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# Expression Profile of S100A2 and its Clinicopathological Significance in Renal Cell Carcinoma

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**Abstract.** *Background/Aim: Dysregulated expression of S100 protein family members, including S100A2, has been reported in various types of human malignant tumors; however, the functional role of S100A2 in renal cell carcinoma (RCC) remains unknown. The objective of this study was to assess the S100A2 expression pattern and its clinicopathological significance in RCC. Materials and Methods: This study investigated the expression profiles of S100A2 mRNA, S100A2 protein and p53 mRNA in addition to S100A2 DNA methylation levels in 3 human RCC cell lines and 81 surgically resected RCC specimens. These findings were analyzed according to several clinicopathological parameters. Results: In all RCC cell lines, both S100A2 mRNA and protein were barely detectable, and the S100A2 promoter was considered to be methylated. S100A2 mRNA expression levels in RCC tissues were markedly lower than those in normal kidney tissues. The rate in clear cell RCC (ccRCC) specimens with higher expression of S100A2 mRNA was significantly lower than that in non-ccRCC specimens (29.9 versus 71.4%, respectively). Furthermore, S100A2 expression in ccRCC specimens was significantly correlated with p53 mRNA expression, but not conventional clinicopathological parameters. Conclusion: These findings suggest limited roles of S100A2 in the progression of RCC, particularly ccRCC.*

In humans, the S100 gene family has been shown to be composed of at least 21 members with marked structural similarity (1). This family of proteins is characterized by the ability to modulate a wide variety of cellular responses by functioning as intracellular $Ca^{2+}$ sensors as well as extracellular factors (2). In recent years, a number of studies demonstrated the involvement of multiple members of this family in various biological events mediating tumorigenesis and cancer progression (3). In fact, the dysregulated expression of S100 protein family members has been reported as a common feature of human malignant tumors, with each type of tumor exhibiting a unique expression profile of S100 proteins (4).

In human renal cell carcinoma (RCC) as well, intensive investigations focusing on the S100 gene family have been performed, and several interesting findings have been accumulated (5-10). For example, Küper *et al*. revealed that S100A4 is induced by the nuclear factor of activated T-cells 5 and stimulates proliferation and migration activities in human RCC cells (5), while Li *et al*. reported that immunohistochemical assessments of S100A1 could help distinguish renal oncocytoma and chromophobe RCC (10). We also previously reported the usefulness of S100A10 evaluation as a biomarker for diagnosing RCC (11). To date, however, no studies have investigated the significance of S100A2, one of the members of the S100 gene family, in relation to RCC.

Considering these findings, we evaluated the expression profiles of S100A2 mRNA, S100A2 protein and p53 mRNA in addition to S100A2 DNA methylation levels in human RCC cell lines and clinical RCC specimens in order to comprehensively characterize the role of S100A2 in RCC.

## Materials and Methods

*Tumor cell lines*. Two human RCC cell lines, Caki-1 and 769P, a human RCC cell line, Caki-2, and a human bladder cancer cell line, T24, were purchased from the American Type Culture Collection (Manassas, VA, USA), Riken Cell Bank (Tsukuba, Japan) and European Collection of Authenticated Cell Cultures (Salisbury, UK), respectively. These cell lines were maintained in RPMI 1640 (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum.

*Human RCC specimens*. Clinical specimens were obtained from a total of 81 patients who underwent nephrectomy and were pathologically diagnosed with RCC. The study protocol using these specimens was approved by the institutional review board of the Hamamatsu University School of Medicine (14-076), and written informed consent for the use of these specimens was obtained from all patients.

