine residue). Finally, 3, 3'-diaminobenzidine tetrahydrochloride was used as a chromogen for detection, and the tissues were counterstained with Mayer's hematoxylin.

Interpretation of immunohistochemical staining. Nuclear staining of the tumour cells was assessed using the H-score method (staining intensity×percentage of positive cells for each intensity score). Staining intensity was graded as follows: none=0, weak=1, moderate=2, and strong=3. Representative micrographs are shown in Figure 1. ROC curve analysis was performed to determine the cut-off score for low SSBP2 expression for survival endpoints (21). Expression below the diagnostic cut-off, *i.e.* an H-score<100, was defined as low SSBP2 expression.

Statistical analysis. The statistical analysis was performed using SPSS software, version 21 (IBM, Armonk, NY, USA). The chi-square test was used to evaluate the correlations between SSBP2 expression and the clinicopathologic parameters of tumour size, WHO/ISUP grade, lymphovascular invasion, sinus fat invasion, perirenal soft tissue involvement, tumour necrosis, sarcomatoid change, and pT AJCC stage. Recurrence-free survival and cancer-specific survival were determined using Kaplan-Meier survival curves, and the log-rank test was used to compare the differences. A *p*-value less than 0.05 was considered statistically significant.

Results

Patient characteristics. Baseline characteristics of patients are summarized in Table I. The median patient age was 58 years (range=28-83 years), and the male to female ratio was 2.46:1. Pathologic evaluation revealed that the mean tumour size was 3.63 cm (\pm 2.31 cm). According to the WHO/ISUP grading system, 22 cases (12.7%) were grade 1, 95 (54.9%) were grade 2, 46 (26.6%) were grade 3, and 10 (5.8%) were grade 4. According to the 8th AJCC staging system, 133 cases

