



Association of Expression Levels or Activation Status of STAT3 with Treatment Outcomes of Sunitinib in Patients with Renal Cell Carcinoma

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Association of Tumor Expression or Activation of STAT3 with Treatment

Outcomes of Sunitinib in Patients with Renal Cell Carcinoma

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Abstract

Background: The expression of signal transducer and activator of transcription 3 (STAT3) in tumor cells is correlated to the survival time in various type of cancer and chemotherapeutic outcomes.

Objective: This retrospective study aimed to elucidate the association of STAT3 expression in tumor cells with the therapeutic outcomes of sunitinib in patients with renal cell carcinoma (RCC).

Patients and Methods: Patients with metastatic RCC who received sunitinib therapy were enrolled in this study. All patients underwent radical nephrectomy for RCC, and nephrectomy specimens were stained for STAT3 and phosphorylated STAT3 (p-STAT3) by immunohistochemistry.

Results: We assessed 51 patients receiving sunitinib as a first-line therapy. The progression-free survival (PFS) and overall survival (OS) did not differ as per the STAT3 expression levels; however, patients with p-STAT3-positive tumors exhibited significantly worse PFS compared with those with p-STAT3-negative lesions (log-rank test, $P = 0.034$). Moreover, the OS tended to be prolonged in patients with p-STAT3-negative tumors. Conversely, the objective response rate or disease control rate based on the best overall response did not show a significant association with STAT3 or p-STAT3 expression. Furthermore, univariate Cox proportional hazard regression analyses for clinical predictors revealed that p-STAT3 positivity significantly correlated with shorter PFS (hazard ratio [HR], 2.22, $P = 0.041$), whereas p-STAT3 expression was not related to the OS.

Conclusions: Activated STAT3 in tumor tissues shows a significant association with poor prognosis in patients with RCC who received sunitinib as a first-line therapy, and positive p-STAT3 expression could be a potential biomarker for refractory to sunitinib therapy.

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6 **Key points**
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9 · Activated STAT3 in tumor tissues is significantly associated with poor prognosis in patients with
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11 RCC who received sunitinib as a first-line therapy.
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14 · The expression of p-STAT3 in RCC cells could be used as a biomarker for the refractory to
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16 sunitinib therapy.
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1. Introduction

Multiple tyrosine kinase inhibitors (mTKIs) are the standard therapeutic option for patients with renal cell carcinoma (RCC) and are known to extend the progression-free survival (PFS) and overall survival (OS) significantly compared with interferon therapy in patients with metastatic RCC (mRCC) [1,2]. For patients with mRCC with a good performance status (PS) according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for Kidney Cancer, sunitinib serves as a first-line therapy option [3]. However, approximately 20% of the patients receiving sunitinib are diagnosed with a progressive disease (PD) [1]. In addition, sunitinib has been reported to be severely toxic leading to complications including hematological disorders, diarrhea, and fatigue. Thus, determining prognostic and individualized markers for ensuring its safety and efficacy is imperative. Although several prognostic markers have been reported in patients with RCC receiving sunitinib, no clinical application of prognostic factors signifying the tumor cell profile has been reported.

Signal transducer and activator of transcription 3 (STAT3) is a signal transducer and transcriptional factor that is activated by growth factors and cytokines through phosphorylation by receptor or non-receptor tyrosine kinases [4]. STAT3 plays an important role in proliferation, apoptosis, and migration of cancer and is an important target for cancer therapy [5-7]. In addition, preliminary studies have suggested that inhibition of STAT3 activation in tumor cells is one of antitumor effects of sunitinib [8-11].

Moreover, STAT3 is known to play a role in the mechanism of resistance to sunitinib in tumor cells [12]. In particular, STAT3 decreases the intracellular accumulation of sunitinib by enhancing P-glycoprotein expression. In addition, several previous reports suggest that genetic polymorphisms in *STAT3* are

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3 correlated to the therapeutic outcomes of interferon therapy in Japanese patients with RCC [13,14]. In our
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6 previous study, we reported that genetic polymorphisms in *STAT3* are associated with the overall response
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9 to mTKIs therapy in patients with RCC [15]. Hence, we further hypothesized that specific polymorphisms
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12 associated with lower *STAT3* protein expression could be associated with a better prognosis following
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15 chemotherapy in patients with RCC. Although genomic DNA extracted from peripheral blood was
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18 analyzed in these studies, the correlation of actual *STAT3* expression levels in tumor cells with
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21 therapeutic outcomes of sunitinib remains unclear.
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23 This retrospective analysis using nephrectomy specimens from patients with RCC aims to elucidate the
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26 correlation between actual *STAT3* expression level in tumor cells and clinical outcomes after sunitinib
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29 therapy.
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31 32 33 34 35 36 **2. Materials and Methods**

37 38 *2.1 Patients*

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40 In this study, we retrospectively examined 62 consecutive patients with mRCC who were treated with
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42 sunitinib at the Department of Urology, Kobe University Hospital (Kobe, Japan) between January 2013
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44 and December 2014. All enrolled patients underwent radical nephrectomy for RCC, and initially received
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46 either 50 mg once daily in repeated 6-week cycles comprising 4 weeks on treatment followed by 2 weeks
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48 off treatment. The sunitinib administration was continued until the disease progression or intolerable
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50 adverse events (AEs) occurred. As a rule, dose modification was considered in patients with treatment-
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52 associated AEs that were grade-3 or higher as follows: the initial sunitinib dose was reduced from 50 to
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54 37.5 mg once daily and then to 25 mg once daily and patients who failed sunitinib treatment before the
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56 evaluation using computed tomography (CT) were excluded from the analysis of the best overall
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58 response. This study protocol was approved by the ethical committee at Kobe University Graduate of
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Medicine, and we obtained informed consent from each enrolled patient. The medical records of all patients were collected in cooperation with an attending urologist. In addition, all procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

2.2 Immunohistochemical staining

We used nephrectomy sections from the RCC operation for analysis in this study, and formaldehyde-fixed, paraffin-embedded tissue was deparaffinized and rehydrated. After blocking endogenous peroxidase, sections were incubated with STAT3 and phosphorylated STAT3 (p-STAT3) antibodies (Cat. No. #8768 and #9145; Cell Signaling Technology, Boston, MA) overnight at 4°C. Next, the samples were incubated with the corresponding secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After incubation in an avidin–biotin–peroxidase complex, we exposed the sections to diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin. Staining findings were interpreted and scored by two independent observers who were blinded to the clinicopathological data of patients. Positive staining for STAT3 or p-STAT3 in >50% of tumor cells was indicative of the positive expression for these proteins with reference to a previous study that evaluated the survival using pSTAT3 immunohistochemistry of histological specimens[16,17].