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*Reverse-transcription polymerase chain reaction (RT-PCR)*. The RT-PCR assay was performed to evaluate the expression of S100A2, peptidyl-prolyl cis-trans isomerase A (PPIA) and p53 mRNAs, as previously described (12). Briefly, total RNA was extracted, and first-strand cDNA was synthesized with a reverse transcriptase (Thermo Fisher Scientific, MA, USA). The synthesized cDNA and a recombinant Taq DNA polymerase (Thermo Fisher Scientific) were added to the standard PCR reaction mixture containing primers. The PCR reactions were performed as follows: samples were heated to 94˚C for 5 min, followed by 20, 25, 30 or 35 cycles, consisting of 3 reaction phases: 94˚C for 30 s, 63˚C for 30 s, and 72˚C for 30 s, in each cycle. In this study, we used the following primers: sense, 5'-TGCCACCTGGTCTGCCACAGAT-3' and anti-sense, 5'-TCCAGGCTGCCCATCAGCTTCT-3' for the analysis of S100A2, sense, 5'-AGACAAGGTCCCAAAGAC-3' and anti-sense, 5'-ACCACCCTGACA- CATAAA-3' for that of PPIA, and sense, 5'-GCCCCTCCTCAGCATC-3' and anti-sense, 5'-TCCCAGGA CAGGCACA-3' for that of p53. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining under ultraviolet (UV) transillumination.

The resulting images were density scanned, band intensities with areas corresponding to the amplimers of S100A2 were then quantified with the NIH image program (Wayne Rasband Analytics, NIH, Bethesda, MD, USA), and the expression level of S100A2 mRNA in RCC tissue relative to that in T24 cells used as a positive control was calculated.

*Combined bisulfite restriction analysis (COBRA)*. The methylation status of S100A2 was assessed using COBRA, as previously described (13). Briefly, genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Irving, CA, USA), and the bisulfite-converted DNA was used as a template in PCR with the sense primer (5'-GTTTATTATGTTAGTTAGGATGG-3'), and the anti-sense primer (5'-AAAAC-CCCTAAACTAAAATATCC-3'), which are designed to target the regulatory region of S100A2. Amplified products were then reacted with the restriction enzyme, *BstUI* (New England BioLabs, Ipswich, MA, USA). The digested DNA was electrophoresed on 3% agarose gels and visualized by ethidium bromide staining under UV transillumination. Methylated and non-methylated DNA sets (Zymo Research) were used as a control.

*Western blot analysis*. Western blotting was performed as previously reported (14). Equal amounts of protein samples from cell lines were subjected to electrophoresis using SDS-polyacrylamide gel and transferred to Immobilon®-P PVDF Membrane (Merck, Darmstadt, Germany). The membranes were incubated for 1 h, with human S100A2 and PPIA antibodies (Cell Signaling Technology, Inc., Tokyo, Japan). These membranes were subsequently incubated for 30 min, with secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA), and specific proteins were detected using an enhanced chemiluminescence system (Merck).

*Immunohistochemical staining*. An immunohistochemical study of clinical specimens was conducted as previously described (15). In brief, sections from paraffin-embedded tissues were deparaffinized and stained with human S100A2 antibody (R&D Systems, Minneapolis, MN, USA) for 1 h. Sections were then incubated with secondary antibody for 30 min, using Histofine Simple Stain Kit (Nichirei Biosciences, Tokyo, Japan), and counterstained with hematoxylin.

*Statistical analysis*. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), and a $p$-value<0.05 was considered significant. Differences in the expression levels of S100A2 according to several parameters were assessed by Fisher's exact $t$-test. Spearman's rank correlation coefficient analysis was performed to evaluate the association between S100A2 and p53 expression levels in RCC specimens.

## Results

*S100A2 expression and its epigenetic regulation in human RCC cell lines*. Expression of S100A2 mRNA in 3 human RCC cell lines was initially examined by RT-PCR. As shown in Figure 1A, S100A2 mRNA expression was confirmed in all 3 human RCC cell lines; however, after amplification of 20 cycles by PCR, this was present but barely detectable in 769P cells, but absent in the 2 remaining RCC cell lines. Western blot analysis showed findings consistent with RT-PCR (Figure 1B); that is, S100A2 protein expression was detected in only 769P cells. Furthermore, COBRA clearly showed that the promotor region of S100A2 was methylated in all 3 human RCC cell lines (Figure 2).

