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ORIGINAL PAPER

Expression level of phosphorylated-4E-binding protein 1 in radical nephrectomy specimens as a prognostic predictor in patients with metastatic renal cell carcinoma treated with mammalian target of rapamycin inhibitors

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Abstract The objective of this study was to analyze the expression levels of multiple components in the mammalian target of rapamycin (mTOR) signaling pathway in radical nephrectomy specimens from patients with metastatic renal-cell carcinoma (RCC) treated with mTOR inhibitors in order to identify factors predicting susceptibility to these agents. This study retrospectively included a total of 48 consecutive patients undergoing radical nephrectomy, who were diagnosed with metastatic RCC and subsequently treated with an mTOR inhibitor (everolimus or temsirolimus) as either first- or second-line systemic therapy. Expression levels of 5 molecular markers involved in the signaling pathway associated with mTOR, including PTEN, phosphorylated (p)-Akt, p-mTOR, p-p70 ribosomal S6 kinase, and p-4E-binding protein 1 (4E-BP1), were measured by immunohistochemical staining of primary RCC specimens. Of several factors examined, bone metastasis, liver metastasis, and the expression level of p-4E-BP1 were shown to have significant impacts on the response to the mTOR inhibitors. Progression-free survival (PFS) was significantly correlated with the expression levels of PTEN and p-4E-BP1 in addition to the presence of bone metastasis on univariate analysis. Of these significant factors, p-4E-BP1 expression and bone metastasis appeared to be independently associated with PFS on multivariate analysis. These findings suggest that it would be useful to consider the expression levels of potential molecular markers in the mTOR signaling pathway, particularly p-4E-BP1, as well as conventional clinical parameters when selecting patients with metastatic RCC who are likely to benefit from treatment with mTOR inhibitors.

Keywords Renal-cell carcinoma · mTOR inhibitors · 4E-BP1 · Radical nephrectomy · Prognosis

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Introduction

Mammalian target of rapamycin (mTOR), an evolutionally conserved multiprotein complex, including mTOR complex 1 (mTORC1) and mTORC2, is regarded as a potential downstream effector of the phosphoinositide 3-kinase/Akt signaling pathway, which has been shown to play crucial roles in several pathophysiological conditions [1]. In response to upstream stimuli through Akt-mediated inhibition of the tuberous sclerosis complex, mTORC1 has been shown to directly phosphorylate p70 ribosomal S6 kinase (p70S6K) and 4E-binding protein 1 (4E-BP1), resulting in the enhanced synthesis of proteins mediating a wide variety of cellular functions, such as proliferation, differentiation, metabolism, migration, and angiogenesis, while mTORC2 functions via the phosphorylation of its substrates, including Akt, to promote cell survival [2]. Therefore, deregulation of the mTOR signaling pathway has been proposed to promote the progression of malignant tumors by facilitating acquisition of the aggressive phenotype [1, 2].

Rapamycin and its analogs are known to be able to inactivate mTOR, thereby preventing the phosphorylation of its downstream molecules, such as p70S6K and 4E-BP1. Several previous studies have shown that mTOR inhibitors exhibit potent preclinical activities against various types of cancers, including renal-cell carcinoma (RCC) [3]. Two mTOR inhibitors, everolimus and temsirolimus, were

recently approved for the treatment of patients with advanced RCC [4, 5]. Everolimus was compared to a placebo in a randomized phase III study targeting patients with metastatic clear-cell RCC who had progressed following tyrosine kinase inhibitors (TKIs), and a significant improvement was observed in progression-free survival (PFS) [4]. In contrast, the phase III study of temsirolimus, which compared its efficacy with that of interferon (IFN)-α in patients with previously untreated, poor-prognosis metastatic RCC, demonstrated a significant improvement in overall survival with temsirolimus than with IFN-α [5]. Based on these findings, both everolimus and temsirolimus are currently considered the standard agents of choice for the treatment of patients with advanced RCC.