2.3 Evaluation of clinical outcomes with sunitinib

Baseline evaluations at the start of a first-line therapy with sunitinib comprised the PS and clinicopathological examinations, which were assessed on the basis of the Karnofsky PS scale. In addition, the risk classification was performed using the Memorial Sloan–Kettering Cancer Center (MSKCC) score for mRCC [18,19]. Before the introduction of sunitinib, all patients were evaluated through radiological examinations, including CT of the brain, chest, and abdomen, or radionuclide bone scans, or both. Typically, tumor measurements were performed using CT every 8–12 weeks after initiating sunitinib therapy. All responses and AEs were assessed by a treating physician based on the Response Evaluation Criteria in Solid Tumors version 1.1 and the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0, respectively.

2.4 Statistical analysis

Categorical data across groups in this study compared using the Fischer's exact test. Continuous data across groups compared using the unpaired Student's *t*-test. In addition, the PFS and OS were calculated using the Kaplan–Meier method, and significant differences were determined using the log-rank test. The significance of predictive factors was assessed using the Cox proportional hazards regression model. Furthermore, we considered $P < 0.05$ (two-tailed) as statistically significant in this study. All statistical analyses were performed using SPSS software version 24.

3. Results

3.1 Patient characteristics according to STAT3 or p-STAT3 expression patterns

In this study, we enrolled 62 patients (Fig. 1). However, time-to-event analyses were performed with 51 patients after excluding those treated with sunitinib post-first-line therapy. In addition, the best overall response analysis was performed in 48 patients after excluding patients who failed sunitinib treatment prior to diagnosis using CT. Immunohistochemical staining revealed that positive p-STAT3 expression and positive STAT3 expression were observed in 14 (27.5%) and 22 patients (43.1%), respectively. Figure 2 shows representative images of immunohistochemical staining, and Table 1 represents the characteristics of 51 patients according to the STAT3 and p-STAT3 expression patterns. Patients with the positive p-STAT3 expression were related to the lower body mass index (BMI; $P = 0.037$) relative to the findings in patients with the negative p-STAT3 expression.

3.2 Association of the STAT3 and p-STAT3 expression in tumor cells with treatment outcomes

In this study, the median PFS and OS in 51 patients were 10.3 (95% confidence interval [CI]: 5.8–16.3) and 38.0 (95% CI: 15.4 to not reached) months, respectively. Figure 3 shows the Kaplan–Meier curves for the PFS and OS according to the STAT3 and p-STAT3 expression levels. We observed no significant difference in the PFS and OS between the positive and negative STAT3 expression groups; however, patients with positive p-STAT3 expression exhibited significantly shorter PFS than those with negative p-STAT3 expression ($P = 0.037$). In addition, p-STAT3 negativity correlated with a prolonged OS, although the differences were not statistically significant. Furthermore, we observed no significant associations in the objective response and disease control rates based on the overall best response with STAT3 or p-STAT3 expression levels (Table 2).

3.3 Predictive effects of the STAT3 or p-STAT3 expression on the clinical outcomes of the PFS and OS

Univariate Cox proportional hazards regression analyses for the PFS and OS revealed that the positive p-STAT3 expression was significantly associated with a shorter PFS, and multiple metastatic sites and poor MSKCC risk were also associated with shorter PFS (Table 3). Conversely, younger age, lower BMI, and poor MSKCC risk significantly correlated with the shorter OS. Furthermore, the positive p-STAT3 expression did not show significant association with a shorter OS. Multivariate analyses to evaluate predictive factors for PFS and OS were not performed because of its small sample size.

4. Discussion

STAT3 is activated by phosphorylation and enhances the transcription of the target genes related to cell proliferation, apoptosis suppressors, and migration factors [5]. Reportedly, the apoptosis- and cell growth-inhibiting effects of sunitinib were reduced in constitutively-active STAT3-transfected RCC cell lines; conversely, the cell growth-inhibitory effects of sunitinib were enhanced in STAT3-knockdown RCC cells [10]. This study supports the previous findings and demonstrates that the activated STAT3, not the total STAT3, expression in tumor cells is correlated to a shorter PFS of sunitinib therapy in patients with RCC. In contrast, the OS did not significantly differ between the positive and negative p-STAT3 expression groups, although the OS in the early phase after the initiation of sunitinib was significantly higher in p-STAT3-negative patients compared with that in p-STAT3-positive patients. This study included patients treated with sunitinib therapy as a first-line RCC therapy, and most p-STAT3-positive patients were in the disease progression at 10 months after sunitinib therapy, and their therapy were changed to others from sunitinib. Moreover, there were no significant associations in the objective response and disease control rates based on the best overall response with STAT3 or p-STAT3 expression levels because there was no significant difference of number of patients at the end of follow-up period in PFS and OS between high and low p-STAT3 expression groups. Thus, the positive p-STAT3 expression efficiently predicts the refractory to sunitinib therapy in an early phase. P-STAT3 is a refractory factor and not associate with a responsiveness because p-STAT3 associates with chemotherapy resistance and migration relating to EMT. In addition, some studies have suggested the correlation between p-STAT3

