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ORIGINAL ARTICLE

Influence of Rictor and Raptor Expression of mTOR Signaling on Long-Term Outcomes of Patients with Hepatocellular Carcinoma

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Abstract

Background Aberrant signaling mediated by the mammalian target of rapamycin (mTOR) occurs at high frequency in hepatocellular carcinoma (HCC), indicating that mTOR is a candidate for targeted therapy. mTOR forms two complexes called mTORC1 (mTOR complexed with raptor) and mTORC2 (mTOR complexed with rictor). There are minor studies of the expression kinetics of mTORC1 and mTORC2 in HCC.

Methods We studied 62 patients with HCC who underwent curative resection. We used univariate and multivariate analyses to identify factors that potentially influence disease and overall survival after hepatectomy. The mRNA and protein levels of mTOR, rictor and raptor in cancer and non-cancer tissues were analyzed using quantitative RT-PCR, immunohistochemistry and Western blotting.

Results/Conclusion High ratio of the levels of rictor and raptor mRNAs in tumors was identified as independent prognostic indicators for disease-free survival. Low and high levels of preoperative serum albumin and mTOR mRNA in the tumor, respectively, were identified as independent indicators of overall survival. HCC is likely to recur early after hepatic resection in patients with high levels of mTOR and rictor mRNAs and high rictor/raptor ratios in cancer tissues. We conclude that analysis of mTOR expression in cancer tissues represents an essential strategy to predict HCC recurrence after curative treatment.

Keywords Hepatocellular carcinoma · Hepatic resection · mTOR · Rictor · Raptor · Rictor/raptor ratio · Prognostic factor

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide [1]. Although the incidence of HCC is highest in Asia and Africa, its incidence and mortality rates are rising in North America and Europe [2, 3]. Surgical resection is typically the first-line treatment for patients with small tumors and underlying chronic liver disease; however, the long-term survival rate after potentially curative resection of HCC is unsatisfactory because of the high rate of recurrence [4]. To improve prognosis, it is important to prevent the recurrence of HCC after initial resection, but there is no standard therapy for intrahepatic metastasis.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates protein synthesis, autophagy, endocytosis and metabolism in response to growth factors, nutrients, energy and stress [5]. Evidence indicates

that signaling pathways that activate mTOR are frequently dysregulated in most human cancers [6–14]. Because rapamycin inhibits mTOR activity, blocks tumor growth and kills cancer cells [15–17], the use of mTOR inhibitors was considered candidates for cancer therapy. However, the success of therapy using mTOR inhibitors is limited [18].

mTORC1 (mTOR complex 1), which is a downstream target of AKT, comprises mTOR, raptor (a regulatory protein associated with mTOR) and the mammalian LST8/G-protein β-subunit-like protein. mTORC1 acts as a central regulator of cell growth and proliferation by activating S6 kinase, which in turn regulates protein synthesis and allows progression from the G1 to S phase of the cell cycle [19]. In contrast, mTORC2 (mTOR complex 2), formed by mTOR, rictor (rapamycin-insensitive companion of mTOR), and proline-rich protein 5/G-protein β-subunit-like protein, is primarily responsible for the activation of AKT through phosphorylation of AKT Ser473 [20].

Abnormal activation of mTOR signaling occurs at high frequency in HCC. For example, the activation of mTOR signaling pathways in HCC ranged from 15 to 41 % [21–24], and evidence from clinical and basic studies indicates that mTOR signaling mediated by rictor plays a critical role in the pathogenesis of HCC [25]. However, there are no published studies on the kinetics of mTORC1 and mTORC2 expression. To fill this gap in our knowledge, here we conducted a retrospective study of the levels of expression of the mRNAs encoding mTOR, rictor and raptor in cancer and non-cancer tissues from surgical specimens. We assessed whether the data, taken together with the rictor/raptor ratios, correlate with the recurrence of HCC and long-term survival after potentially curative resection.

