Expression of total and phospho 4EBP1 in metastatic and non-metastatic renal cell carcinoma

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Abstract. Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) is phosphorylated and activated by mammalian target of rapamycin complex 1, which serves as a regulator of cell growth, cell survival, metastasis and angiogenesis in many types of cancer. The aim of this study was to evaluate the role of phosphorylated 4EBP1 (p4EBP1) in primary renal cell carcinoma (RCC) as a biomarker in metastatic RCC (mRCC) and non-mRCC cohorts. Primary tumor tissue from 254 non-mRCC and 60 mRCC patients were immunohistochemically stained for t4EBP1 and p4EBP1. The disease-free interval (DFI) categorized by the expressions and clinical parameters were assessed by univariate and multivariate analysis in the non-mRCC cohort. Then, the cause-specific survival (CSS) was assessed in the mRCC cohort by the same methods as used in the non-mRCC cohort. In the non-mRCC cohort, patients with t4EBP1 expression had no RCC recurrence. Patients with p4EBP1 expression had the shorter DFI in univariate analysis (P=0.037). p4EBP1 and pT1b-4 expression levels were independent predictors for *de novo* metastasis. In the mRCC cohort, intermediate/poor MSKCC risk, non-clear cell RCC, and no p4EBP1 expression were correlated with poor CSS on multivariate analysis. Expression of p4EBP1 could be a predictive biomarker for *de novo* metastasis in non-mRCC patient cohort. By contrast, mRCC patients showing no p4EBP1 expression had shorter CSS than patients with p4EBP1 expression.

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Introduction

An estimated 338,000 new cases of kidney cancer are diagnosed annually, and 143,000 patients die each year worldwide (1). Renal cell carcinoma (RCC) accounts for approximately 90% of kidney cancer cases, and half of the patients eventually develop metastatic disease (2). In the case of metastatic RCC (mRCC), remission remains exceptionally infrequent (3).

Several systemic pharmacotherapies, which target vascular endothelial growth factor (VEGF) and mammalian target of rapamycin complex 1 (mTORC1), have prolonged the survival of patients with mRCC for the past decade. Most recently, the immuno-oncology (I-O) drug nivolumab was developed and established as a treatment for mRCCs that are resistant to VEGF-targeted agents (3). A guideline by the European Association of Urology recommends nivolumab and VEGF-targeted agents, including axitinib and cabozantinib, as candidates for second line treatment (4). However, no indicators are clinically available to determine whether to administer nivolumab, axitinib, or cabozantinib. The development of I-O drugs has further stimulated the interest in predictive biomarkers.

The most common RCC subtype is clear cell RCC (ccRCC; 70-75%). Approximately 90% of ccRCCs harbor inactivation of both copies of the von Hippel Lindau (VHL) tumor

suppressor gene (5). Loss of function of VHL protein (pVHL) leads to the accumulation of hypoxia inducible factors (HIFs), which promote the transcription of numerous genes, including VEGF (6). Therefore, VEGF could rationally be a therapeutic target in mRCC treatment, and is the most frequently targeted molecule in clinical treatment. HIFs also interact with mTORC1, which promotes their stability and translation of HIF mRNAs. mTORC1 is a central crossroad of many intracellular signaling pathways. It can be regulated upstream by growth factors (GFs), the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, and the nutrient/5' adenosine monophosphate-activated protein kinase (AMPK) pathway, and it regulates downstream pathways including those involving stabilizing HIFs and those promoting cap-dependent translation through the phosphorylation of S6 kinase and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (7,8). Since pVHL also suppressed Akt like HIFs, Akt/mTORC1 pathway is presumed to be activated in ccRCC (9).

In a number of *in vivo* and *in vitro* cancer cell line studies, aberrant activation of the Akt/mTORC1/4EBP1 pathways contributed to tumor growth, cell survival, angiogenesis, and metastasis. 4EBP1 binds and suppresses eukaryotic initiation factor 4E (eIF4E). Phosphoryltion of 4EBP1 promotes to dissociate eIF4E/4EBP1 assembly, which leads to eIF4E-dependent translation initiation (7). In RCC cell line studies, inhibition of mTORC1 suppressed tumor growth, cell survival, angiogenesis, and metastasis (10,11). Furthermore, our previous studies demonstrated that activation of the PI3K/Akt/mTORC1 pathway enhanced resistance to VEGF-targeted agents in RCC cell lines (12,13). Resistance to the VEGF-targeted agent sunitinib is correlated with phosphatase and tensin homolog deleted from chromosome 10 (PTEN) expression, and restoration of PTEN expression restores sensitivity to sunitinib (12). Akt activation by low-density lipoprotein (LDL) addition in RCC cell lines counteracts the anti-tumor effects of the VEGF-targeted agents sunitinib and sorafenib (13). In adition, we have previously reported that high levels of 4EBP1/eIF4E activeation predict higher recurrence rate (14).

Hence, we hypothesized that increased phosphorylation of 4EBP1 could cause progression of metastasis in non-mRCC patients and precipitate resistance to VEGF-targeted agents in mRCC patients. As expected, our results showed that non-mRCC patients with high phosphorylation ratio had a shorter disease-free interval (DFI). However, lack of 4EBP1 phosphorylation correlated with worse cause-specific survival (CSS) in mRCC patient cohort, contrary to our expectations.

Materials and methods

Patients. We retrospectively collected information on patient and tumor characteristics, pathological data, recurrence, treatments, response, and survival from hospital's electronic database and from patients' medical records in Yamagata University Hospital and hospitals where the patients had been followed up. The date of data collection was December 2017.