To date, several model systems predicting the survival of patients with metastatic RCC have been reported [6, 7]; however, these prognostic profiles were developed based on data from patients who participated in clinical trials using cytokine therapies. Therefore, in the era of molecular-targeted agents, it appears to be important to identify novel factors associated with susceptibility to these agents in order to provide individualized risk-directed treatments for metastatic RCC. Considering these findings, we retrospectively reviewed clinicopathological data from a total of 48 consecutive patients undergoing radical nephrectomy for RCC who were diagnosed with metastatic diseases and subsequently treated with either everolimus or temsirolimus. We then evaluated the expression levels of multiple potential molecular markers involved in the mTOR signaling pathway, including PTEN, phosphorylated (p)-Akt, p-mTOR, p-p70S6K, and p-4E-BP1, in radical nephrectomy specimens from these patients with immunohistochemical staining and analyzed outcomes according to several conventional parameters.

Materials and methods

This study retrospectively included a total of 48 consecutive patients undergoing radical nephrectomy for RCC who were diagnosed with metastatic diseases and subsequently treated with an mTOR inhibitor (everolimus or temsirolimus) as either first- or second-line systemic therapy between May 2010 and October 2012. Informed consent was obtained from each patient before participating in this study. Prior to entry, all patients were evaluated by computed tomography (CT) of the brain, chest, and abdomen as well as a radionucleotide bone scan. Performance status, clinicopathological examinations, and risk classification were performed using the Karnofsky performance status scale, UICC TNM classification system, and Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic factor model [7], respectively. Tumor measurements were generally performed with CT before and every 8 weeks after the initiation of treatment with an mTOR inhibitor. Response and progression were assessed by treating physician based on the Response Evaluation Criteria in Solid Tumors. At our institution, cytoreductive nephrectomy was generally performed in patients with synchronous metastatic diseases who were physiologically able to tolerate surgical treatment. In addition, the indications of treatment with an mTOR inhibitor as first-line systemic agent included patients who were classified into poor-risk group, those with non-clear-cell carcinoma, and those with severe comorbidity and/or poor performance status prior to the initiation of systemic therapy.

In this series, all patients were pathologically diagnosed with RCC, including 40 with clear-cell RCC, 6 with papillary RCC, and 2 with chromophobe RCC. Of the 48 patients, 21 were treated with an mTOR inhibitor as first-line agent, while the remaining 27 received an mTOR inhibitor following the administration of first-line TKI for the median interval of 11.2 months. When an mTOR inhibitor was introduced, 12 patients received 10 mg everolimus once daily on a continuous dosing schedule, and the remaining 36 patients were treated with 25 mg of intravenous temsirolimus weekly. However, dose modification of these agents was performed based on adverse events in accordance with the manufacturer's recommendations.

Immunohistochemical staining of radical nephrectomy specimens was performed as previously described [8]. Briefly, formaldehyde-fixed and paraffin-embedded tissue sections from 48 radical nephrectomy specimens were deparaffinized and rehydrated. After blocking endogenous peroxidase with 3 % hydrogen peroxidase, sections were boiled in 0.01 M citrate buffer for 10 min and incubated with 5 % normal blocking serum for 20 min. These sections were then incubated with the following anti-human antibodies: PTEN mouse monoclonal antibody (Abcam, Cambridge, United Kingdom), p-Akt rabbit monoclonal antibody, p-mTOR rabbit monoclonal antibody, p-p70S6K mouse monoclonal antibody, and p-4E-BP1 rabbit monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA). Sections were then incubated with biotinylated anti-mouse or rabbit IgG (Vector Laboratories, Burlingame, CA, USA). After incubation in an avidin–biotin peroxidase complex for 30 min, samples were exposed to diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin.