Materials and Methods

Patients

Two hundred and seventy-five patients with HCC underwent curative resection (defined as macroscopic removal of all tumors) at our institution between January 2000 and December 2006. Seven patients died while hospitalized, and the remaining 268 were followed as outpatients. Among the 268 patients, total RNA extraction for RT-PCR analysis was available from paraffin-embedded tissues in the 62 patients and retrospectively reviewed. All patients provided written informed consent for participation in this study, and the protocol was approved by the institutional Ethics Committee.

Clinical Variables and Surgery

Before surgery, each patient underwent conventional liver function testing and measurement of the indocyanine green retention rate at 15 min (ICGR15). Patients were tested for

hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (HCVAb), α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP). Surgical procedures were classified according to the Brisbane terminology proposed by Strasberg et al. [26]. Anatomic resection was defined as resection of the tumor together with the related portal vein branches and corresponding hepatic territory and was classified as hemihepatectomy (resection of half of the liver), extended hemihepatectomy (right trisectionectomy, or similar procedures on the left or right for smaller resections), sectionectomy (resection of two Couinaud sub-segments [27]) or segmentectomy (resection of one Couinaud sub-segment). All other procedures were classified as non-anatomic resection, which was frequently performed for peripheral or central tumors. Peripheral tumors and those with extrahepatic growth were treated by partial hepatectomy, because this procedure achieved a sufficient surgical margin. Central tumors located near the hepatic hilum or major vessels were treated by enucleation only, because it was too difficult, dangerous or both to remove enough liver tissue to obtain adequate margins. A consulting pathologist reviewed the histology of the specimens to confirm the diagnosis.

Follow-Up

Perioperative and postoperative complications and deaths were recorded to determine morbidity and mortality following hepatectomy. All surviving patients were examined at 3-month or fewer intervals after discharge. Follow-up included a physical examination, liver function tests, chest radiographs to detect pulmonary metastases and ultrasonography, computed tomography or magnetic resonance imaging to detect intrahepatic recurrence. Computed tomography of the chest was performed if the chest radiograph showed abnormalities. Bone metastases were diagnosed using bone scintigraphy. When analyses of tumor markers or imaging studies detected recurrence of HCC, recurrence limited to the remnant liver was treated using transarterial chemoembolization, lipiodolization, repeat resection or percutaneous local ablative therapy such as radiofrequency ablation. When extrahepatic metastases such as lung or bone were detected, the treatment with sorafenib was undertaken in patients with good hepatic reserve function (Child-Pugh class A or B) and good ECOG performance status (0 or 1), while other patients received only radiation therapy to relieve symptoms of bone metastases. Patients with a solitary extrahepatic metastasis and no intrahepatic recurrence underwent surgical resection.

Extraction of Total RNA from Paraffin-Embedded Tissue and mRNA Quantitation

Three 10-mm sections were prepared from blocks of primary tumors that contained at least 50 % tumor cells and

were placed in a microcentrifuge tube. The sections were formalin-fixed, paraffin-embedded and analyzed for the presence of cancer tissue using hematoxylin–eosin staining. Nuclear fast red staining was used to facilitate RNA extraction according to a published method with minor modifications [28]. Quantitation of the relative levels of cDNAs encoding mTOR, rictor, raptor, epithelial cell adhesion protein (EpCAM) and β-actin (*ACTB*, internal control) was performed using a fluorescence real-time detection method (ABI PRISM 7900 Sequence Detection System; TaqMan; Applied Biosystems, Foster City, CA, USA) as described previously [29–31]. Primers and probe sequences are as follows: mTOR, 5′-GACTGCTTTGAG GTTGCTATGAC-3′ and 5′-CCTTTGGTATTTGTGTC CATCAGC-3′; Rictor, 5′-AACACCAAGCAGGTTCAT GAAAGC-3′ and 5′-CAGATGGAAGACCTCCTGCATC A-3′; Raptor, 5′-TGACGGCCACAGACGATGGTGCC-3′ and 5′-CGTAGGGATGTCCTGCACCTTCA-3′; EpCAM; and 5′-CATTTGCTCA AAGCTGGCTG CCAA-3′ and 5′-TGATGATCCA GTAGGTTCTC ACTC-3′ and β-actin, 5′-GAGCGCGGCTACAGCTT-3′ and 5′-TCCTTAATGT CACGCACGATTT-3′. The PCR mixtures (25 µL) contained 600 nM of each primer, 200 nM probe, 2.5 units of AmpliTaq Gold polymerase, 200 µM each dATP, dCTP and dGTP and 400 µM dUTP, 5.5 mM MgCl$_2$, and 1× Taqman Buffer A containing a reference dye (all from Applied Biosystems). PCR conditions were as follows: 50 °C for 10 s, 95 °C for 10 min, 46 cycles at 95 °C for 15 s and 60 °C for 1 min. All gene expression analyses were performed by investigators who unaware of the clinical data. Relative levels of expression of genes encoding mTOR, rictor, raptor and EpCAM were determined according to their threshold cycles compared with that of *ACTB* mRNA [32]. Positive controls (samples of known value) and negative controls (samples without cDNA templates) were performed in parallel for each PCR experiment to assure equivalent assay conditions.