We retrospectively analyzed two different cohorts. The first cohort consisted of 254 non-mRCC patients who underwent radical nephrectomy or nephron sparing surgery in the Yamagata University Hospital between 2003 and 2010. All patients were diagnosed using chest and abdominal computer tomography before surgery, and patients with lymph node metastases, or distant metastases at surgery were excluded from the non-mRCC cohort. We included only clear cell RCC into the non-mRCC cohort. Patients who received adjuvant interferon-alpha treatment after primary surgery were included if they had no metastatic lesions at surgery.

The second cohort consisted of 60 mRCC patients with available pre-treatment primary tumor tissues and distinct clinical outcomes who underwent systemic therapy for mRCC in the Yamagata University Hospital between 2008 and 2015.

Immunohistochemistry. The expression of total 4EBP1 (t4EBP1) and p4EBP1 were retrospectively evaluated by immunohistochemistry (IHC) as described. A monoclonal anti-4EBP1 and anti-p4EBP1 (Thr37/46) (Cell Signaling Technology, Osaka, Japan) were used. The primary tumors were fixed in 10% buffered formalin and embedded in paraffin. A 3-μm-thick paraffin section was mounted on silanized glass slides (Dako Cytomation, Tokyo, Japan). After deparaffination and rehydration, epitopes were reactivated by autoclaving the sections in 10 mM citric acid buffer (pH 6.0) for 10 min. The slides were incubated with the primary antibody overnight at 4°C in a moist chamber. After washing with phosphate buffered saline, the bound antibody was detected by the peroxidase method using the Histofine simple stain MAZ-PO (Nichirei, Tokyo, Japan). The staining reaction was developed by diaminobenzidine in the presence of H_2O_2. Nuclear counterstaining was performed using hematoxylin. Positive and negative controls were included in each staining series.

Two investigators (HK and TN), who were both blinded to the patient data, evaluated the expression of t4EBP1 and p4EBP1 in tumor cells was determined (Fig. 1A).

Statistical analysis. In the non-mRCC cohort, the endpoint of interest was DFI from primary surgery to the date of metastatic diagnosis or last follow-up. Firstly, DFIs which were categorized by expression score of t4EBP1, p4EBP1, and 4EBP1 phosphorylation status were overviewed. The phosphorylation status was defined as following: No Substrate; no expression of t4EBP1, No Phosphorylation; expression of t4EBP1 and no expression of p4EBP1, Phosphrylation; expression of t4EBP1 and p4EBP1. Then 4EBP1 phosphorylation status, sex, age (<55 vs. 56-60 vs. 61-65 vs. 66-70 vs. 71-75 vs. 76-80 vs. >80 years), age (≤80 vs. >80 years), laterality, pT stage (pT1a vs. pT1b-4), pathological grade (grade 1-2 vs. 3-4), and v (- vs. +). The factors that significantly related to DFI in univariate analyses were entered into multivariate analyses.

In the mRCC cohort, the endpoint of interest was CSS from the date of metastatic diagnosis to the date of death or last follow-up. Firstly, CSSs which were categorized by expression of p4EBP1 were overviewed. Then univariate analyses were undertaken with the patients categorized by age (≤60 vs. 61-70 vs. >70 years), age (≤60 vs. >60 years), sex, Memorial Sloan Kettering Cancer Center (MSKCC) risk group (favorable vs. intermediate vs. poor risk group), MSKCC risk group (favorable vs. intermediate or poor risk group), subtype (clear cell vs. papillary vs. chromophobe vs. collecting duct cell vs. mixed vs. Xp11.2 translocation vs. unclassified vs. other), subtype (clear cell vs. non-clear cell), pathological grade

(1 vs. 2. vs. 3. vs. 4), and pathological grade (1-3 vs. 4). Then the factors that statistically related to CSS in the univariate analyses and p4EBP1 expression (no expression vs. expression) were entered into multivariate analyses. Factors that were divided over two categories were excluded as candidates in multivariate analysis.

Finally, we investigated the correlations of p4EBP1 expression with best objective response (BOR) in first line therapy, first VEGF-targeted therapy, first mTORC1 inhibitor treatment, and treatment with each VEGF-targeted agent (sunitinib, sorafenib, and axitinib). BOR was determined by Response Evaluation Criteria in Solid Tumor version 1.1 (RECIST v1.1) (15). Complete response (CR) and partial response were determined as clinical response. In cases where patients had no target lesion, such as bone metastasis, that had undergone radiotherapy, and where patients changed regimens before evaluation of BOR, they were defined as 'unevaluable'.

T classification and pathological grade were determined according to the 2016 World Health Organization classification (2). MSKCC risk group was determined according to a report by Motzer *et al* (16). Univariate analyses were calculated using the Kaplan-Meier method, with the significance determined using log-rank test. Multivariate analyses were calculated using the Cox proportional hazard model with step-wise regression procedures. Distribution was analyzed using the Fisher's exact test. All statistical analyses were two-sided with a significant level of 0.05, and were performed using a free statistical software package R version 3.3.1.

Validation study using TCGA database. To validate the value as prognostic indicator, we collected clinical data and p4EBP1 protein level of Kidney Renal Clear Cel Carcinoma in the Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov/) via cBioPortal (Http://www.cbioportal.org/, accessed on 28 June 2018). TCGA patients who were diagnosed M0 at pathological diagnosis, were divided into two groups with high or low expression level using the following cutoff level for each protein; -0.1 for t4EBP1 and 0 for p4EBP1. Then we calculated DFI using the Kaplan-Meier method and compared DFI with the significance determined using log-rank test. M1 patients were divided into two groups with high or low p4EBP1 expression using a cutoff level of -0.7, and calculated and compared OS.