Staining results were evaluated by two independent investigators who were blinded to the data of each patient. If discordant interpretations occurred, differences were resolved by a joint review and/or consultation with a third observer familiar with immunohistochemical pathology. PTEN expression was scored according to staining

intensity (1 weak; 2 medium; or 3 strong) multiplied by the proportion of immunoreactive cells (1, 0–5; 2, 6–25; 3, 26–75; or 4, 75–100 %), and a score of >6 was considered to represent strong expression, as previously described [9]. The expression levels of p-Akt, p-mTOR, p-p70S6K, and p-4E-BP1 were evaluated as follows: tumors with <10 % cells with weak staining were scored as 0, with >10 % cells with weak staining or <20 % cells with intermediate to strong staining were scored as 1, and with >20 % cells with intermediate to strong staining were scored as 2. A staining score of either 1 or 2 was considered to represent strong expression for these four markers, as previously reported [10, 11].

All statistical analyses were performed using Statview 5.0 software (Abacus Concepts, Berkeley, CA, USA). The χ^2 test was used to analyze associations between response to the mTOR inhibitors and several parameters. PFS rates were calculated by the Kaplan–Meier method, and differences were determined by the log-rank test. The prognostic significance of certain factors was assessed by the Cox proportional hazards regression model. Probability (P) values <0.05 were considered significant.

Results

Of the 48 patients included in this study, 22 (45.8 %), who presented metastases at the time of initial diagnosis, underwent cytoreductive nephrectomy, while the remaining 26 (55.2 %) developed metastatic disease with the median interval of 9.6 months after surgery. In addition, the median time from surgery to the introduction of first-line agent was 2.7 months and that to the introduction of an mTOR inhibitor was 16.5 months. Table 1 shows the clinicopathological characteristics of the 48 patients included in this study according to their responses to their mTOR inhibitor. All these patients were evaluated for their best response to these agents, and 1 (2.1 %), 31 (64.6 %), and 16 (33.3 %) patients were judged to partial response (PR), stable disease (SD) at least for 6 weeks, and progressive disease (PD), respectively. Of several clinicopathological factors, bone metastasis and liver metastasis were significantly related to the response to the mTOR inhibitor. In addition, of the 5 molecular markers investigated in this study, the expression level of p-4E-BP1 had a significant impact on the response to treatment with the mTOR inhibitor (Table 1). However, there were no significant effects of the time from surgery to the development of metastases on the expression levels of all 5 molecular markers in the 48 patients, and the response to initial TKI in 27 who received an mTOR inhibitor as second-line agents had no significant impact on the expression of all molecular markers (data not shown).

During the median observation period of 13.8 months (range 3.5–29.2 months), 33 patients (68.8 %) developed disease progression, and the median duration of PFS was 7.4 months (range 1.0–26.0 months). As shown in Fig. 1a, the 1- and 2-year PFS rates of the 48 patients were 34.1 and 16.4 %, respectively. To identify parameters associated with PFS in patients treated with the mTOR inhibitor, univariate and multivariate analyses were performed using the Cox proportional hazard regression model. Of the 5 molecular markers, the expression levels of PTEN and p-4E-BP1 were identified as significant predictors of PFS on univariate analysis. In addition to molecular markers, the presence of bone metastasis was also significant among several conventional factors examined. Moreover, these three significant factors on univariate analysis were further evaluated by multivariate analysis to determine the predictive value for PFS, and the expression level of p-4E-BP1 and presence of bone metastasis appeared to be independently associated with PFS (Table 2). Representative findings of the immunohistochemical study to detect p-4E-BP1 expression are shown in Fig. 2. As shown in Fig. 1b, c, significant differences were observed in PFS among the 48 patients with respect to bone metastasis and p-4E-BP1 expression levels.