Immunohistochemistry

Paraffin-embedded 5-µm-thick tissue sections were stained using the streptavidin–peroxidase technique [33]. After deparaffinization and inhibition of endogenous peroxidase activity, the sections were incubated for 20 min at 23 °C with 1 % normal horse serum and for 20 h at 4 °C with mouse monoclonal antibodies against mTOR, rictor (Cell Signaling, Beverly, MA, USA) or raptor (Bethyl Laboratories, Montgomery, TX, USA). Bound antibodies were detected using biotinylated horse universal secondary antibodies and streptavidin–peroxidase complex with diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer's hematoxylin. Immunoreactivity was undetectable in the presence of mouse non-immunized serum or the absence of primary antibodies.

Western Blot Analysis

Frozen liver samples, which were obtained from surgical specimens and kept at −80 °C, were homogenized in ten volumes of cell solubilizing buffer (10 mM Tris–HCl, pH 7.4; containing 1 % Triton X-100, 0.5 % Nonidet P-40, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF), phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and centrifuged (16,500×*g* for 15 min). The supernatant was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final 125 mM Tris–HCl, pH 6.8; containing 5 % glycerol, 2 % SDS and 1 % 2-mercaptoethanol), subjected to SDS-PAGE (6 % gel) and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies against human mTOR (ab2732, Abcam, Tokyo, Japan), rictor (53A2, Cell Signaling, Bervery, MA, USA), phospho-rictor (Thr1135) (Cell Signaling),

Table 1 Perioperative characteristics of patients with HCC

Age (years)	63 ± 11
Gender (male/female)	55/7
HBV/HCV/NBC	19/31/12
Child-Pugh class (A/B)	58/4
ICGR15 (%)	18 ± 11
Albumin (g/dL)	3.9 ± 0.4
Total bilirubin (mg/dL)	0.9 ± 0.3
Prothrombin time (%)	90 ± 15
Platelet count (×10^4/µL)	16 ± 6
AST (U/L)	42 ± 17
ALT (U/L)	49 ± 27
AFP (ng/mL)	773 ± 2,271
DCP (mAU/mL)	323 ± 918
Esophageal and/or gastric varices (±)	15/47
Surgical procedure (limited/anatomic)	40/22
Operation time (min)	273 ± 86
Operative blood loss (mL)	1,019 ± 903
Blood transfusion (±)	23/39
Tumor size (cm)	3.3 ± 0.9
Associated liver disease	
(normal/fibrosis or hepatitis/cirrhosis)	9/36/17
Morbidity (±)	5/57
Mortality (±)	0/62

Data represent the mean ± SD or the number of patients. *HBV* hepatitis B virus, *HCV* hepatitis C virus, *NBC* non-hepatitis B or C virus, *WBC* white blood cell, *ICGR15* indocyanine green retention rate at 15 min, *ALT* alanine aminotransferase, *AFP* α-fetoprotein, *DCP* des-γ-carboxy prothrombin

Table 2 Univariate analysis of factors potentially associated with disease-free and overall survival after hepatectomy for HCC