Results

Non-mRCC cohort
Baseline characteristics in the non-mRCC cohort. The median age at primary surgery in 254 non-mRCC patients was 64.5 years (range: 28-86 years). Twenty-seven patients had recurrent RCC lesions during the follow-up period, and 227 did not. The median follow-up period was 7.11 years [95% confidential interval (CI) 6.71-7.52 years] as estimated by the Kaplan-Meier method. Other baseline features are shown in Table I.

Expression of t4EBP1 and p4EBP1 in the non-mRCC cohort. Total of 226 and 64 tumors expressed t4EBP1 and p4EBP1, respectively. Almost all tumors (except one tumor) without t4EBP1 expression did not express p4EBP1 as expected (Fig. 1B). While 11.9% of patients with t4EBP1 expression had recurrent disease during follow-up period, no patients

without t4EBP1 had a recurrent disease (P=0.049) (Fig. 1C and Table I). Patients with p4EBP1 expression had shorter DFI (P=0.037) (Fig. 1D and Table I). When patients were divided into three groups (no substrate; no expression of t4EBP1, no phosphorylation; expression of t4EBP1 and no expression of p4EBP1, Phosphorylation; expression of t4EBP1 and p4EBP1), patients in no phosphorylation group had statistically worse prognosis (P=0.029) (Fig. 1E).

Correlation of clinical factors and 4EBP1 phosphorylation status with DFI in the non-mRCC cohort. Table I shows the correlations of clinical factors with DFI in the non-mRCC cohort, analyzed with univariate and multivariate methods. Patients with age over 80 years, pT1b to 4, grade, grade 3 or 4 disease, and v+ had statistically shorter DFI by univariate analyses (P=0.045, <0.001, <0.001 and <0.001). In the resulting univariate analyses, six factors-age over 80 years, pT1b to 4, grade 3 or 4 disease, v+, t4EBP1 expression, and p4EBP1 expression-were included in the multivariate analysis. Expression of p4EBP1, pT1b to 4, and grade 3 or 4 disease were independent factors associated with shorter DFI in the non-mRCC cohort by multivariate analysis (Table II).

Validation study using TCGA database with regards to non-Mrcc. To validate the value of p4EBP1 as biomarker for DFI in non-mRCC patients, we analyzed M0 patients in TCGA database. Patients with high expression of t4EBP1, p4EBP1, and phosphorylation level had shorter DFI (P=0.002, Fig. 1F; P=0.036, Fig. 1G; P=0.039, Fig. 1H, respectively). These results corresponded with our study.

mRCC cohort
Baseline characteristics in the mRCC cohort. The median age at primary surgery in the 60-patient mRCC cohort was 64 years (range: 34-81 years). At the last follow-up, 26 patients were alive, 32 had died due to RCC, and two had died from RCC-unrelated causes. The median follow-up period was 4.96 years (95% CI; 3.97-6.78 years) as estimated by the Kaplan-Meier method. Other baseline features are shown in Table III. At the commencement of treatment, 27 patients had metastases. The median duration from diagnosis of RCC to start of treatment was 30.8 months (range: 0.9-285.8 months).

Expression of t4EBP1 and p4EBP1 in the mRCC cohort. In the mRCC cohort, all tumors showed t4EBP1 expression. Patients with p4EBP1 expression had relatively longer survival (Table III and Fig. 2A). The distributions of patients with p4EBP1 expression was not statistically different by MSKCC risk group, pathological grade, or subtype (P=0.760, 0.560 and >0.99, respectively). Patients without p4EBP1 expression did not belong to the MSKCC poor risk group and grade 4, and all had ccRCC (Table SI).