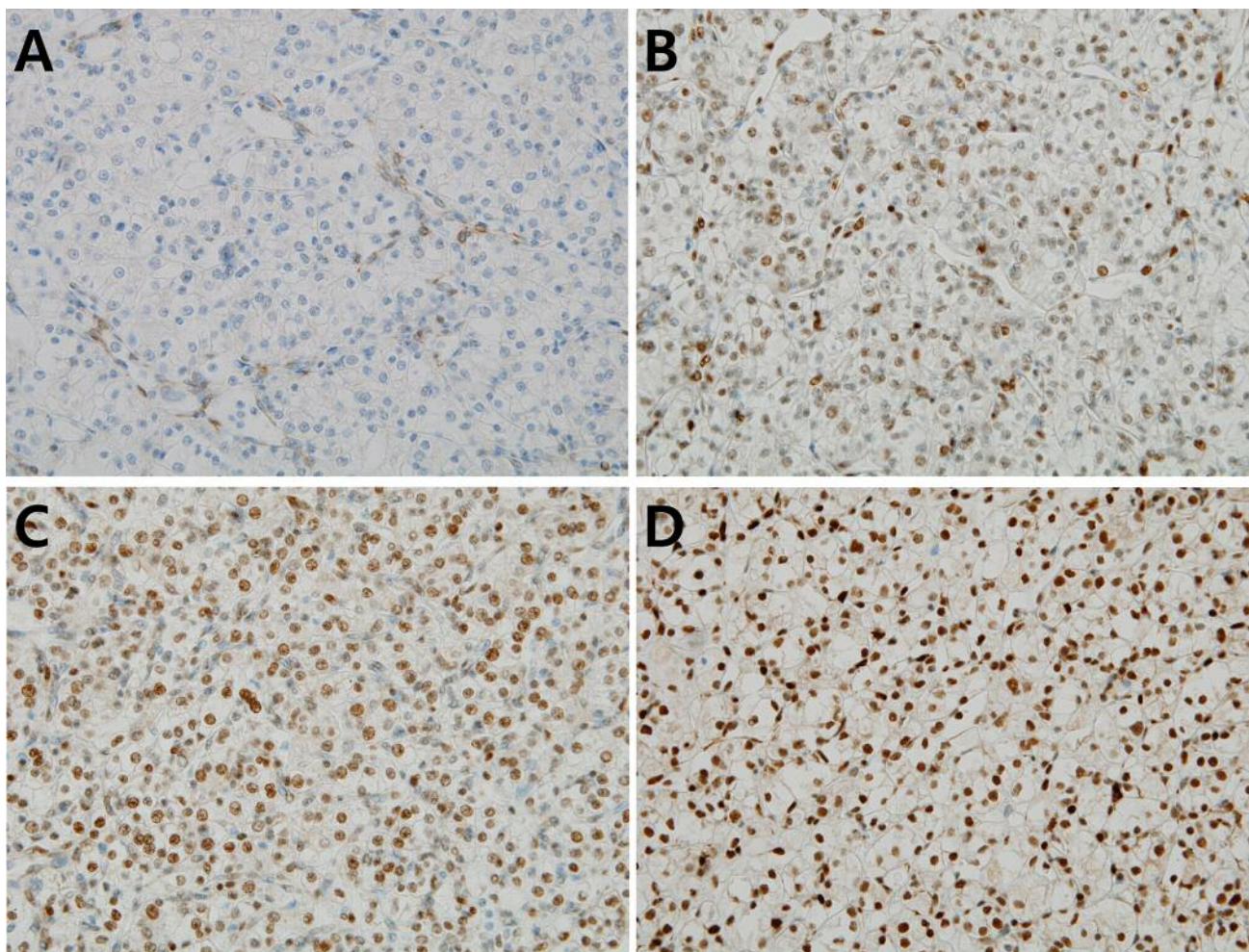


Figure 1. Representative micrographs of immunohistochemical staining for SSBP2 in clear cell renal cell carcinoma tissues. The intensity of nuclear staining was scored as negative (A), weakly positive (B), moderately positive (C), and strongly positive (D) (A-D: Original magnification, $\times 200$).

(76.9%) were pT1, 6 (3.5%) were pT2, 34 (19.6%) were pT3, and none was pT4.

Correlations between SSBP2 expression and clinicopathological characteristics. High SSBP2 expression was observed in 34.1% (59/173) of the ccRCC samples, and low SSBP2 expression was observed in 65.9% (114/173) of the ccRCC samples. Low SSBP2 expression was significantly correlated with larger tumour size ($p=0.005$, Chi-square test), higher WHO/ISUP histologic grade ($p<0.001$, Chi-square test), tumour necrosis ($p=0.008$, Chi-square test), sarcomatoid change ($p=0.021$, Chi-square test), and higher pT AJCC stage ($p=0.002$, Chi-square test) (Table II).

Correlations between SSBP2 expression and patient outcomes. Kaplan-Meier survival curves showed that patients with low SSBP2 expression had worse recurrence-free survival

($p=0.041$, log-rank test) (Figure 2A). There also was a tendency toward worse cancer-specific survival for patients with negative SSBP2 expression, but it was not statistically significant ($p=0.061$, log-rank test) (Figure 2B). In multivariate survival analyses, a loss of SSBP2 expression was not an independent prognostic factor for recurrence-free or cancer-specific survival (data not shown).

Discussion

SSBP2 is a well-known tumour suppressor in acute myelogenous leukaemia. However, the role of SSBP2 as a tumour promoter or suppressor in various human malignancies is controversial (14). Most studies in many human cancers, such as prostate, oesophageal, ovarian, and gallbladder cancers, have reported SSBP2 as a tumour suppressor that is silenced by promoter hypermethylation. Liu *et al.* detected SSBP2

Table II. Correlation between *SSBP2* expression and clinicopathologic factors in clear cell renal cell carcinoma.