Variable		*p* value	
		Disease-free survival	Overall survival
Gender	Male versus female	0.2351	0.2880
Age (years)	<65 versus ≥65	0.0517	0.3018
HBsAg	Positive versus negative	0.1240	0.2337
HCVAb	Positive versus negative	0.3119	0.0714
Alcohol abuse	No versus yes	0.3389	0.5970
Child-Pugh class	A versus B	0.0550	0.6270
ICGR15 (%)	<15 versus ≥15	0.1537	0.0239
Albumin (g/dL)	<3.8 versus ≥3.8	0.0300	0.0075
Platelet ($\times 10^4$/mm^3)	<15 versus ≥15	0.3868	0.2745
AST (U/L)	<37 versus ≥37	0.0284	0.0637
ALT (U/L)	<41 versus ≥41	0.4353	0.6293
Total bilirubin (mg/dL)	<0.9 versus ≥0.9	0.4254	0.2018
Cholinesterase (U/L)	<123 versus ≥123	0.1077	0.0312
Prothrombin time (%)	<90 versus ≥90	0.6536	0.3197
ALP (U/L)	<250 versus ≥250	0.0455	0.2459
γ-GTP (U/L)	<50 versus ≥50	0.6827	0.3153
AFP (ng/mL)	<17 versus ≥17	0.0119	0.0527
PIVKA-II (mAU/mL)	<53 versus ≥53	0.1139	0.2402
mRNA in tumor			
mTOR (/‰Actin)	<2.4 versus ≥2.4	0.0371	0.0148
Raptor (/‰Actin)	<1.0 versus ≥1.0	0.2798	0.1972
Rictor (/‰Actin)	<0.4 versus ≥0.4	0.0346	0.1953
Rictor/raptor	<0.3 versus ≥0.3	0.0251	0.0407
EpCAM (/‰Actin)	<1.8 versus ≥1.8	0.5551	0.7901
mRNA in non-tumor			
mTOR (/‰Actin)	<2.1 versus ≥2.1	0.0286	0.0882
Raptor (/‰Actin)	0 versus >0	0.9283	0.9355
Rictor (/‰Actin)	0 versus >0	0.4446	0.9869
Rictor/raptor	<0.5 versus ≥0.5	0.6691	0.8661
EpCAM (/‰Actin)	<7.1 versus ≥7.1	0.1693	0.0928
Surgical procedure	Anatomic versus limited	0.8289	0.1502
Operating time (min)	<300 versus ≥300	0.6804	0.7655
Operative blood loss (mL)	<1,000 versus ≥1,000	0.2197	0.1352
Blood transfusion	No versus yes	0.2231	0.4443
Tumor size (cm)	<3.2 versus ≥3.2	0.2723	0.1970
Histology	Well or moderately versus poorly	0.4229	0.3273
Microvascular invasion	Present versus absent	0.2894	0.0927
Associated liver disease	Normal or hepatitis versus cirrhosis	0.5043	0.8289
Tumor stage	I or II versus III or IV	0.4888	0.4909

The Kaplan–Meier life table method was used to calculate the disease-free and overall survival rates, and differences in survival were estimated using the generalized log-rank test

HBsAg hepatitis B surface antigen, *HCVAb* hepatitis C virus antibody, *ICGR15* indocyanine green retention rate at 15 min, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *γ-GTP* γ-glutamyltransferase, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence/antagonism-II, *mTOR* mammalian target of rapamycin, *EpCAM* epithelial cell adhesion molecule

raptor (24C12, Cell Signaling), phospho-raptor (Ser792) (Cell Signaling) and rat β-tubulin (internal control; Clone TUB2.1; Sigma Chemical Co., St. Louis, MO, USA), followed by visualization with an enhanced chemiluminescence (ECL) blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

Table 3 Prognostic factors for disease-free survival and overall survival identified by multivariate analysis