Correlation of clinical factors and p4EBP1 expression with CSS in the mRCC cohort. Table II shows the correlations of clinical factors and p4EBP1 expression with CSS, analyzed with univariate and multivariate methods. Patients in the intermediate or poor MSKCC risk group, those with non-clear cell subtype, grade 4, and no p4EBP1 expression had statistically worse CSS by univariate analyses (P=0.005, 0.076, <0.001 and 0.023,
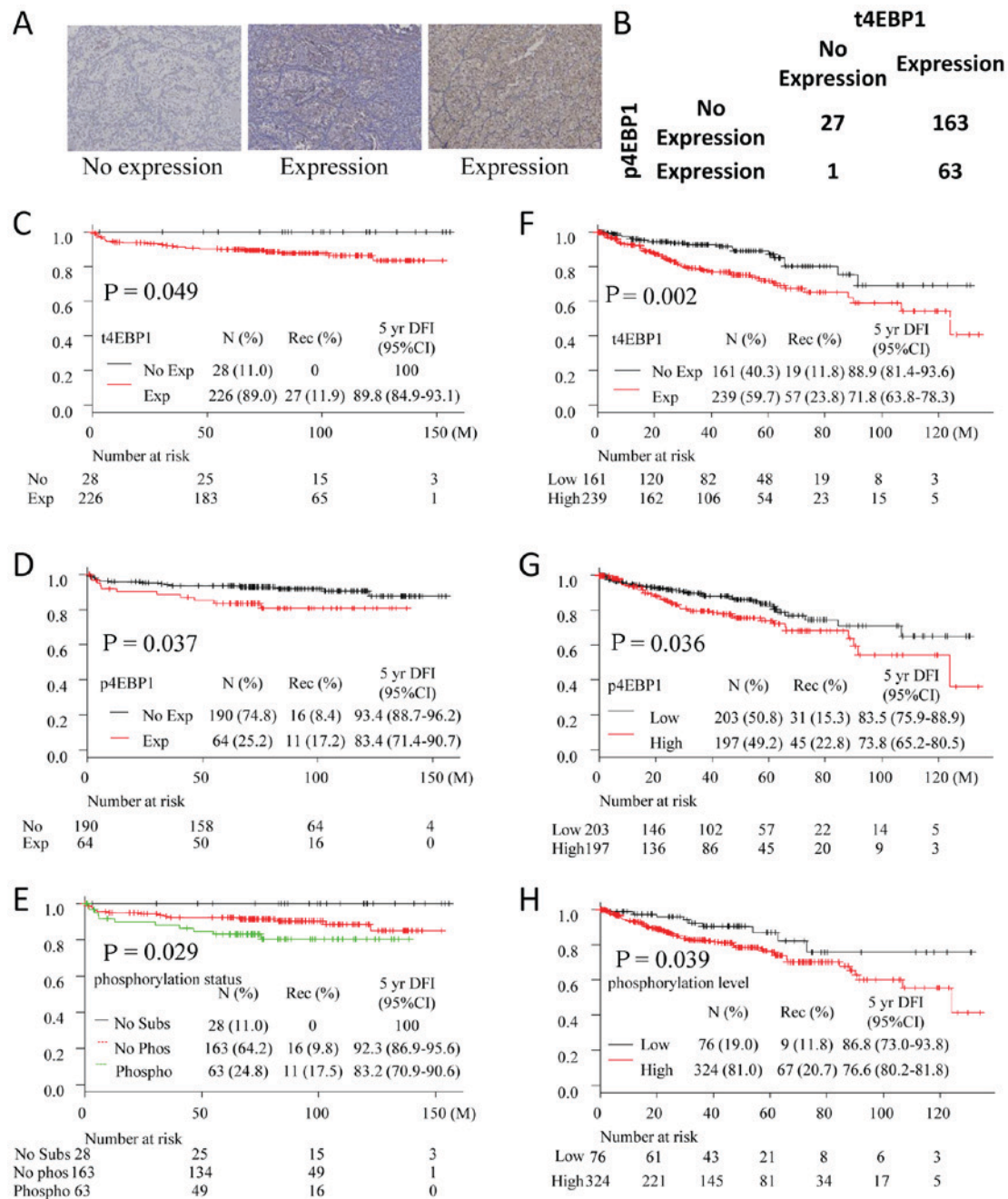
Figure 1. (A) Representative sample of no p4EBP1 expression and p4EBP1 expression. (B) Distribution of patients with t4EBP1 and p4EBP1. (C-E) Kaplan-Meier curves for disease-free survival in non-mRCC patients in Yamagata University (C, divided by t4EBP1 expression; D, divided by p4EBP1 expression; and E, divided by phosphorylation status). (F-H) Kaplan-Meier curves for disease free survival in non-mRCC patients in TCGA cohort (F, divided by t4EBP1 expression; G, divided by p4EBP1 expression; and H, divided by phosphorylation status). No subs, no t4EBP1 expression patients; no Phos, no p4EBP1 expression patients; phospho, p4EBP1 expression patients; t4EBP1, total eukaryotic translation initiation factor 4E-bidnding protein 1; p4EBP1, phosphorylated eukaryotic translation initiation factor 4E-binding protein 1; mRCC, metastatic renal cell carcinoma.

respectively). No p4EBP1 expression and grade 4 were independently predictive of worse CSS in the mRCC cohort (Table III).

Validation study using TCGA database with regards to mRCC. To validate the value of p4EBP1 as biomarker for DFI in mRCC patients, we analyzed M1 patients in TCGA database. Patients with high expression of p4EBP1 had numerically longer OS, but it was not statistically different (P=0.073) (Fig. 2B).

Correlation of p4EBP1 expression with best overall response to each treatment. Finally, we investigated the correlation of

p4EBP1 expression with BOR in first line, first VEGF-targeted agent, first mTORC1 inhibitor, and sunitinib, sorafenib, and axitinib treatments. In patients showing no p4EBP1 expression, no treatments other than sorafenib induced clinical response. Only one of these patients achieved CR with sorafenib (Table IV).

Discussion

Firstly our study have elucidated that both t4EBP1 and p4EBP1 expression predict recurrence after nephrctomy, especially p4EBP1 is an independent predictor (Fig. 1 and TableII).

Table I. Baseline characteristics and DFR in the non-metastatic renal cell carcinoma cohort.