*S100A2 expression in clinical RCC specimens*. Expression of S100A2 mRNA in a total of 81 human clinical RCC specimens was assessed by RT-PCR. In normal kidney tissues from all 81 specimens, S100A2 mRNA was expressed. However, despite being markedly lower than those in the positive control, expression levels of S100A2 mRNA in RCC tissues varied. Thus, the expression level of S100A2 mRNA in RCC tissue relative to that in normal kidney tissue was calculated in each specimen, and its median value among those of 81 included specimens was used as a cut-off value. According to this definition, the median value was 0.2, and 30 (37.0%) and 51 (63.0%) specimens were judged to show high and low relative expression of S100A2 mRNA, respectively (Figure 3). Immunohistochemical staining revealed findings similar to those in RT-PCR; that is, positive S100A2 protein was observed in normal kidney tissues, while either no or scant S100A2 protein expression was detected in RCC tissues (Figure 4).

Of the 81 specimens, 67 (82.7%) and 14 (17.3%) were pathologically diagnosed as clear cell RCC (ccRCC) and non-ccRCC, respectively. The proportion in ccRCC specimens with a higher relative expression of S100A2 mRNA was significantly lower than that in non-ccRCC specimens (29.9 *vs*. 71.4%, respectively). However, there were no significant differences in the proportion in specimens with a higher relative expression of S100A2 mRNA according to several other clinicopathological parameters, including age, serum albumin, serum calcium, C-reactive protein, hemoglobin, Furman grade and clinical T stage.

*Association between S100A2 and p53 expression in clinical RCC specimens*. p53 mRNA expression levels in ccRCC