Clinicopathologic Factors	n	SSBP2 expression		p-Value
		Low (%) (n=114)	High (%) (n=59)	
Tumor size				0.005
≤4 cm	121	72 (59.5%)	49 (40.5%)	
>4 cm	52	42 (80.8%)	10 (19.2%)	
WHO/ISUP grade				<0.001
Grade 1 & 2	117	63 (53.8%)	54 (46.2%)	
Grade 3 & 4	56	51 (91.1%)	5 (8.9%)	
Lymphovascular invasion*				0.433
Absent	171	112 (65.5%)	59 (34.5%)	
Present	2	2 (100.0%)	0 (0.0%)	
Sinus fat invasion				0.567
Absent	171	113 (66.1%)	58 (33.9%)	
Present	2	1 (50.0%)	1 (50.0%)	
Perirenal soft tissue involve				0.433
Absent	171	112 (65.5%)	59 (34.5%)	
Present	2	1 (50.0%)	1 (50.0%)	
Tumor necrosis				0.008
Absent	148	92 (62.2%)	56 (37.8%)	
Present	25	22 (88.0%)	3 (12.0%)	
Sarcomatoid change				0.021
Absent	164	105 (64.0%)	59 (36.0%)	
Present	9	9 (100.0%)	0 (0.0%)	
pT AJCC stage				0.002
pT1	133	80 (60.2%)	53 (39.8%)	
pT2 & pT3	40	34 (85.0%)	6 (15.0%)	

*Renal vein tumor thrombus included.

hypermethylation in 61.4% (54/88) of prostate cancers and 0% (0/23) of benign prostatic hyperplasias. Hypermethylation of *SSBP2* was associated with higher stage, and in a colony formation assay, *SSBP2* expression inhibited tumour cell proliferation and induced cell cycle arrest (15). Huang *et al.* (16) reported that promoter methylation and down-regulation of *SSBP2* were frequently detected in squamous cell carcinomas of the oesophagus and suggested that *SSBP2* functions as a tumour suppressor that acts by inhibiting the Wnt signalling pathway. Brait *et al.* (17) detected promoter methylation at 13 genes, including *SSBP2*, in ovarian cancer. Although hypermethylation of *SSBP2* was observed in 9% (3 of 33 cases) of ovarian cancers, it was not statistically significant. Tsukamoto *et al.* (18) found that methylation of the *SSBP2* promoter was more frequently in gallbladder cancer than in cholecystitis. In addition, the oncogenic role of *SSBP2* as a tumour promoter has also been suggested in glioblastoma. Using genotyping, Xiao *et al.* (19) identified a single-nucleotide polymorphism (Rs7732320), located in the intronic region of *SSBP2*, with prognostic significance. They investigated whether patient outcome was correlated with the transcript levels of *SSBP2* in 619 glioblastoma patients (from

3 publicly available gene expression data sets) (22-24). There was a strong and significant association between *SSBP2* gene expression and poor overall survival in glioblastoma patients (19). In this study, we observed low *SSBP2* expression in 65.9% of ccRCC tissues and showed that *SSBP2* loss was significantly associated with aggressive phenotypes, including larger tumour size, higher WHO/ISUP histologic grade, tumour necrosis, sarcomatoid change, higher pT AJCC stage, and worse recurrence-free survival.

To date, there have been no studies on *SSBP2* expression in RCC. Dormoy *et al.* reported that the developmental marker *Lim1* functions as an oncogene in ccRCC cells and suggested targeting *Lim1* as an innovative therapeutic intervention for human ccRCC (20). *SSBP2* and *Lim1* are two of the various factors involved in regulating the transcriptional activity of LIM-homeodomain proteins, and their interactions are important in development (11, 25). Further molecular investigations are needed to provide a plausible mechanism for their function in oncogenesis.