Factor	Number (%)	Number of recurrence (%)	% DFR at 5 year (95% CI)	P-value for DFI[a]
All	254	27 (10.6)	90.9 (86.5-93.9)	
Age				
≤55	57 (22.4)	4 (7.0)	92.9 (82.2-97.3)	0.285
56-60	52 (20.5)	9 (17.3)	89.8 (77.1-95.6)	
61-65	23 (9.1)	2 (8.7)	91.3 (69.5-97.8)	
66-70	42 (16.5)	3 (7.1)	94.7 (80.6-98.7)	
71-75	44 (17.3)	4 (9.1)	90.7 (77.0-96.4)	
76-80	23 (9.1)	2 (8.7)	90.2 (66.2-97.5)	
80<	13 (5.1)	3 (23.1)	75.0 (40.8-91.2)	
≤80	241 (94.9)	24 (10.0)	91.7 (87.4-94.7)	0.0451
>80	13 (5.1)	3 (23.1)	75.0 (40.8-91.2)	
Sex				
Male	157 (61.8)	17 (10.8)	92.1 (6.5-95.4)	0.959
Female	97 (38.2)	10 (10.3)	89.0 (80.4-93.9)	
Laterality				
Right	139 (54.7)	18 (12.9)	90.2 (83.8-94.2)	0.201
Left	115 (45.3)	9 (7.8)	91.7 (84.6-95.6)	
pT				
1a	158 (62.2)	3 (1.9)	98.6 (94.6-99.7)	<0.001
1b	48 (18.9)	8 (16.7)	86.8 (72.9-93.9)	
2a	10 (3.9)	4 (40.0)	80.0 (40.9-94.6)	
2b	6 (2.4)	2 (33.3)	66.7 (19.5-90.4)	
3a	19 (7.5)	4 (21.1)	87.3 (51.9-91.3)	
3b	9 (3.5)	4 (44.4)	50.0 (15.2-77.5)	
3c	2 (0.8)	1 (50.0)	0 (N.A-N.A)	
4	2 (0.8)	1 (50.0)	50.0 (35.4-0.6)	
1a	158 (62.2)	3 (1.3)	98.6 (94.6-99.7)	<0.001
1b-4	96 (37.8)	24 (25.0)	77.8 (67.7-85.1)	
Grade				
1	113 (44.5)	6 (5.3)	95.2 (88.9-98.0)	<0.001
2	110 (44.3)	11 (10.0)	93.3 (86.4-96.7)	
3	24 (9.4)	8 (33.3)	65.2 (42.3-80.8)	
4	7 (2.8)	2 (28.6)	71.4 (25.8-92.0)	
1 or 2	223 (87.8)	17 (7.6)	94.3 (90.1-96.7)	<0.001
3 or 4	31 (12.2)	10 (32.3)	65.8 (45.7-80.0)	
Vessel invasion				
-	224 (88.2)	18 (8.0)	93.4 (89.1-96.1)	<0.001
+	30 (11.8)	9 (30.0)	71.3 (50.6-84.5)	

[a]P-values and the hazard ratio were calculated with DFI. DFR, disease-free rate; DFI, disease-free interval.

Several previous reports have mentioned that high phosphorylation of 4EBP1 indicates poor prognosis in prostate, colon, ovarian, and breast cancer, as well as ccRCC and Xp11.2 translocated RCC (11,17-20). Our findings and the validation results regarding to p4EBP1 in the non-mRCC cohort agree with the previous studies indicated in other cancers. One supposed reason for the result is that phosphorylation of 4EBP1 promotes to dissociate 4EBP1/eIF4E assembly (7,8). Actually, we previously elucidated that high levels of 4EBP1/eIF4E activation predicts RCC recurrence (14). Another reason is the consequence of Akt/mTORC1 pathway activation. Akt/mTORC1 pathway activates another molecules including S6K (7,8).

While p4EBP1 predictably indicates high RCC recurrence, t4EBP1 was contrary to our expectations. Since 4EBP1 is a suppressor of eIF4E which promotes cap-dependent translation, cell proliferation, and metastasis (8), we had assumed that patients without t4EBP1 expression could have frequently developed recurrence. A possible reason for this unexpected

Table II. Multivariate analyses for disease-free interval in non-metastatic renal cell carcinoma cohort (N=254).

			Univariate		Multivariate	
Factor	Number (%)	Number of recurrence (%)	% DFR at 5 years (95% CI)	P-value for DFI[a]	HR (95% CI) for DFI[a]	P-value for DFI[a]
Age						
≤80	241 (94.9)	24 (10.0)	91.7 (87.4-94.7)	0.0451	Withdrawn stepwise	
80<	13 (5.1)	3 (23.1)	75.0 (40.8-91.2)			
pT						
1a	158 (62.2)	3 (1.3)	98.6 (94.6-99.7)	<0.001		
1b-4	96 (37.8)	24 (25.0)	77.8 (67.7-85.1)		14.79 (4.40-49.75)	<0.001
Grade						
1 or 2	223 (87.8)	17 (7.6)	94.3 (90.1-96.7)	<0.001		
3 or 4	31 (12.2)	10 (32.3)	65.8 (45.7-80.0)		4.24 (1.88-9.61)	<0.001
v						
-	224 (88.2)	18 (8.0)	93.4 (89.1-96.1)	<0.001	Withdrawn stepwise	
+	30 (11.8)	9 (30.0)	71.3 (50.6-84.5)			
t4EBP1						
No expression	28 (11.0)	0	100	0.049	Withdrawn stepwise	
Expression	226 (89.0)	27 (11.9)	89.8 (84.9-93.1)			
p4EBP1						
No expression	190 (74.8)	16 (8.4)	93.4 (88.7-96.2)	0.037		
Expression	64 (25.2)	11 (17.2)	83.4 (71.4-90.7)		2.77 (1.13-6.78)	0.026