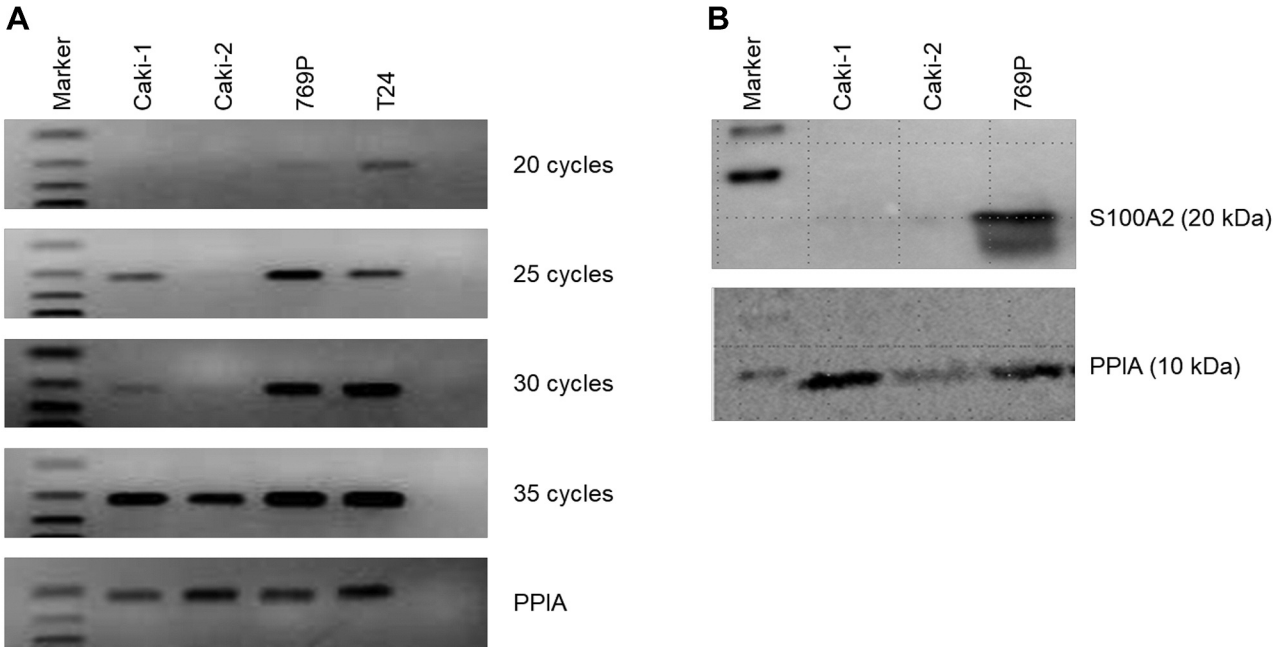


Figure 1. *(A) Reverse transcription-polymerase chain reaction (RT-PCR) using the set of primers for the amplification of S100A2 or peptidyl-prolyl cis-trans isomerase A (PPIA) in human renal cell carcinoma (RCC). The polymerase chain reaction products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining under ultraviolet transillumination. A human bladder cancer cell line, T24, was used as a positive control for the expression of S100A2. (B) Western blot analysis for the assessment of expression levels of S100A2 in human RCC cell lines. Protein was extracted from each cell line, and western blotting was performed to analyze the expression levels of S100A2 and PPIA in these cell lines.*

tissues from 64 specimens were measured by RT-PCR, and the variable expression status of p53 mRNA was observed in these RCC tissues. Based on the median p53 mRNA level as a cut-off value, 32 (50%) and 32 (50%) were classified into high and low p53 mRNA expression groups, respectively. Furthermore, there was a significant correlation between p53 mRNA and S100A2 mRNA expression levels in these specimens (Figure 5).

## Discussion

The S100 gene family consists of at least 21 members, which have been demonstrated to be involved in the regulation of various intra- and extracellular physiological events, such as calcium homeostasis, cell proliferation, apoptosis, chemotaxis, protein phosphorylation, cytoskeleton interactions, inflammation and autoimmunity (1, 2). The members of this family have also been shown to exhibit a wide variety of functions that help modulate the major phenotypes associated with the pathogenesis and progression of malignant tumors (3, 4). Furthermore, S100 gene family members have been reported to show altered expression in several types of malignant tumors, including RCC (4-11). However, accumulated findings on the association between S100A2 and cancer are contradictory (3, 4, 16-21). For example, S100A2 overexpression in a squamous cell carcinoma cell line induced a decrease in colony formation *via* the suppression of cyclooxygenase-2 and *in vivo* tumor growth (16), whereas S100A2 stimulated *in vivo* growth of lung cancer cells through the interaction of Smad3/TGF-β (17). Although these molecules are recognized as inflammatory mediators, an association between inflammatory signals and disease progression has been reported in RCC (22). Therefore, assessments of the expression of S100A2 in RCC may address the role of S100A2 in the pathophysiology of RCC. To our knowledge, no study has invested the role of S100A2 focusing on the association with RCC; accordingly, in this study, we comprehensively investigated the expression pattern of S100A2 in human RCC cell lines and clinical RCC specimens in order to assess its clinicopathological significance.

In this study, we initially characterized the expression profile of S100A2 in human RCC cell lines, and revealed that the S100A2 expression levels in these cell lines were not high. Thus, we subsequently investigated the association of epigenetic regulation with low expression of S100A2 in these cell lines, and found the definitive methylation of the S100A2 promotor in all cell lines. To date, there have been several studies showing epigenetic deregulation of S100 gene family members by differential hypo- and hypermethylation events in various types of malignant tumors (23). As for