Variable	Coefficient	SE	Relative risk	p value
(A) Disease-free survival				
Rictor/raptor in tumor ≥0.3/ ‰Actin	2.045	0.812	7.752	0.0118
AFP ≥ 17 ng/mL	1.893	0.595	6.623	0.0371
(B) Overall survival				
mTOR in tumor ≥2.4/ ‰Actin	2.161	1.071	8.696	0.0436
Albumin <3.8 g/dL	2.070	1.122	7.937	0.0470

SE standard error, *AFP* α-fetoprotein, *mTOR* mammalian target of rapamycin

Prognostic Factors

We performed univariate and multivariate analyses of 37 clinical factors to identify independent variables related to disease-free and overall survival. Patient characteristics investigated were as follows: gender, age, HBsAg, HCVAb, alcohol abuse (alcohol consumption ≥50 g/day for more than 3 years) and liver function (albumin, platelet count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, cholinesterase, prothrombin time, alkaline phosphatase (ALP), γ-glutamyltransferase (γ-GTP), ICGR15 and Child-Pugh class. The tumor factors studied were AFP, PIVKA-II, mTOR, raptor, rictor, rictor/raptor and EpCAM, and histologic features (including tumor diameter), differentiation, microvascular invasion, grade of fibrosis and tumor stage according to the TNM classification [34]. The surgical factors investigated were as follows: procedure, operating time, blood loss and perioperative blood transfusion. All variables identified as significantly associated with disease-free and overall survival using univariate analyses were then evaluated using multivariate logistic regression analysis to identify variables that were independently associated with disease-free and overall survival.