[a]P-values and hazard ratios were calculated with DFI. DFR, disease-free rate; DFI, disease-free interval; HR, hazard ratio; CI, confidence interval; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1.
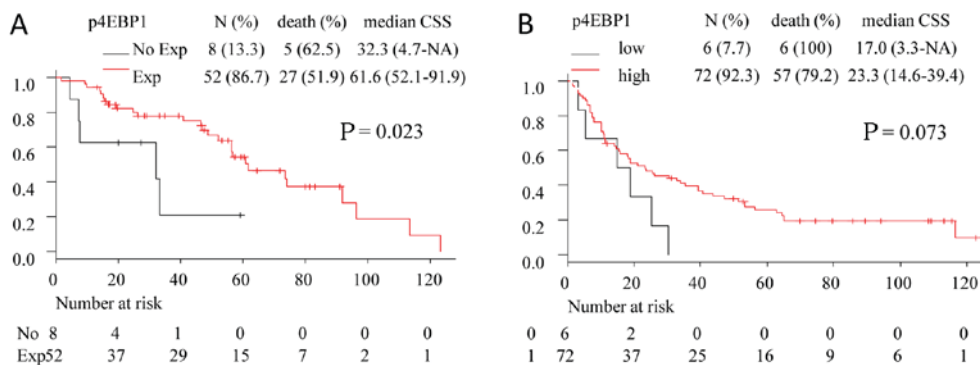

Figure 2. (A) Kaplan-Meier curve for cause-specific survival divided by p4EBP1 expression in mRCC patients in Yamagata University. (B) Kaplan-Meier curve for overall survival divided by p4EBP1 expression in mRCC patients in TCGA cohort. p4EBP1, phosphorylated eukaryotic translation initiation factor 4E-binding protein 1; mRCC, metastatic renal cell carcinoma.

result is compensation by other molecules such as fragile X mental retardation protein (FMRP). FMRP binds eIF4E and inhibits its function (8). This eIF4E inhibition by FMRP is reported to suppress cancer cell proliferation and metastasis (21). If low t4EBP1 expression induces compensatory FMRP activation, t4EBP1 expression could be compatible with high RCC recurrence rate. More detailed work is necessary to resolve this issue.

The patients in the mRCC cohort with expression of p4EBP1 showed longer survival than those without it in univariate and multivariate analyses, on the contrary to the

non-mRCC cohort (Fig. 2 and Table II). To our knowledge, no other studies have evaluated the correlation between p4EBP1 and OS in mRCC. Besides, some reports evaluated p4EBP1 expression as a predictor for drug effectiveness. One report evaluated that genetic knock-down of 4EBP1 increased susceptibility for sorafenib, sunitinib, and everolimus. Furthermore, they mentioned that p4EBP1 predicted worse PFS in sunitinib, but did not in sorafenib. Regarding to everolimus, patients with low p4EBP1 expression had relatively worse PFS, which did not show statistical difference (22). Our results also showed that p4EBP1 expression indicates effectiveness for

Table III. Univariate and multivariate analyses for cause-specific survival in metastatic renal cell carcinoma cohort.

| Factor | N. (%) | RCC death (%) | Univariate (N=60) | | Multivariate (N=41) | |
			Median CSS, year (95% CI)	P-value	HR (95% CI)	P-value
Age						
≤60	19 (31.7)	10 (52.6)	7.66 (2.69-NA)	0.270		
61-70	24 (40.0)	11 (45.8)	4.73 (2.08-NA)			
>70	17 (28.3)	11 (64.7)	4.23 (1.29-NA)			
≤60	19 (31.7)	10 (52.6)	7.66 (2.69-NA)	0.110		
>60	41 (68.3)	22 (53.7)	4.73 (3.89-6.14)			
Sex						
Male	47 (78.3)	24 (51.1)	5.07 (4.07-6.17)	0.994		
Female	13 (21.7)	8 (61.5)	2.69 (1.29-NA)			
MSKCC risk group						
Favorable	22 (38.1)	6 (23.8)	8.03 (4.71-NA)	0.017		
Intermediate	28 (50.9)	19 (67.9)	3.89 (1.60-6.14)			
Poor	6 (10.9)	4 (66.7)	2.58 (0.15-NA)			
Unknown	4					
Favorable	22 (38.1)	6 (23.8)	8.03 (4.71-NA)	0.005	Withdrawn by stepwise	
Int. or poor	34 (61.8)	23 (67.6)	3.89 (1.60-4.73)			
Subtype						
Clear	56 (93.3)	28 (50.0)	5.07 (4.07-8.03)	<0.001		
Papillary	1 (1.7)	1 (100)	1.29			
Mixed	1 (1.7)	1 (100)	2.20			
Xp11.2	1 (1.7)	1 (100)	7.66			
Other	1 (1.7)	1 (100)	0.15			
Clear	56 (93.3)	28 (50.0)	5.07 (4.07-8.03)	0.076	Withdrawn by stepwise	
Non-clear	4	4 (100)	1.74 (0.15-NA)			
Grade						
1	4 (9.5)	2 (50.0)	4.73 (4.71-NA)	0.002		
2	18 (42.9)	10 (55.6)	4.34 (2.69-NA)			
3	15 (35.7)	7 (46.7)	4.07 (2.08-NA)			
4	5 (11.9)	4 (80.0)	1.60 (9.15-NA)			
Unknown	18					
1-3	37 (88.1)	19 (51.4)	4.71 (3.89-6.17)	<0.001	1	
4	5 (11.9)	4 (80.0)	1.60 (9.15-NA)		10.72 (2.80-41.00)	<0.001
t4EBP1						
No expression	0					
Expression	60	32 (53.3)	5.07 (3.90-7.66)			
p4EBP1						
No expression	8 (13.3)	5 (62.5)	2.69 (0.39-NA)	0.023	1	
Expression	52 (86.7)	27 (51.9)	5.14 (4.34-7.66)		0.210 (0.052-0.845)	0.028