Discussion

The recent introduction of novel molecular-targeted agents has resulted in a dramatic paradigm shift in therapeutic strategy for metastatic RCC [12]. Of these, two agents have been recognized to exhibit the ability to inhibit the activity of the mTOR signaling pathway. Based on the promising outcomes of pivotal clinical trials, two mTOR inhibitors, everolimus and temsirolimus, are currently recommended as first-line therapy for the treatment of patients with metastatic RCC refractory to TKI and those classified into poor prognosis, respectively, in the major clinical guidelines [4, 5]. However, several limitations associated with the treatment of metastatic RCC using the mTOR inhibitors have been documented, including the extremely low proportion of patients achieving a complete response, the short interval of durable response and high incidence of severe adverse events, such as drug-induced pneumonitis [3]. Collectively, these findings suggest that it may be necessary to properly select patients with metastatic RCC who are likely to receive therapeutic benefits from the mTOR inhibitors prior to the administration of this type of agent. Therefore, we evaluated the association in expression levels of multiple molecular markers involved in the mTOR signaling pathway in radical nephrectomy specimens in addition to several clinicopathological parameters with a response to either everolimus or temsirolimus in

Table 1 Relationship between several parameters and the response to mTOR inhibitors

Variables	PR ($n = 1$)	SD ($n = 31$)	PD ($n = 16$)	P value	Variables	PR ($n = 1$)	SD ($n = 31$)	PD ($n = 16$)	P value
Age (years)				0.22	PTEN				0.54
<70	1	22	14		Weak expression	1	16	7	
70<	0	9	2		Strong expression	0	15	9	
Gender				0.58	p-Akt				0.15
Male	0	26	14		Weak expression	1	18	6	
Female	1	5	2		Strong expression	0	13	10	
Pathological T stage				0.13	p-mTOR				0.68
pT1 or pT2	1	12	3		Weak expression	0	12	3	
pT3 or pT4	1	19	13		Strong expression	1	19	13	
Grade				0.83	p-p70S6 K				0.26
1 or 2	1	20	11		Weak expression	0	11	3	
3	0	11	5		Strong expression	1	20	13	
Histological subtype				0.27	p-4E-BP1				0.021
CCC	0	28	12		Weak expression	1	22	6	
Non-CCC	1	3	4		Strong expression	0	9	10	
MSKCC risk group				0.057					
Favorable or intermediate	1	22	7						
Poor	0	9	9						
Lung metastasis alone				0.81					
Negative	1	24	12						
Positive	0	7	4						
Bone metastasis				0.005					
Negative	1	24	6						
Positive	0	7	10						
Lymph-node metastasis				0.28					
Negative	0	23	9						
Positive	1	8	7						
Liver metastasis				< 0.001					
Negative	1	26	6						
Positive	0	5	10						
Pretreatment C-reactive protein				0.83					
Normal	1	12	6						
Abnormal	0	19	10						
Presence of metastasis at diagnosis				0.42					
Negative	1	15	6						
Positive	0	16	10						
mTOR inhibitor				0.22					
Everolimus	1	8	3						
Temsirolimus	0	23	13						
Timing of mTOR inhibitor treatment				0.15					

Table 1 continued

Variables	PR ($n = 1$)	SD ($n = 31$)	PD ($n = 16$)	P value	Variables	PR ($n = 1$)	SD ($n = 31$)	PD ($n = 16$)	P value
First-line	1	16	5						
Second-line	0	15	11						

mTOR mammalian target of rapamycin, *PR* partial response, *SD* stable disease, *PD* progressive disease, *CCC* clear-cell carcinoma, *MSKCC* memorial Sloan-Kettering cancer center, *p* phosphorylated, *p70S6K* p70 ribosomal S6 kinase, *4E-BP1* 4E-binding protein 1