Statistical Analysis

Continuous variables are presented as the mean ± standard deviation (SD), and the two groups were compared using the Mann–Whitney U test. The patients were divided into groups according to the median values of continuous variables. The Kaplan–Meier method was used to calculate rates of disease-free and overall survival as of December 2012, and the significance of differences in survival rates was estimated using the generalized log-rank test. The Cox proportional hazards regression model (stepwise method) was used for multivariate analyses. Differences were considered significant when $P < 0.05$.
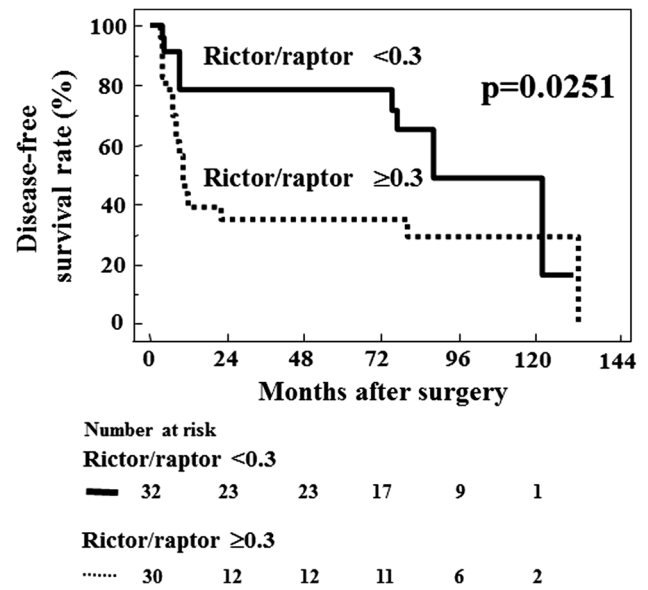
Fig. 1 Comparison of disease-free survival rates of HCC patients after hepatectomy. Rictor/raptor <0.3/ ‰Actin (*black line*), rictor/raptor ≥0.3/ ‰Actin (*dotted line*). There was a significant difference in disease-free survival between the two groups ($P = 0.0251$)
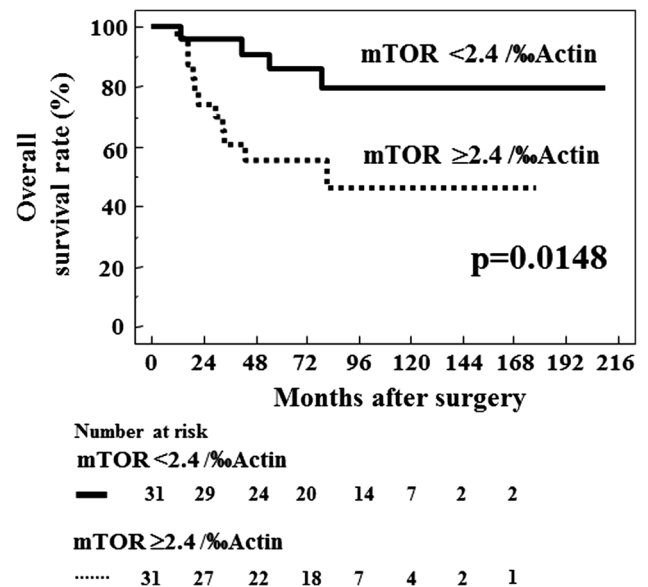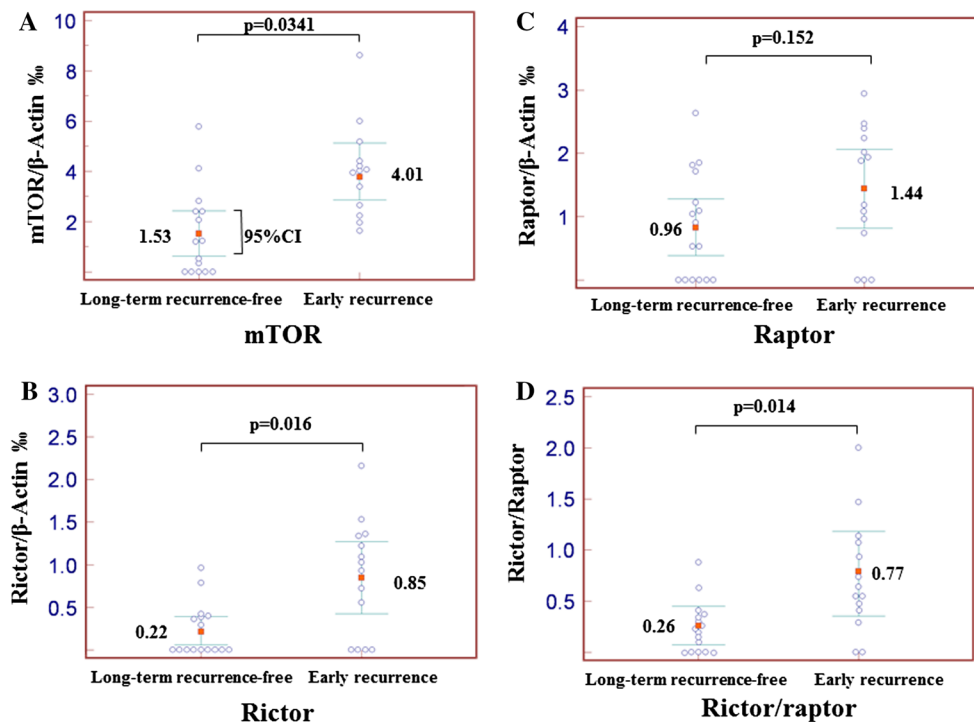

Fig. 2 Comparison of overall survival in HCC patients after hepatectomy. mTOR <2.4/ ‰Actin (*black line*), mTOR ≥2.4/ ‰Actin (*dotted line*). There was a significant difference in overall survival between the two groups ($P = 0.0148$)

Results

The present study included 62 patients (55 men and 7 women), mean age 63 ± 11 years, and Table 1 lists their perioperative characteristics.