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3 levels in RCC specimens and survival times for patients with RCC regardless of the type of treatment
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6 [17,20]. In addition, survival times in patients with prostate cancer are demonstrated to be associated with
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9 p-STAT3 levels more than RCC [21]. Perhaps, the p-STAT3 tumor expression level could be a prognostic
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12 marker in various types of cancers. These studies corroborate our hypothesis that p-STAT3 could possibly
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15 predict the OS after sunitinib therapy in RCC.
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17 Our previous study revealed that functional single-nucleotide polymorphisms (SNPs) that can
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20 potentially lower the STAT3 expression are associated with the good overall best response or short time to
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23 response with TKI therapy for mRCC, but not with PFS [15]. However, this study demonstrates that the
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26 STAT3 expression in tumors was not related to the best overall response to sunitinib therapy. In addition,
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29 our previous study determined that functional SNPs in *STAT3* did not correlate with the tumor expression
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32 of STAT3 in patients with RCC (data not shown). Thus, these findings suggest that genomic
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35 polymorphisms of *STAT3* do not affect the STAT3 tumor expression. The correlation of functional *STAT3*
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38 SNPs with treatment response to sunitinib could be illustrated by the difference of the STAT3 expression
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41 in systemic immune cells according to the polymorphism; however, further studies are warranted to
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44 certify this hypothesis.
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46 Reportedly, the STAT3 activation in tumor cells is induced by Interleukin (IL)-6 [22]. Some studies
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49 have reported that higher serum IL-6 levels induced the therapeutic resistance to sunitinib and resulted in
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52 the worse OS and PFS [23,24]. Briefly, the tumor p-STAT3 expression could be a response to higher
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55 serum IL-6 levels, causing the sunitinib resistance. In addition, the IL-6–STAT3 pathway activation
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58 induced the epithelial–mesenchymal transition (EMT), which is the most crucial process for the sunitinib
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3 resistance and acquirement of the migratory mesenchymal phenotype in RCC [25,26]. Thus, association
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6 with the STAT3 activation and prognosis of sunitinib therapy could mediate the EMT process; however,
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9 further investigation is needed to verify this hypothesis.

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11 The median PFS among 51 patients who received the first-line sunitinib therapy in this study was 10.3
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13 months, which corroborated the median PFS (11 months) in patients with RCC who received the first-line
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15 sunitinib therapy in an international, multicenter, randomized, phase III trial [27]. In contrast, the median
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17 OS in this study was 38 months, although it was 26.4 months in the aforementioned international trial.
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22 Apparently, the therapeutic outcome of sunitinib is better in Japanese patients than in other populations.
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26 Phase II trials of Japanese patients with untreated RCC demonstrated that the median PFS and OS were
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28 12.2 and 33.1 months, respectively [28,29], which was consistent with our study. In addition, Japanese
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30 patients with mRCC currently have more options after a first-line therapy than those available when the
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32 international trial was conducted. Perhaps, these findings could explain the longer OS observed in our
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35 study.
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41 MSKCC scores are classified on the basis of poor prognostic factors for cytokine therapy, and some
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43 studies suggested that the MSKCC risk is also associated with the prognosis of sunitinib therapy in clear-
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45 cell RCC but not other than clear-cell RCC [27,30]. In this study, we could not establish a significant
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47 correlation between the PFS and the MSKCC risk score because patients with non-clear-cell RCC were
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49 enrolled in this study. In addition, the presence of multiple metastatic sites correlated with a poor
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51 prognosis for sunitinib therapy [27,31]. However, younger patients exhibited poor prognoses following
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53 sunitinib therapy in our univariate analysis to assess predictive factors for the OS. Furthermore, subgroup
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3 analyzes and updated OS results from the Sunitinib Treatment Of Renal Adjuvant Cancer (S-TRAC)
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6 study, in which sunitinib was tested as an adjuvant treatment, illustrated that patients aged 45–64 years
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9 who were treated with sunitinib did not experience superior outcomes compared to placebo-treated
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12 patients [32]. Some studies have reported that patients with RCC and lower BMI exhibited poorer cancer-
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15 specific survival [33]. As described previously, the association of known prognostic factors with the OS in
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18 this study supported the published data.

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20 This study has several limitations. First, this was a small-sample, retrospective study. Thus, multivariate
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23 analysis of various parameters as predictors of the PFS and OS were not performed. In addition, the study
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26 protocol was not adequate to obtain definitive conclusions. Second, poorly standardized and validated
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29 procedures of STAT3 immunohistochemical staining were used in this study. The immunohistochemical
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32 analysis could be typically affected by the variation in antibodies and thresholds for judgment of positive
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35 expression. Finally, immunostaining was performed using primary tumor specimens, although metastatic
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38 tumor specimens would be more suitable for investigating the correlation between mTKI therapeutic
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41 outcomes and the expression of specific factors.

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43 In conclusion, activated STAT3 in tumor tissues shows significant correlation with poor prognosis in
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46 patients with RCC who received sunitinib as a first-line therapy. In addition, the positive p-STAT3
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49 expression is likely correlated with a shorter OS in the early treatment phase. Hence, this study suggests
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52 that the STAT3 activation in tumor tissues could be a predictive marker of refractory to sunitinib therapy
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55 in patients with mRCC.
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Compliance with Ethical Standards

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Conflict of interest

H. Miyake and M. Fujisawa have received lecture fees from Pfizer, whereas the other authors disclosed no potential conflicts of interest.

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Figure Legends

Fig. 1 Distribution of patients.

Fig. 2 Examples of immunohistochemical staining of RCC tumor cells using antibodies against signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 (p-STAT3).

Positive staining for STAT3 or p-STAT3 in >50% of tumor cells indicated positive expression of these proteins.