CSS, cause specific survival; HR, hazard ratio; MSKCC, Memorial Sloan Kettering Cancer Center; t4EBP1, total eukaryotic translation initiation factor 4E-binding protein 1; p4EBP1, phosphorylated-eukaryotic translation initiation factor 4E-binding protein 1.

both mTORC1 inhibitors and VEGF-targeted agents, except for sorafenib (Table III). Li *et al* also demonstrated that phosphorylation of mTOR and ribosomal protein S6, which is downstream of mTORC1, were associated with statistically longer PFS in 18 patients treated with mTORC1 inhibitor (23). Thir and our results indicate that p4EBP1 expression could

be an indicator for response to mTORC1 inhibitors and VEGF-targeted agents, except sorafenib. In addition, patients having no expression of p4EBP1 might be preferred candidates for sorafenib or new treatment strategies, such as I-O drugs. Since these studies, including ours, were based on a small number of patients with highly miscellaneous backgrounds,

Table IV. Optimal objective response of each medication.

Variable	Complete response	Partial response	Stable disease	Progression disease	Unevaluable
First line treatment					
No expression	0	0	5	3	0
Expression	0	5	19	17	11
First VEGF targeted drug					
No expression	0	0	1	3	3
Expression	0	6	16	10	3
First mTORC1 inhibitor					
No expression	0	0	1	1	
Expression	0	1	5	6	
Sunitinib					
No expression	0	0	1	4	1
Expression	0	4	9	11	9
Sorafenib					
No expression	1	0	0	2	2
Expression	0	3	5	9	6
Axitinib					
No expression	0	0	0	3	1
Expression	0	2	10	7	2

VEGF targeted drug, vascular endothelial growth factor targeted drug (includes sunitinib, sorafenib, axitinib, and pazopanib); mTORC1 inhibitor, mammalian target of rapamycine complex 1 inhibitor (includes everolimus and temsirolimus).

a larger cohort or direct comparison are required to confirm a predictive biomarker for drug effectiveness.

We initially hypothesized that p4EBP1 expression could have indicated resistance to VEGF-targeted agents, because our previous cell line studies showed that RCC cells with activated PI3K/Akt/mTORC1 are resistant to sunitinib and sorafenib (12,13). We have several theories for this contrary result. Firstly, 4EBP1 is a substrate of not only mTORC1 but also other kinases. For instance, we previously demonstrated that glycogen synthase kinase-3 directly phosphorylates 4EBP1 (11). Secondly, mTORC1 is activated by the nutrient/AMPK pathway in addition to the GFs/PI3K/Akt pathway (7). Thirdly, factors other than 4EBP1 can also regulate eIF4E like FMRP (8). Lastly, VEGF-targeted agents could have both anti-tumor and anti-angiogenic effects (12,24). The serum level of clinically available sorafenib suppresses RCC cell proliferation in *in vitro* studies, while sunitinib does not suppress cell proliferation at clinical serum levels (13). Sorafenib not only targets VEGF but also Raf kinase, which is a molecule in another major pathway activated by GFs (25). These findings appear to indicate that sorafenib, in particular, possesses anti-tumor action, while the anti-tumor mechanism of sunitinib is weak. In addition, mTORC1 activation facilitates VEGF expression. Since phosphorylation of p4EBP1 could partially represent mTORC1 activation, it is reasonable to suppose that VEGF-targeted agents and mTORC1 inhibitors exhibited no effect in patients who showed no p4EBP1 expression, and these patients had a shorter CSS as a result.

This study had several limitations. First, it was a retrospective study. Second, these cohorts had incomplete data. We were unable to determine MSKCC risk group in 5 of 60 patients in the mRCC cohort because of insufficient data. Third, the non-mRCC cohort was too small, especially when we analyzed the efficacy of each medication. Fourth, since some of the medications are part of sequential therapy, the tumors could have been affected by prior medications. Last, IHC was performed on primary lesions, and these pathological findings would be different from those of metastatic lesions.