Fig. 3 Comparison of mRNA levels encoding mTOR, rictor, raptor and rictor/raptor in HCC patients with early recurrence and long-term recurrence-free survival. Patients with early recurrence expressed significantly higher levels of mRNA encoding **a** mTOR ($P = 0.0341$), **b** rictor ($P = 0.016$) and **d** rictor/raptor ($P = 0.014$) in cancer tissue compared with those with long-term recurrence-free survival
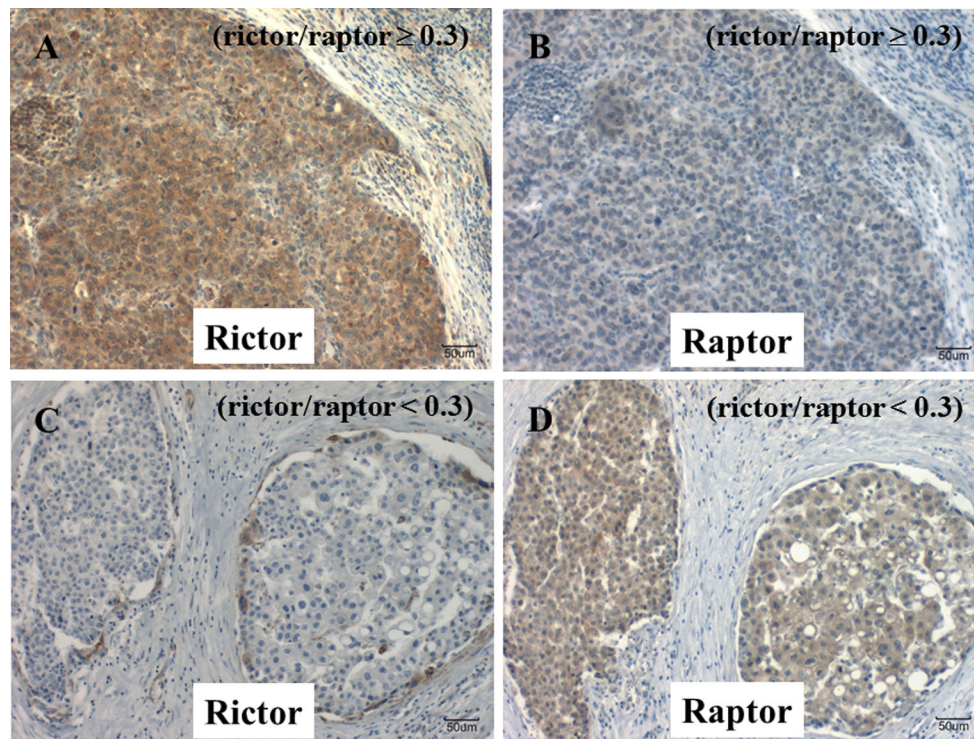
Fig. 4 Immunohistochemical analyses of mTOR and rictor in patients with HCC with early recurrence. Specimens with high-level expression of mRNAs encoding mTOR or rictor were probed with a monoclonal antibody against mTOR or rictor (**a** ×40; **b** ×400) or rictor (**c** ×40; **d** ×400), respectively, in patients with early recurrence ($n = 6$–7)