Fig. 3. Correlation of the signal transducer and activator of transcription 3 (STAT3) expression status with progression-free survival (PFS) and overall survival (OS). A, the PFS in 51 patients with renal cell carcinoma (RCC) following a first-line treatment with sunitinib based on their STAT3 and p-STAT3 expression status. B, the OS in 51 patients with RCC following a first-line treatment with sunitinib based on their STAT3 and p-STAT3 expression status. Gray line, negative expression; black line, positive expression. *P*-values were calculated using the log-rank test.

Association of Tumor Expression or Activation of STAT3 with Treatment

Outcomes of Sunitinib in Patients with Renal Cell Carcinoma

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Abstract

Background: The expression of signal transducer and activator of transcription 3 (STAT3) in tumor cells is correlated to the survival time in various type of cancer and chemotherapeutic outcomes.

Objective: This retrospective study aimed to elucidate the association of STAT3 expression in tumor cells with the therapeutic outcomes of sunitinib in patients with renal cell carcinoma (RCC).

Patients and Methods: Patients with metastatic RCC who received sunitinib therapy were enrolled in this study. All patients underwent radical nephrectomy for RCC, and nephrectomy specimens were stained for STAT3 and phosphorylated STAT3 (p-STAT3) by immunohistochemistry.

Results: We assessed 51 patients receiving sunitinib as a first-line therapy. The progression-free survival (PFS) and overall survival (OS) did not differ as per the STAT3 expression levels; however, patients with p-STAT3-positive tumors exhibited significantly worse PFS compared with those with p-STAT3-negative lesions (log-rank test, $P = 0.034$). Moreover, the OS tended to be prolonged in patients with p-STAT3-negative tumors. Conversely, the objective response rate or disease control rate based on the best overall response did not show a significant association with STAT3 or p-STAT3 expression. Furthermore, univariate Cox proportional hazard regression analyses for clinical predictors revealed that p-STAT3 positivity significantly correlated with shorter PFS (hazard ratio [HR], 2.22, $P = 0.041$), whereas p-STAT3 expression was not related to the OS.

Conclusions: Activated STAT3 in tumor tissues shows a significant association with poor prognosis in patients with RCC who received sunitinib as a first-line therapy, and positive p-STAT3 expression could be a potential biomarker for refractory to sunitinib therapy.

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6 **Key points**
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9 · Activated STAT3 in tumor tissues is significantly associated with poor prognosis in patients with
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11 RCC who received sunitinib as a first-line therapy.
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14 · The expression of p-STAT3 in RCC cells could be used as a biomarker for the refractory to
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16 sunitinib therapy.
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1. Introduction

Multiple tyrosine kinase inhibitors (mTKIs) are the standard therapeutic option for patients with renal cell carcinoma (RCC) and are known to extend the progression-free survival (PFS) and overall survival (OS) significantly compared with interferon therapy in patients with metastatic RCC (mRCC) [1,2]. For patients with mRCC with a good performance status (PS) according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for Kidney Cancer, sunitinib serves as a first-line therapy option [3]. However, approximately 20% of the patients receiving sunitinib are diagnosed with a progressive disease (PD) [1]. In addition, sunitinib has been reported to be severely toxic leading to complications including hematological disorders, diarrhea, and fatigue. Thus, determining prognostic and individualized markers for ensuring its safety and efficacy is imperative. Although several prognostic markers have been reported in patients with RCC receiving sunitinib, no clinical application of prognostic factors signifying the tumor cell profile has been reported.

Signal transducer and activator of transcription 3 (STAT3) is a signal transducer and transcriptional factor that is activated by growth factors and cytokines through phosphorylation by receptor or non-receptor tyrosine kinases [4]. STAT3 plays an important role in proliferation, apoptosis, and migration of cancer and is an important target for cancer therapy [5-7]. In addition, preliminary studies have suggested that inhibition of STAT3 activation in tumor cells is one of antitumor effects of sunitinib [8-11].

Moreover, STAT3 is known to play a role in the mechanism of resistance to sunitinib in tumor cells [12]. In particular, STAT3 decreases the intracellular accumulation of sunitinib by enhancing P-glycoprotein expression. In addition, several previous reports suggest that genetic polymorphisms in *STAT3* are

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3 correlated to the therapeutic outcomes of interferon therapy in Japanese patients with RCC [13,14]. In our
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6 previous study, we reported that genetic polymorphisms in *STAT3* are associated with the overall response
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9 to mTKIs therapy in patients with RCC [15]. Hence, we further hypothesized that specific polymorphisms
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12 associated with lower *STAT3* protein expression could be associated with a better prognosis following
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15 chemotherapy in patients with RCC. Although genomic DNA extracted from peripheral blood was
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18 analyzed in these studies, the correlation of actual *STAT3* expression levels in tumor cells with
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21 therapeutic outcomes of sunitinib remains unclear.
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23 This retrospective analysis using nephrectomy specimens from patients with RCC aims to elucidate the
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26 correlation between actual *STAT3* expression level in tumor cells and clinical outcomes after sunitinib
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29 therapy.
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31 32 33 34 35 36 **2. Materials and Methods**

37 38 **2.1 Patients**

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40 In this study, we retrospectively examined 62 consecutive patients with mRCC who were treated with
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42 sunitinib at the Department of Urology, Kobe University Hospital (Kobe, Japan) between January 2013
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44 and December 2014. All enrolled patients underwent radical nephrectomy for RCC, and initially received
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46 either 50 mg once daily in repeated 6-week cycles comprising 4 weeks on treatment followed by 2 weeks
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48 off treatment. The sunitinib administration was continued until the disease progression or intolerable
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50 adverse events (AEs) occurred. As a rule, dose modification was considered in patients with treatment-
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52 associated AEs that were grade-3 or higher as follows: the initial sunitinib dose was reduced from 50 to
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54 37.5 mg once daily and then to 25 mg once daily and patients who failed sunitinib treatment before the
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56 evaluation using computed tomography (CT) were excluded from the analysis of the best overall
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58 response. This study protocol was approved by the ethical committee at Kobe University Graduate of
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2 **Medicine**, and we obtained informed consent from each enrolled patient. The medical records of all
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4 patients were collected in cooperation with an attending urologist. In addition, **all procedures performed**
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6 **in this study involving human participants were in accordance with the ethical standards of the**
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8 **institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later**
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10 **amendments or comparable ethical standards.**
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14 **2.2 Immunohistochemical staining**