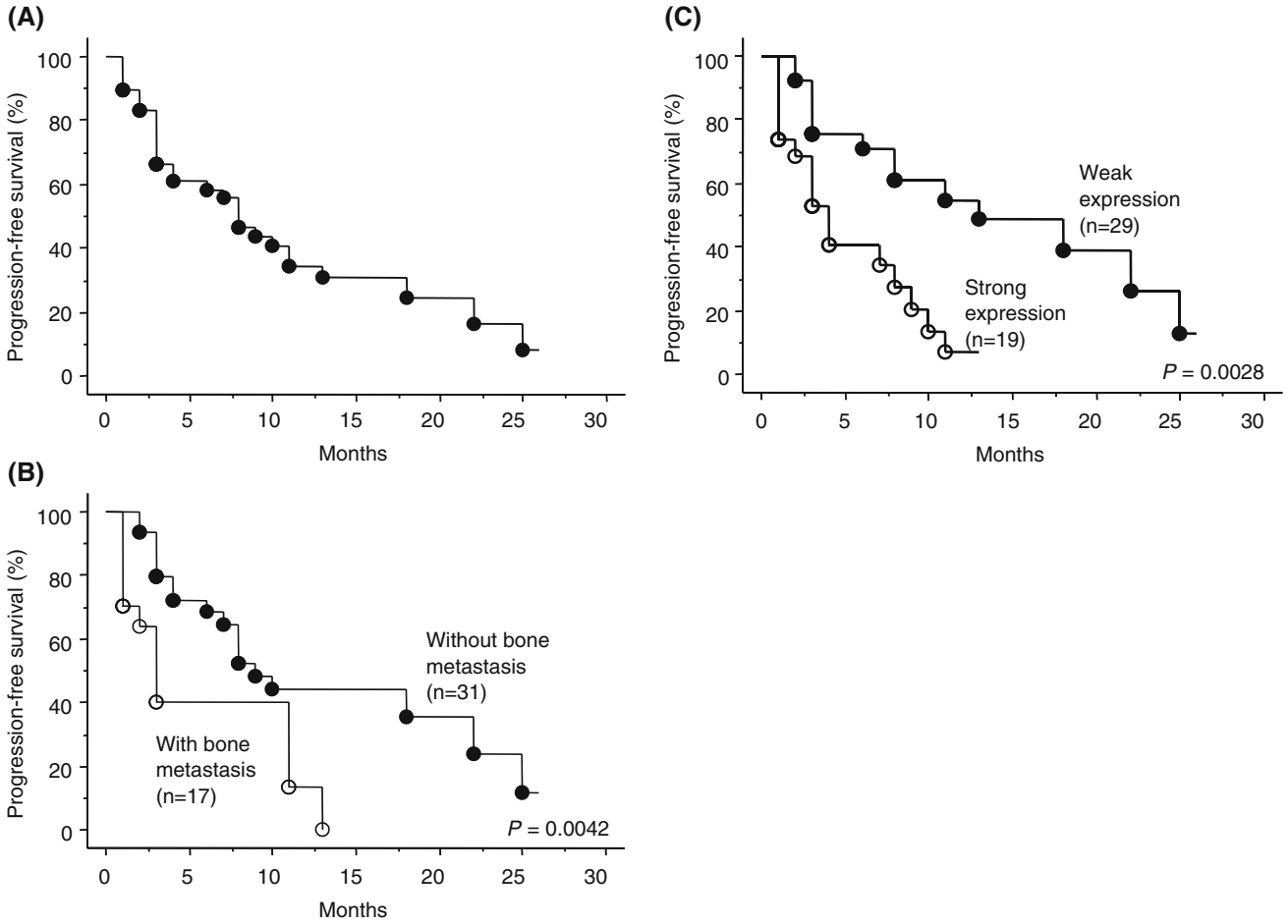

Fig. 1 **a** Progression-free survival (PFS) of patients undergoing radical nephrectomy, who were diagnosed with metastatic renal-cell carcinoma (RCC) and treated with either everolimus or temsirolimus. **b** PFS of these patients according to the presence of bone metastasis. **c** PFS of these patients according to the expression level of phosphorylated-4E-binding protein 1

order to identify factors precisely predicting clinical courses in patients with metastatic RCC treated with the mTOR inhibitor.