In summary, t4EBP1 and p4EBP1 expression correlated with *de novo* metastasis. In contrast, patients with metastatic disease who showed no p4EBP1 expression had shorter CSS. This could be a predictive biomarker for the clinical efficacy of mTORC1 inhibitors and VEGF-targeted agents, other than sorafenib.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SN, OI, HI, ToK, PM, VMK and NT participated in the design of the present study. SN and OI drafted the manuscript and figures, and performed statistical analyses. TaK and MiY assessed the pathological features. HI and HF performed immunohistochemistry procedures. HidK and TN assessed the immunohistochemistry data. MaY, AY, YK, TS, HN, HisK, and TY collected data. ToK and NT participated in the coordination and helped with drafting the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Yamagata University Faculty of Medicine (approval no. 421, 2016). The methods were carried out in accordance with the approved guidelines. The need for consent to participate in this study has been waived by the same institutional review board.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
2. Moch H, Cubilla AL, Humphrey PA, Reuter VE and Ulbright TM: The 2016 WHO classification of tumours of the urinary system and male genital organs-part A: Renal, penile, and testicular tumours. Eur Urol 70: 93-105, 2016.
3. Greef B and Eisen T: Medical treatment of renal cancer: New horizons. Br J Cancer 115: 505-516, 2016.
4. Powles T, Staehler M, Ljungberg B, Bensalah K, Canfield SE, Dabestani S, Giles R, Hofmann F, Hora M, Kuczyk MA, et al: Updated EAU guidelines for clear cell renal cancer patients who fail VEGF targeted therapy. Eur Urol 69: 4-6, 2016.
5. Sato Y, Yoshizato T, Shiraishi Y, Maekawa S, Okuno Y, Kamura T, Shimamura T, Sato-Otsubo A, Nagae G, Suzuki H, et al: Integrated molecular analysis of clear-cell renal cell carcinoma. Nat Genet 45: 860-867, 2013.
6. Schödel J, Grampp S, Maher ER, Moch H, Ratcliffe PJ, Russo P and Mole DR: Hypoxia, hypoxia-inducible transcription factors and renal cancer. Eur Urol 69: 646-657, 2016.
7. Pópulo H, Lopes JM and Soares P: The mTOR signalling pathway in human cancer. Int J Mol Sci 13: 1886-1918, 2012.
8. Bramham CR, Jensen KB and Proud CG: Tuning specific translation in cancer metastasis and synaptic memory: Control at the MNK-eIF4E axis. Trends Biochem Sci 41: 847-858, 2016.
9. Guo J, Chakraborty AA, Liu P, Gan W, Zheng X, Inuzuka H, Wang B, Zhang J, Zhang L, Yuan M, et al: pVHL suppresses kinase activity of Akt in a proline-hydroxylation-dependent manner. Science 353: 929-932, 2016.
10. Luan FL, Ding R, Sharma VK, Chon WJ, Lagman M and Suthanthiran M: Rapamycin is an effective inhibitor of human renal cancer metastasis. Kidney Int 63: 917-926, 2003.
11. Achermann C, Stenner F and Rothschild SI: Treatment, outcome and prognostic factors in renal cell carcinoma-a single center study (2000-2010). J Cancer 7: 921-927, 2016.
12. Makhov PB, Golovine K, Kutikov A, Teper E, Canter DJ, Simhan J, Uzzo RG and Kolenko VM: Modulation of Akt/mTOR signaling overcomes sunitinib resistance in renal and prostate cancer cells. Mol Cancer Ther 11: 1510-1517, 2012.
13. Naito S, Makhov P, Astsaturov I, Golovine K, Tulin A, Kutikov A, Uzzo RG and Kolenko VM: LDL cholesterol counteracts the antitumour effect of tyrosine kinase inhibitors against renal cell carcinoma. Br J Cancer 116: 1203-1207, 2017.
14. Ichiyanagi O, Naito S, Ito H, Kabasawa T, Narisawa T, Kanno H, Kurota Y, Kurokawa M, Fukuhara H, Sakurai T, et al: Levels of 4EBP1/eIF4E activation in renal cell carcinoma could differentially predict its early and late recurrence. Clin Genitourin Cancer 16: e1029-e1058, 2018.
15. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, et al: New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer 45: 228-247, 2009.
16. Motzer RJ, Bacik J, Murphy BA, Russo P and Mazumdar M: Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma. J Clin Oncol 20: 289-296, 2002.
17. Armengol G, Rojo F, Castellvi J, Iglesias C, Cuatrecasas M, Pons B, Baselga J and Ramón y Cajal S: 4E-binding protein 1: A key molecular 'funnel factor' in human cancer with clinical implications. Cancer Res 67: 7551-7555, 2007.
18. No JH, Jeon YT, Park IA, Kim YB, Kim JW, Park NH, Kang SB, Han JY, Lim JM and Song YS: Activation of mTOR signaling pathway associated with adverse prognostic factors of epithelial ovarian cancer. Gynecol Oncol 121: 8-12, 2011.
19. Coleman LJ, Peter MB, Teall TJ, Brannan RA, Hanby AM, Honarpisheh H, Shaaban AM, Smith L, Speirs V, Verghese ET, et al: Combined analysis of eIF4E and 4E-binding protein expression predicts breast cancer survival and estimates eIF4E activity. Br J Cancer 100: 1393-1399, 2009.
20. Campbell L, Jasani B, Griffiths DF and Gumbleton M: Phospho-4e-BP1 and eIF4E overexpression synergistically drives disease progression in clinically confined clear cell renal cell carcinoma. Am J Cancer Res 5: 2838-2848, 2015.
21. Joshi S and Platanias LC: Mnk kinase pathway: Cellular functions and biological outcomes. World J Biol Chem 5: 321-333, 2014.
22. Nakai Y, Miyake M, Morizawa Y, Hori S, Tatsumi Y, Anai S, Onishi S, Tanaka N and Fujimoto K: Potential biomarkers for the therapeutic efficacy of sorafenib, sunitinib and everolimus. Oncol Rep 37: 227-234, 2017.
23. Beuselinck B, Vano YA, Oudard S, Wolter P, De Smet R, Depoorter L, Teghom C, Karadimou A, Zucman-Rossi J, Debruyne PR, et al: Prognostic impact of baseline serum C-reactive protein in patients with metastatic renal cell carcinoma (RCC) treated with sunitinib. BJU Int 114: 81-89, 2014.
24. Xin H, Zhang C, Herrmann A, Du Y, Figlin R and Yu H: Sunitinib inhibition of Stat3 induces renal cell carcinoma tumor cell apoptosis and reduces immunosuppressive cells. Cancer Res 69: 2506-2513, 2009.
25. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, et al: BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64: 7099-7109, 2004.