16 We used nephrectomy sections from the RCC operation for analysis in this study, and formaldehyde-
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18 fixed, paraffin-embedded tissue was deparaffinized and rehydrated. After blocking endogenous
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20 peroxidase, sections were incubated with STAT3 and phosphorylated STAT3 (p-STAT3) antibodies (Cat.
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22 **No. #8768 and #9145**; Cell Signaling Technology, Boston, MA) overnight at 4°C. Next, the samples were
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24 incubated with the corresponding secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 min
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26 at room temperature. After incubation in an avidin–biotin–peroxidase complex, we exposed the sections
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28 to diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin. Staining findings
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30 were interpreted and scored by two independent observers who were blinded to the clinicopathological
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32 data of patients. Positive staining for STAT3 or p-STAT3 in >50% of tumor cells was indicative of the
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34 positive expression for these proteins **with reference to a previous study that evaluated the survival using**
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36 **pSTAT3 immunohistochemistry of histological specimens[16,17].**
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41 **2.3 Evaluation of clinical outcomes with sunitinib**

43 Baseline evaluations at the start of a first-line therapy with sunitinib comprised the PS and
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45 clinicopathological examinations, which were assessed on the basis of the Karnofsky PS scale. In
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47 addition, the risk classification was performed using the Memorial Sloan–Kettering Cancer Center
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49 (MSKCC) score for mRCC [18,19]. Before the introduction of sunitinib, all patients were evaluated
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51 through radiological examinations, including CT of the brain, chest, and abdomen, or radionuclide bone
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53 scans, or both. Typically, tumor measurements were performed using CT every 8–12 weeks after initiating
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55 sunitinib therapy. All responses and AEs were assessed by a treating physician based on the Response
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57 Evaluation Criteria in Solid Tumors version 1.1 and the National Cancer Institute Common Terminology
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59 Criteria for Adverse Events version 3.0, respectively.
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2.4 Statistical analysis

Categorical data across groups in this study compared using the Fischer's exact test. Continuous data across groups compared using the unpaired Student's *t*-test. In addition, the PFS and OS were calculated using the Kaplan–Meier method, and significant differences were determined using the log-rank test. The significance of predictive factors was assessed using the Cox proportional hazards regression model. Furthermore, we considered $P < 0.05$ (two-tailed) as statistically significant in this study. All statistical analyses were performed using SPSS software version 24.

3. Results

3.1 Patient characteristics according to STAT3 or p-STAT3 expression patterns

In this study, we enrolled 62 patients (Fig. 1). However, time-to-event analyses were performed with 51 patients after excluding those treated with sunitinib post-first-line therapy. In addition, the best overall response analysis was performed in 48 patients after excluding patients who failed sunitinib treatment prior to diagnosis using CT. Immunohistochemical staining revealed that positive p-STAT3 expression and positive STAT3 expression were observed in 14 (27.5%) and 22 patients (43.1%), respectively. Figure 2 shows representative images of immunohistochemical staining, and Table 1 represents the characteristics of 51 patients according to the STAT3 and p-STAT3 expression patterns. Patients with the positive p-STAT3 expression were related to the lower body mass index (BMI; $P = 0.037$) relative to the findings in patients with the negative p-STAT3 expression.

3.2 Association of the STAT3 and p-STAT3 expression in tumor cells with treatment outcomes

In this study, the median PFS and OS in 51 patients were 10.3 (95% confidence interval [CI]: 5.8–16.3) and 38.0 (95% CI: 15.4 to not reached) months, respectively. Figure 3 shows the Kaplan–Meier curves for the PFS and OS according to the STAT3 and p-STAT3 expression levels. We observed no significant difference in the PFS and OS between the positive and negative STAT3 expression groups; however, patients with positive p-STAT3 expression exhibited significantly shorter PFS than those with negative p-STAT3 expression ($P = 0.037$). In addition, p-STAT3 negativity correlated with a prolonged OS, although the differences were not statistically significant. Furthermore, we observed no significant associations in the objective response and disease control rates based on the overall best response with STAT3 or p-STAT3 expression levels (Table 2).

3.3 Predictive effects of the STAT3 or p-STAT3 expression on the clinical outcomes of the PFS and OS

Univariate Cox proportional hazards regression analyses for the PFS and OS revealed that the positive p-STAT3 expression was significantly associated with a shorter PFS, and multiple metastatic sites and poor MSKCC risk were also associated with shorter PFS (Table 3). Conversely, younger age, lower BMI, and poor MSKCC risk significantly correlated with the shorter OS. Furthermore, the positive p-STAT3 expression did not show significant association with a shorter OS. Multivariate analyses to evaluate predictive factors for PFS and OS were not performed because of its small sample size.