A total of 48 patients with metastatic RCC who underwent radical nephrectomy were included in this study. Following treatment with either everolimus or temsirolimus, 1, 31, and 16 patients were classified as showing PR, SD, and PD, respectively; the clinical benefit of these agents could be achieved in 66.7 % of patients, which is similar to that reported in previous clinical trials [4, 5]. Of the several conventional parameters examined, bone metastasis and liver metastasis were significantly correlated with the response to the mTOR inhibitors. Furthermore, the immunohistochemical study showed that five molecular components in the mTOR signaling pathway were detectable in the majority of primary RCC tissues; however, only p-4E-BP1 was identified as a marker with a significant relationship to the response to the mTOR inhibitor. To date, the findings of several studies support the present findings with respect to phosphorylation of 4E-

Table 2 Uni- and multivariate analyses of associations between various parameters with progression-free survival

Variables	Univariate analysis Hazard ratio	*P* value	Multivariate analysis Hazard ratio	*P* value
Age (years) (<70 vs 70<)	1.32	0.51	–	–
Gender (male vs female)	1.25	0.64	–	–
Pathological stage (pT1 or pT2 vs pT3 or pT4)	2.02	0.16	–	–
Grade (1 or 2 vs 3)	1.39	0.37	–	–
Histological subtype (CCC vs non-CCC)	2.05	0.15	–	–
MSKCC risk group (favorable or intermediate vs poor)	1.36	0.36	–	–
Lung metastasis alone (negative vs positive)	1.16	0.76	–	–
Bone metastasis (negative vs positive)	2.82	0.0088	2.63	0.013
Lymph-node metastasis (negative vs positive)	1.80	0.12	–	–
Liver metastasis (negative vs positive)	1.68	0.18	–	–
Pretreatment c-reactive protein (normal vs abnormal)	1.51	0.21	–	–
Presence of metastasis at diagnosis(negative vs positive)	1.42	0.25	–	–
mTOR inhibitor (everolimus vs temsirolimus)	1.48	0.29	–	–
Timing of mTOR inhibitor treatment (first-line vs second-line)	1.19	0.48	–	–
PTEN (weak vs strong expression)	2.66	0.019	1.50	0.42
p-Akt (weak vs strong expression)	1.83	0.11	–	–
p-mTOR (weak vs strong expression)	1.48	0.32	–	–
p-p70S6K (weak vs strong expression)	1.78	0.18	–	–
p-4E-BP1 (weak vs strong expression)	3.28	0.0063	2.42	0.045

CCC clear-cell carcinoma, *MSKCC* memorial Sloan-Kettering cancer center, *mTOR* mammalian target of rapamycin, *p* phosphorylated, *p70S6K* p70 ribosomal S6 kinase, *4E-BP1* 4E-binding protein 1

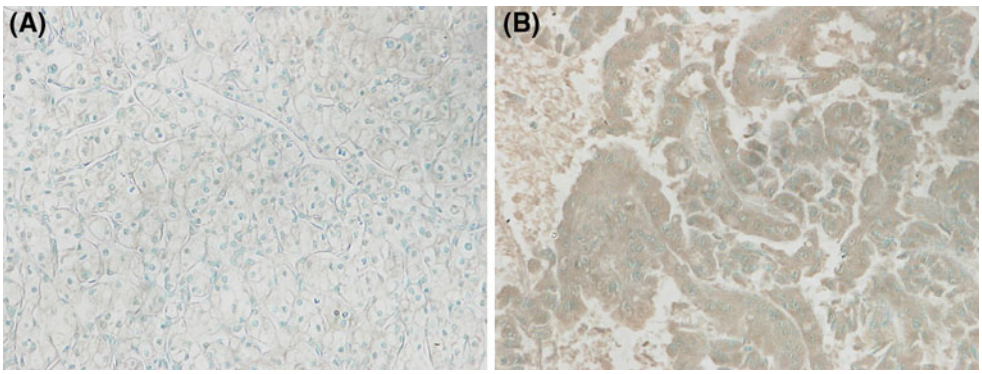

Fig. 2 Typical outcomes of immunohistochemical staining of primary renal-cell carcinoma with the phosphorylated-4E-binding protein 1 (p-4E-BP1) antibody. **a** Weak expression of p-4E-BP1. **b** Strong expression of p-4E-BP1

BP1 as an indicator of the response to the mTOR inhibitors [13–15]. For example, Zhang and Zheng identified mTOR-independent 4E-BP1 phosphorylation in colorectal cancer cells that was proportional to the degree of resistance to the mTOR inhibitors [13]. Considering these findings, patients with weak expression of p-4E-BP1 in the primary specimen may obtain greater clinical benefits from treatment with mTOR inhibitors than those that strongly express p-4E-BP1.