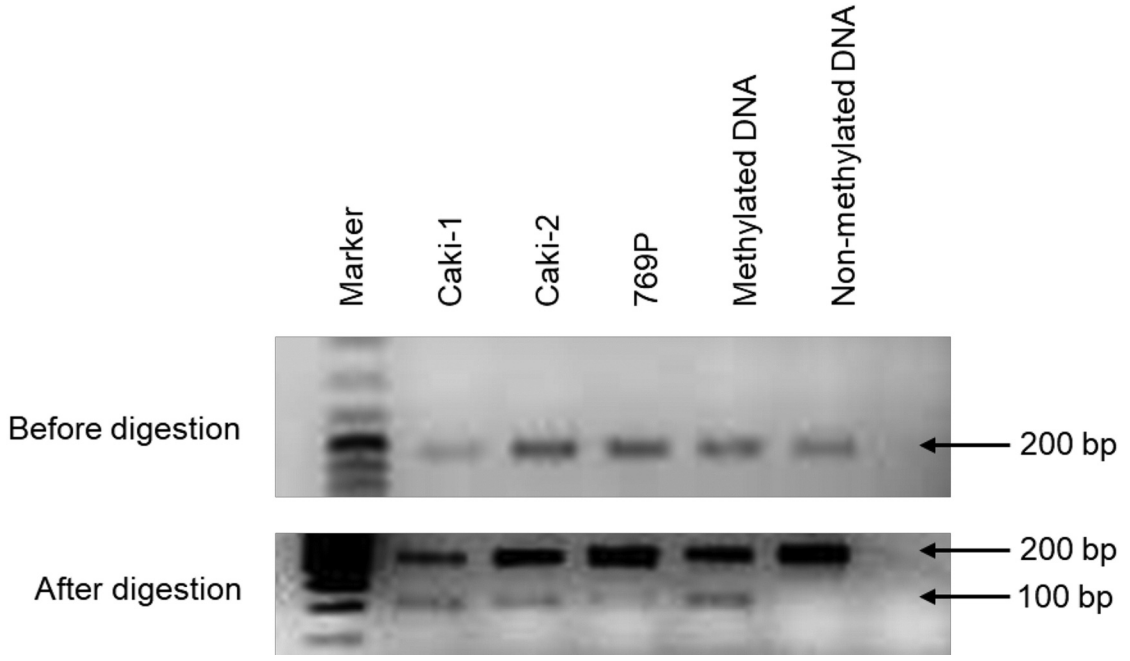


Figure 2. *Combined bisulfite restriction analysis to investigate the methylation status of S100A2 in human renal cell carcinoma (RCC) cell lines. Amplified products of genomic DNA from RCC cell lines by the polymerase chain reaction with the set of primers designed to target the regulatory region of S100A2 were reacted with the restriction enzyme, BstUI. The DNAs before and after digestion were electrophoresed on 3% agarose gel and visualized by ethidium bromide staining under ultraviolet transillumination.*