4. Discussion

STAT3 is activated by phosphorylation and enhances the transcription of the target genes related to cell proliferation, apoptosis suppressors, and migration factors [5]. Reportedly, the apoptosis- and cell growth-inhibiting effects of sunitinib were reduced in constitutively-active STAT3-transfected RCC cell lines; conversely, the cell growth-inhibitory effects of sunitinib were enhanced in STAT3-knockdown RCC cells [10]. This study supports the previous findings and demonstrates that the activated STAT3, not the total STAT3, expression in tumor cells is correlated to a shorter PFS of sunitinib therapy in patients with RCC. In contrast, the OS did not significantly differ between the positive and negative p-STAT3 expression groups, although the OS in the early phase after the initiation of sunitinib was significantly higher in p-STAT3-negative patients compared with that in p-STAT3-positive patients. This study included patients treated with sunitinib therapy as a first-line RCC therapy, and most p-STAT3-positive patients were in the disease progression at 10 months after sunitinib therapy, and their therapy were changed to others from sunitinib. Moreover, there were no significant associations in the objective response and disease control rates based on the best overall response with STAT3 or p-STAT3 expression levels because there was no significant difference of number of patients at the end of follow-up period in PFS and OS between high and low p-STAT3 expression groups. Thus, the positive p-STAT3 expression efficiently predicts the refractory to sunitinib therapy in an early phase. P-STAT3 is a refractory factor and not associate with a responsiveness because p-STAT3 associates with chemotherapy resistance and migration relating to EMT. In addition, some studies have suggested the correlation between p-STAT3

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3 levels in RCC specimens and survival times for patients with RCC regardless of the type of treatment
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6 [17,20]. In addition, survival times in patients with prostate cancer are demonstrated to be associated with
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9 p-STAT3 levels more than RCC [21]. Perhaps, the p-STAT3 tumor expression level could be a prognostic
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12 marker in various types of cancers. These studies corroborate our hypothesis that p-STAT3 could possibly
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15 predict the OS after sunitinib therapy in RCC.
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18 Our previous study revealed that functional single-nucleotide polymorphisms (SNPs) that can
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21 potentially lower the STAT3 expression are associated with the good overall best response or short time to
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24 response with TKI therapy for mRCC, but not with PFS [15]. However, this study demonstrates that the
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27 STAT3 expression in tumors was not related to the best overall response to sunitinib therapy. In addition,
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30 our previous study determined that functional SNPs in *STAT3* did not correlate with the tumor expression
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33 of STAT3 in patients with RCC (data not shown). Thus, these findings suggest that genomic
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36 polymorphisms of *STAT3* do not affect the STAT3 tumor expression. The correlation of functional *STAT3*
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39 SNPs with treatment response to sunitinib could be illustrated by the difference of the STAT3 expression
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42 in systemic immune cells according to the polymorphism; however, further studies are warranted to
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45 certify this hypothesis.
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49 Reportedly, the STAT3 activation in tumor cells is induced by Interleukin (IL)-6 [22]. Some studies
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52 have reported that higher serum IL-6 levels induced the therapeutic resistance to sunitinib and resulted in
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55 the worse OS and PFS [23,24]. Briefly, the tumor p-STAT3 expression could be a response to higher
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58 serum IL-6 levels, causing the sunitinib resistance. In addition, the IL-6–STAT3 pathway activation
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61 induced the epithelial–mesenchymal transition (EMT), which is the most crucial process for the sunitinib
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3 resistance and acquirement of the migratory mesenchymal phenotype in RCC [25,26]. Thus, association
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6 with the STAT3 activation and prognosis of sunitinib therapy could mediate the EMT process; however,
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9 further investigation is needed to verify this hypothesis.

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11 The median PFS among 51 patients who received the first-line sunitinib therapy in this study was 10.3
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13 months, which corroborated the median PFS (11 months) in patients with RCC who received the first-line
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15 sunitinib therapy in an international, multicenter, randomized, phase III trial [27]. In contrast, the median
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17 OS in this study was 38 months, although it was 26.4 months in the aforementioned international trial.
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22 Apparently, the therapeutic outcome of sunitinib is better in Japanese patients than in other populations.
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26 Phase II trials of Japanese patients with untreated RCC demonstrated that the median PFS and OS were
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28 12.2 and 33.1 months, respectively [28,29], which was consistent with our study. In addition, Japanese
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30 patients with mRCC currently have more options after a first-line therapy than those available when the
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32 international trial was conducted. Perhaps, these findings could explain the longer OS observed in our
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38 study.

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41 MSKCC scores are classified on the basis of poor prognostic factors for cytokine therapy, and some
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43 studies suggested that the MSKCC risk is also associated with the prognosis of sunitinib therapy in clear-
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45 cell RCC but not other than clear-cell RCC [27,30]. In this study, we could not establish a significant
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47 correlation between the PFS and the MSKCC risk score because patients with non-clear-cell RCC were
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51 enrolled in this study. In addition, the presence of multiple metastatic sites correlated with a poor
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55 prognosis for sunitinib therapy [27,31]. However, younger patients exhibited poor prognoses following
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58 sunitinib therapy in our univariate analysis to assess predictive factors for the OS. Furthermore, subgroup
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3 analyzes and updated OS results from the Sunitinib Treatment Of Renal Adjuvant Cancer (S-TRAC)
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6 study, in which sunitinib was tested as an adjuvant treatment, illustrated that patients aged 45–64 years
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9 who were treated with sunitinib did not experience superior outcomes compared to placebo-treated
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12 patients [32]. Some studies have reported that patients with RCC and lower BMI exhibited poorer cancer-
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15 specific survival [33]. As described previously, the association of known prognostic factors with the OS in
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18 this study supported the published data.

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20 This study has several limitations. First, this was a small-sample, retrospective study. Thus, multivariate
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23 analysis of various parameters as predictors of the PFS and OS were not performed. In addition, the study
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26 protocol was not adequate to obtain definitive conclusions. Second, poorly standardized and validated
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29 procedures of STAT3 immunohistochemical staining were used in this study. The immunohistochemical
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32 analysis could be typically affected by the variation in antibodies and thresholds for judgment of positive
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35 expression. Finally, immunostaining was performed using primary tumor specimens, although metastatic
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38 tumor specimens would be more suitable for investigating the correlation between mTKI therapeutic
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41 outcomes and the expression of specific factors.