Disease progression occurred in 33 patients (68.8 %) during the observation period in the present study. Of several conventional prognostic factors in addition to five molecular markers involved in the mTOR signaling pathway, the presence of bone metastasis and expression levels of PTEN, and p-4E-BP1 were shown to have significant impacts on PFS. Among these, three factors identified as significant prognostic predictors by univariate analysis, the presence of bone metastasis and p-4E-BP1 expression

appeared to be independently associated with PFS on multivariate analysis. To date, there has been limited information on biomarkers predicting the clinical benefit of the mTOR inhibitors [16, 17]. For example, Lee et al. [16] reported that an increase in cholesterol was associated with longer survival and predicted temsirolimus efficacy in patients with advanced renal-cell carcinoma with poor-risk prognostic factors included in the phase III study of temsirolimus; however, no correlation was found between baseline PTEN and hypoxia inducible factor-1α levels in primary RCC tissues and the treatment effect of temsirolimus with respect to overall survival, PFS, or the objective response rate in these patients [17]. Given the limitations of biomarker development using retrospective datasets, some novel biomarker-driven studies for the mTOR inhibitors are currently planned [18].

It is of interest to address the mechanism whereby only p-4E-BP1, but not other components of the mTOR signaling pathway, had an independent impact on the prognosis of patients treated with the mTOR inhibitors. Although 4E-BP1 is one of the main downstream molecules phosphorylated by mTOR, recently accumulated evidence strongly suggests that other kinases may also be implicated in the phosphorylation of 4E-BP-1, such as those involved in the mitogen-activated protein kinase pathway [19, 20]. Therefore, 4E-BP-1 phosphorylation could be the consequence of several events induced by the activation of different signaling pathways, which suggests the unique functions of 4E-BP-1 as a funneling factor partially independent of upstream signal transduction. Taken together, these findings suggest that inactivation of mTOR alone may not adequately suppress the phosphorylation of 4E-BP1, which could explain the insufficient antitumor effect of the mTOR inhibitors.

Here, we would like to emphasize potential limitations of the present study. This was a retrospective study containing a small number of patients who were not treated with a single agent with a short observation period. In addition, the expression levels of molecular markers were evaluated in radical nephrectomy specimens alone. Although Aziz et al. [21] recently reported that expression of most targets of currently approved agents against RCC was similar in primary and metastatic RCC tissues, findings that more closely reflect the clinical courses of patients treated with the mTOR inhibitors may be achieved by assessing the expression profiles of these markers in metastatic tissues considering clonal evolution that could occur during disease progression. Finally, although five representative molecular markers in the mTOR signaling pathway were analyzed in this study, these were selected by subjective rather than scientifically objective criteria. Accordingly, other molecules may be more closely associated with prognosis in patients with metastatic RCC treated with the mTOR inhibitors.

In conclusion, to the best of our knowledge, this is the first study to analyze the significance of several conventional parameters along with the baseline expression levels of multiple molecular markers associated with the mTOR signaling pathway in radical nephrectomy specimens as prognostic predictors in patients with metastatic RCC treated with either everolimus or temsirolimus. In this study, the presence of bone metastasis and p-4E-BP1 expression were identified as independent factors related to PFS. Therefore, the careful selection of patients with metastatic RCC for treatment with the mTOR inhibitors based on reliable predictive criteria was established considering conventional parameters as well as molecular components in the mTOR signaling pathway would allow this therapy to be offered to patients who are most likely to benefit from it.

Conflict of Interest None.

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