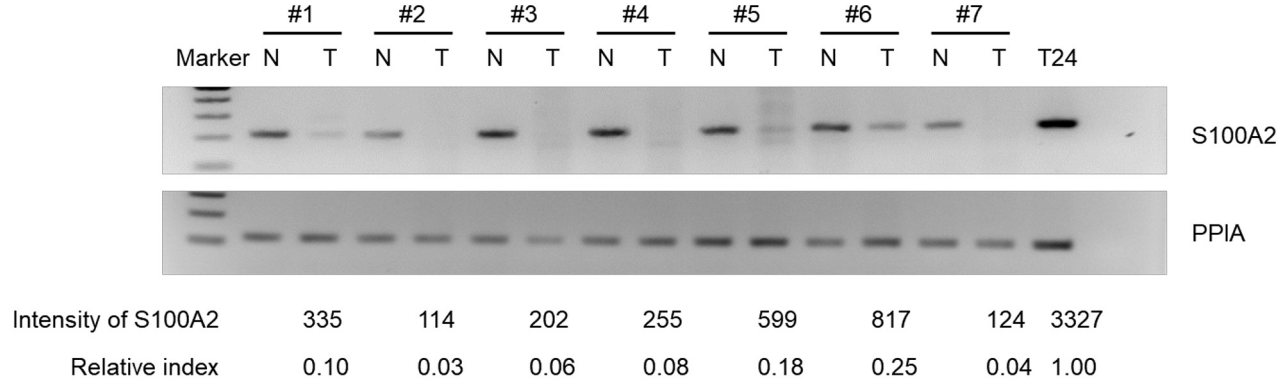


Figure 3. *Reverse transcription-polymerase chain reaction (RT-PCR) using the set of primers for the amplification of S100A2 or peptidyl-prolyl cis-trans isomerase A (PPIA) in surgically resected renal cell carcinoma specimens. The polymerase chain reaction products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining under ultraviolet transillumination. Representative findings (patients #1 – #7) are presented. Intensity of S100A2 mRNA expression and its relative index to that of the positive control quantified with the NIH image program are shown. A human bladder cancer cell line, T24, was used as a positive control for the expression of S100A2.*

S100A2, similar to the findings in this study, several studies reported the involvement of methylation in the down-regulation of S100A2 in multiple cancers, such as prostate, head and neck, and bladder cancer (24, 25).

Consistent with the evaluation of human RCC cell lines, assessments of clinical RCC specimens also showed low expression of S100A2 in tumor tissues compared to that in normal kidney tissues. However, high S100A2 expression was detected in 71.4% of non-ccRCC specimens, whereas only 29.9% of ccRCC specimens expressed high S100A2. These findings suggest the utility of measuring S100A2 expression in differentiating ccRCC and non-ccRCC specimens. Some

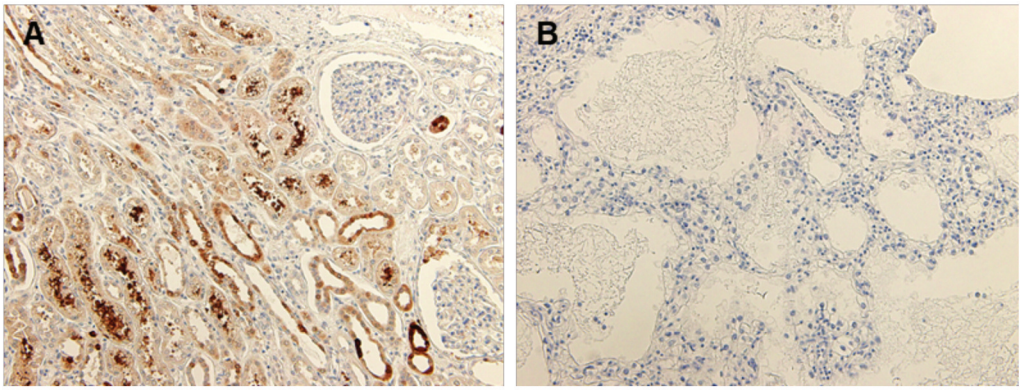


Figure 4. *Representative findings of immunohistochemical staining of surgically resected renal cell carcinoma (RCC) specimens with S100A2 antibody. (A) Normal kidney tissue with strong expression of S100A2. (B) RCC tissue with weak expression of S100A2.*

previous studies reported that evaluation of S100 gene family members could help distinguish non-ccRCC from other renal tumors (6, 9, 10, 26, 27). For example, Kuroda *et al*. showed that immunohistochemical staining of S100A1 may be useful for differentiating chromophobe RCC from oncocytoma (26), while Wang *et al*. showed higher S100A4 mRNA expression in ccRCC, but not other renal tumors, including papillary and chromophobe RCCs (27).