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44 In conclusion, activated STAT3 in tumor tissues shows significant correlation with poor prognosis in
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47 patients with RCC who received sunitinib as a first-line therapy. In addition, the positive p-STAT3
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50 expression is likely correlated with a shorter OS in the early treatment phase. Hence, this study suggests
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53 that the STAT3 activation in tumor tissues could be a predictive marker of refractory to sunitinib therapy
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56 in patients with mRCC.

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Compliance with Ethical Standards

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Conflict of interest

H. Miyake and M. Fujisawa have received lecture fees from Pfizer, whereas the other authors disclosed no potential conflicts of interest.

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Figure Legends

Fig. 1 Distribution of patients.

Fig. 2 Examples of immunohistochemical staining of RCC tumor cells using antibodies against signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 (p-STAT3).

Positive staining for STAT3 or p-STAT3 in >50% of tumor cells indicated positive expression of these proteins.

Fig. 3. Correlation of the signal transducer and activator of transcription 3 (STAT3) expression status with progression-free survival (PFS) and overall survival (OS). A, the PFS in 51 patients with renal cell carcinoma (RCC) following a first-line treatment with sunitinib based on their STAT3 and p-STAT3 expression status. B, the OS in 51 patients with RCC following a first-line treatment with sunitinib based on their STAT3 and p-STAT3 expression status. Gray line, negative expression; black line, positive expression. *P*-values were calculated using the log-rank test.

Table 1 Patient characteristics in each STAT3 expression level

		All patients (n=51)	STAT3			p-STAT3		
			Negative (n=29)	Positive (n=22)	P	Negative (n=37)	Positive (n=14)	P
Mean age, year ± SD		65.0 ± 13.2	66.4 ± 12.0	63.2 ± 14.7	0.41	65.0 ± 14.0	65.2 ± 11.0	0.96
Male, n (%)		40 (78.4%)	25 (86.2%)	15 (68.2%)	0.17	29 (78.4%)	11 (78.6%)	1.00
Mean BMI, kg/m² ± SD		21.4 ± 3.8	21.6 ± 3.9	21.2 ± 3.9	0.66	22.1 ± 3.9	19.6 ± 3.1	0.037
Mean BSA, m² ± SD		1.59 ± 0.17	1.61 ± 0.18	1.58 ± 0.16	0.69	1.61 ± 0.18	1.54 ± 0.15	0.14
Karnofsky Performance Status <80%, n (%)		10 (19.6%)	5 (17.2%)	5 (22.7%)	0.73	7 (18.9%)	3 (21.4%)	1.00
MSKCC risk category, n (%)	Favorable	3 (5.9%)	1 (3.4%)	2 (9.1%)	0.55	1 (2.7%)	2 (14.3%)	0.17
	Intermediate	36 (70.6%)	22 (75.9%)	14 (63.6%)		28 (75.7%)	8 (57.1%)	
	Poor	12 (23.5%)	6 (20.7%)	6 (27.3%)		8 (21.6%)	4 (28.6%)	
Histology	Clear cell	49 (96.0%)	28 (96.6%)	21 (95.5%)	0.68	37 (100%)	12 (85.8%)	0.071
	Papillary	1 (2.0%)	0	1 (4.5%)		0	1 (7.1%)	
	Others	1 (2.0%)	1 (3.4%)	0		0	1 (7.1%)	
Metastatic sites, n (%)	0-1	23 (45.1%)	12 (41.4%)	11 (50.0%)	0.58	17 (45.9%)	6 (42.9%)	1.00
	≥2	28 (54.9%)	17 (58.6%)	11 (50.0%)		20 (54.1%)	8 (57.1%)	
Presence of metastasis, n (%)	Lung	32 (62.7%)	19 (65.5%)	13 (59.1%)	0.77	20 (54.1%)	12 (85.7%)	0.053
	Bone	14 (27.5%)	11 (37.9%)	3 (13.6%)	0.066	11 (29.7%)	3 (21.4%)	0.73
	Lymph node	12 (23.5%)	6 (20.7%)	6 (27.3%)	0.741	8 (21.6%)	4 (28.6%)	0.72
	Liver	11 (21.6%)	5 (17.2%)	6 (27.3%)	0.50	9 (24.3%)	2 (14.3%)	0.71
Nephrectomy , n (%)	Prior sunitinib therapy	49 (96.1%)	28 (96.6%)	21 (95.5%)	1.00	36 (97.3%)	13 (92.9%)	0.48
	Neo-adjuvant sunitinib therapy	2 (3.9%)	1 (3.4%)	1 (4.5%)		1 (2.7%)	1 (7.1%)	

Table 2 Best overall response in the patients with each STAT3 expression level

Best overall response analysis	STAT3		<i>P</i>	p-STAT3		<i>P</i>
	Negative (n=28)	Positive (n=20)		Negative (n=34)	Positive (n=14)	
Objective Response (CR + PR), n (%)	9 (32.1%)	4 (20.0%)	0.51	9 (26.5%)	4 (28.6%)	1.00
Disease Control (CR + PR + SD), n (%)	26 (92.9%)	15 (75.0%)	0.11	29 (85.3%)	12 (85.7%)	1.00

Table 3 Univariate Cox proportional hazards regression analyses of various parameters as predictors of progression-free and overall survivals