As for the strength of this study, this is the only study identifying the significance of *SSBP2* according to protein expression levels in ccRCC. Furthermore, we correlated

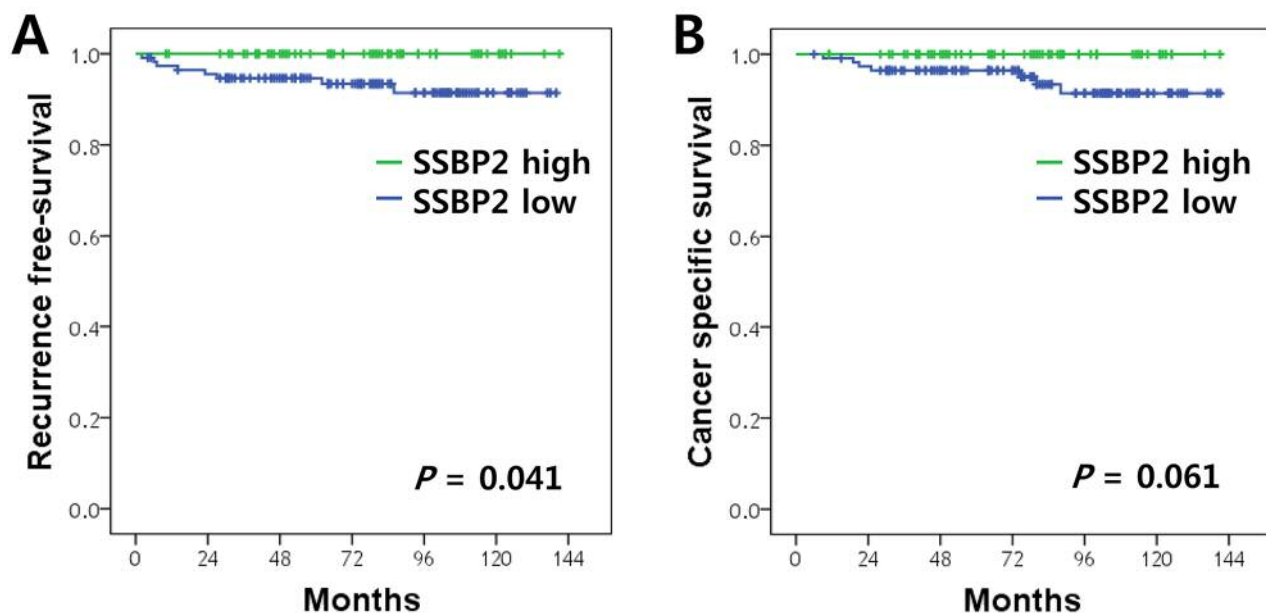


Figure 2. Kaplan-Meier survival curves of patients with clear cell renal cell carcinoma stratified by SSBP2 expression. (A) Recurrence-free survival ($p=0.041$, log-rank test) and (B) cancer-specific survival ($p=0.061$, log-rank test).

various clinical and pathological parameters which practically related to prognosis with SSBP2 expression. However, there are several limitations. Firstly, this study was retrospective and was performed in a single medical centre with a limited number of patients. Secondly, other clinical factors, including Eastern Cooperative Oncology Group (ECOG) performance status, which may affect prognosis, were not considered, and SSBP2 was not statistically significant in multivariate analyses with well-known pathological prognostic factors (26). Thirdly, the detailed molecular mechanism underlying the role of SSBP2 expression in ccRCC was not studied. According to TCGA data (<http://www.cbiportal.org>), 0.4% (0.2%, amplification; 0.2%, missense mutation) of ccRCCs showed genetic alterations in *SSBP2*.

In conclusion, a loss of SSBP2 expression was significantly correlated with aggressive phenotypes and poor recurrence-free survival in ccRCC. Further molecular investigations are needed to clarify the specific pathological mechanism of SSBP2 in ccRCC.

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Conflicts of Interest

The Authors declare that there are no conflicts of interest with regard to the present study.

Authors' Contributions

KJ built the conception and designed the experiments. YK, SB, SP, SJ and JS contributed to acquisition of clinicopathological data and construction of tissue microarray. HK performed the experiments. HK and KJ interpreted the results. SS and SSP assisted with interpretation of data. HK drafted manuscript. KJ, SS and SSP supervised the study. All authors read and approved the final manuscript.

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