It is of interest to explore whether the expression level of S100A2 is correlated with conventional clinicopathological parameters, including prognostic indicators, in RCC patients. In this study, we could not identify any parameters significantly correlated with the expression level of S100A2. To date, there have been several studies reporting conflicting findings regarding the impact of S100A2 on the clinical behavior of malignant tumors (20, 28-32). For example, S100A2 is regarded as a poor prognostic marker for non-small cell lung cancer and colorectal cancer (28, 30), whereas it could be used as a predictor of a favorable prognosis in patients with pancreatic cancer (31, 32). Considering these findings, it is essential to perform a prospective study with a much larger sample size to draw a definitive conclusion concerning the significance of S100A2 as a biomarker of RCC.

Another point of interest is to investigate the possible mechanism underlying the functional role of S100A2 in RCC pathogenesis and progression. It has been well documented that S100 gene family members mediate key cellular functions, and of these, p53 signaling has been regarded as one of the most important pathways interacting with these members (3, 4). In fact, S100 proteins have been shown to affect p53 functions by regulating p53 transcriptional activity, resulting in the involvement of changes in the cell cycle as well as cell growth in several types of cancer cells (4, 33). S100A2 also facilitates interaction with p53 in a $Ca^{2+}$-dependent manner and stimulates p53 transcriptional activity, helping restore the p53 function in the presence of cell arrest and apoptotic cell death (34). In the present study, a significant correlation between S100A2 and p53 expression levels was noted in ccRCC clinical specimens, which may support their possible cooperation with each other.

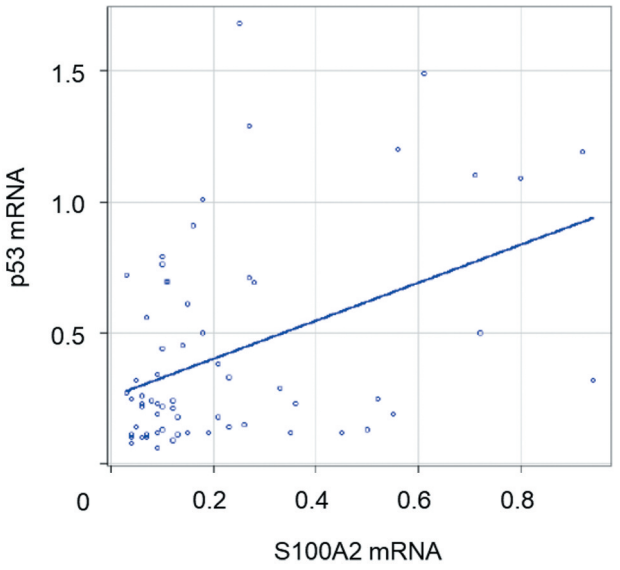


Figure 5. *Correlation of relative S100A2 and p53 expression in resected clear cell renal cell carcinoma (ccRCC) specimens by the reverse transcription-polymerase chain reaction. A significant correlation between S100A2 and p53 mRNA expression levels in ccRCC specimens was noted by Spearman's rank correlation coefficient analysis (p=0.044).*

## Conclusion

In human RCC cell lines, we showed low expression of S100A2 mRNA and protein, which might be due to the

methylation of the S100A2 promoter. In clinical specimens, S100A2 mRNA expression levels in RCC tissues were markedly lower than those in normal kidney tissues, and the rate in ccRCC specimens with high S100A2 expression was significantly lower than that in non-ccRCC specimens. Furthermore, there was a significant correlation between expression levels of S100A2 and p53 mRNAs. Collectively, these findings indicate that S100A2 may play limited roles in the progression of RCC, particularly ccRCC; however, it is necessary to draw a definitive conclusion on this point by conducting a prospective study with a larger sample size.

## Conflicts of Interest

The Authors have no conflicts of interest to declare.

## Authors' Contributions

The types of contribution by each author are as follows: Study conception and design, Takayuki Sugiyama, Ozono Seiichiro, Hideaki Miyake; Acquisition of data, Takayuki Sugiyama; Analysis and interpretation of data, Takayuki Sugiyama, Hideaki Miyake; Drafting of manuscript, Takayuki Sugiyama, Hideaki Miyake.

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