Factors	Progression-free survival		Overall survival	
	HR (95%CI)	<i>P</i>	HR (95%CI)	<i>P</i>
Age (<70)	1.58 (0.68 – 3.65)	0.29	2.93 (1.01 – 8.49)	0.047
Sex (male)	1.45 (0.63 – 3.38)	0.38	2.64 (0.79 – 8.79)	0.11
BMI (≤22)	1.15 (0.58 – 2.29)	0.68	2.63 (1.17 – 5.90)	0.019
BSA (≤1.6)	1.00 (0.50 – 2.00)	0.99	1.69 (0.79 – 3.62)	0.18
Karnofsky PS (<80%)	1.77 (0.70 – 4.44)	0.23	1.41 (0.56 – 3.51)	0.46
MSKCC risk category (Poor)	2.05 (0.93 – 4.53)	0.075	3.52 (1.57 – 7.88)	0.002
Number of metastatic site (≥2)	1.91 (0.92 – 3.94)	0.082	2.28 (1.00 – 5.22)	0.051
Lung metastasis (positive)	1.51 (0.71 – 3.22)	0.28	2.23 (0.93 – 5.33)	0.072
Bone metastasis (positive)	1.06 (0.51 – 2.23)	0.87	2.13 (0.97 – 4.67)	0.059
Lymph node metastasis (positive)	1.58 (0.70 – 3.54)	0.27	1.16 (0.48 – 2.77)	0.74
Liver metastasis (positive)	1.07 (0.48 – 2.39)	0.88	1.02 (0.41 – 2.54)	0.96
STAT3 expression (positive)	1.17 (0.58 – 2.36)	0.67	0.62 (0.28 – 1.39)	0.24
p-STAT3 expression (positive)	2.22 (1.03 – 4.75)	0.041	1.66 (0.74 – 3.72)	0.22

HR: Hazard ratio, CI: confidence interval,

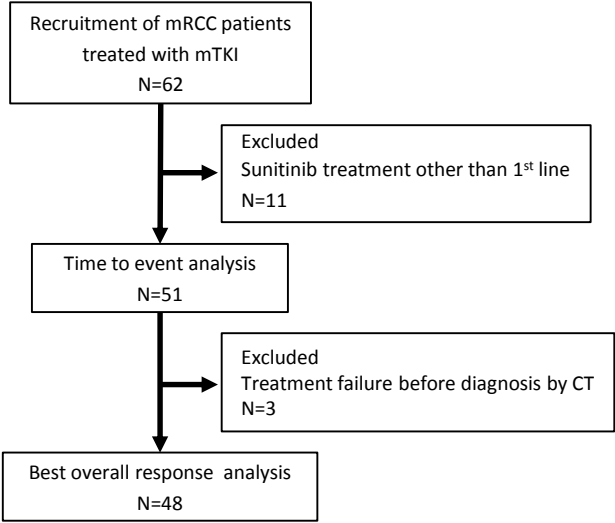


Fig. 1

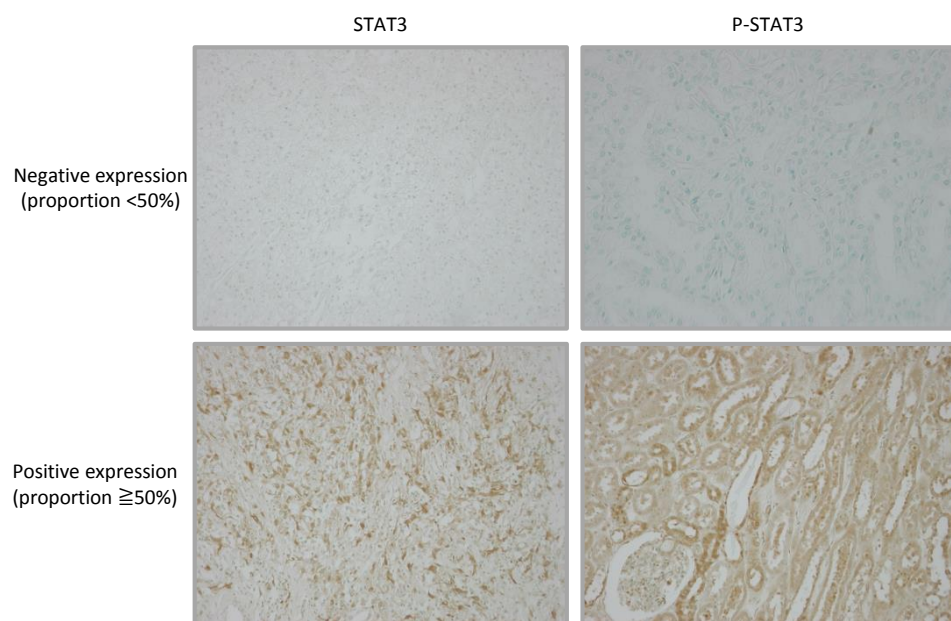


Fig. 2

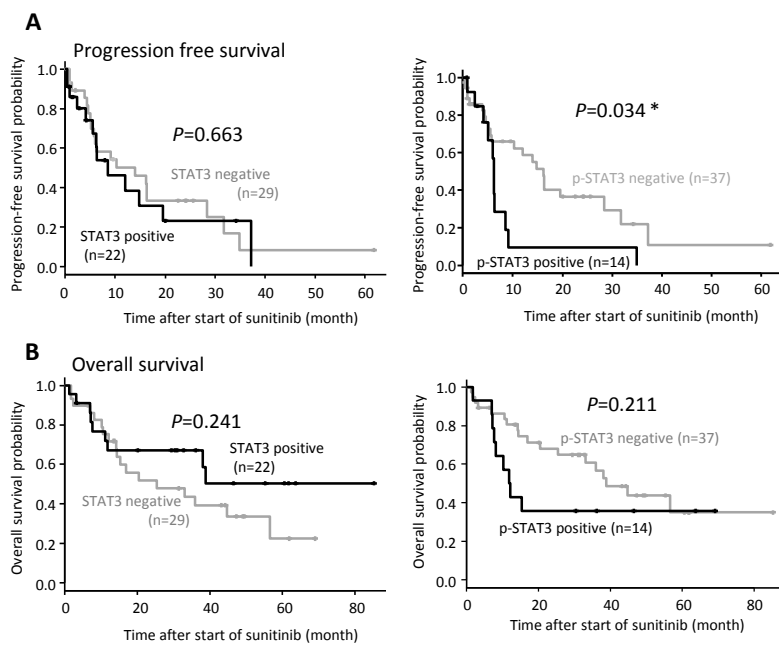


Fig. 3