X 40   **X 400**
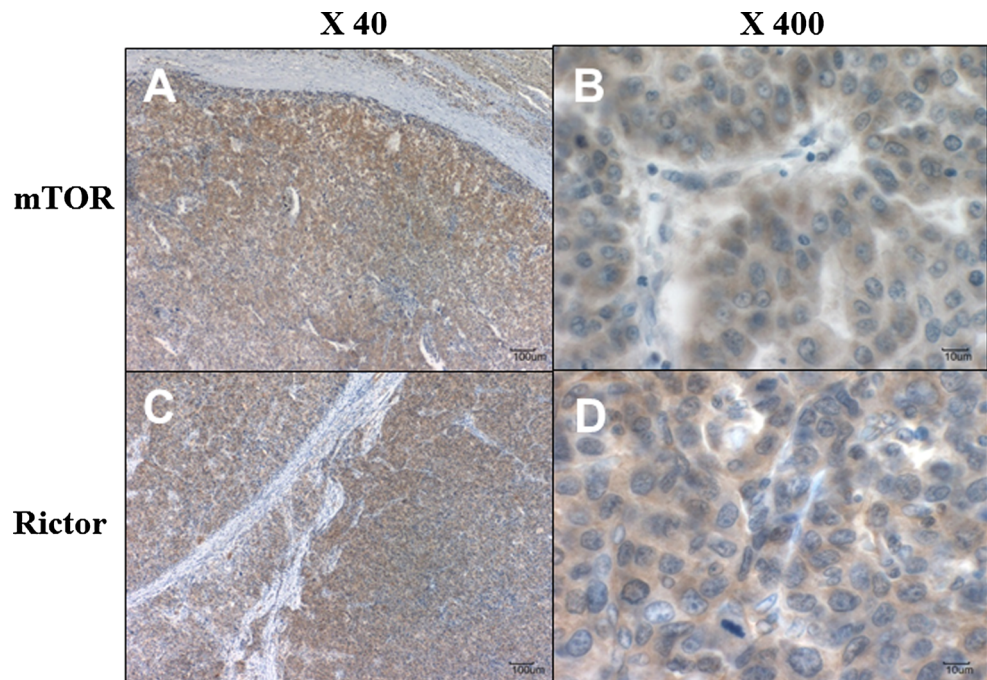
mTOR

Rictor

Factors Affecting Disease-Free and Overall Survival

Univariate analysis identified the factors that associated with worse disease-free survival as follows: expression levels of albumin, AST, ALP, AFP, mTOR, rictor, rictor/ raptor in tumor tissue and mTOR in the non-tumor tissue (Table 2). Factors associated with worse overall survival according to univariate analysis were as follows: ICGR15, albumin, cholinesterase, mTOR and rictor/raptor in tumor tissue (Table 2). Table 3 shows the results of multivariate

Fig. 5 Immunohistochemical analysis of rictor and raptor expression in patients with HCC. Monoclonal antibodies against rictor or raptor were used to probe the same specimens with high rictor (**a**) and low raptor (**b**) mRNA levels (rictor/raptor ≥0.3/ ‰Actin) in patients with early recurrence, and those with low rictor (**c**) and high raptor (**d**) mRNA levels (rictor/raptor <0.3/ ‰Actin) in patients with long-term recurrence-free survival ($n = 5$). Original magnification, ×100

analysis for factors with an influence on disease-free or overall survival. Serum AFP ≥17 ng/mL and rictor/raptor ≥0.3/ ‰Actin in the tumor were selected as independent prognostic indicators for disease-free survival, and serum albumin <3.8 g/dL and mTOR ≥2.4/ ‰Actin in the tumor were identified as factors that influenced overall survival. There was no relationship between the expression levels of mTOR, rictor and raptor mRNA and the malignancy of cancers such as differentiation, portal invasion and metastasis in HCC (data not shown).

Outcomes

There was a significant difference in the disease-free survival rate between patients with rictor/raptor <0.3/ ‰Actin and ≥0.3/ ‰Actin in the tumor ($P = 0.0251$) (Fig. 1). The disease-free survival rates of the patients with rictor/raptor <0.3/ ‰Actin and ≥0.3/ ‰Actin in the tumor were 78 and 34 % at 3 years, 78 and 34 % at 5 years, and 66 and 30 % at 7 years, respectively. There was also a significant difference in overall survival rate between patients with mTOR <2.4/ ‰Actin and ≥2.4/ ‰Actin in the tumor ($P = 0.0148$) (Fig. 2). The overall survival rates of the patients with mTOR <2.4/ ‰Actin and ≥2.4/ ‰ Actin in the tumor were 97 and 60 % at 3 years, 85 and 54 % at 5 years, and 79 and 42 % at 7 years, respectively.

Early Recurrence and Long-Term Survival Without Recurrence

The 62 patients were divided into two groups according to early recurrence within 2 years (early recurrence group, $n = 16$) and no recurrence for 4 years (long-term recurrence-free group, $n = 18$) after hepatic resection. The patients with early recurrence had significantly higher levels of mRNA encoding mTOR and rictor, and higher rictor/raptor ratios in cancer tissue compared with those of patients with long-term recurrence-free survival (Fig. 3a, b, d). There was no significant difference in the levels of raptor mRNA between the two groups (Fig. 3c). Consistent with these observations, there was increased immunoreactivity of mTOR (Fig. 4a, b) and rictor (Fig. 4c, d) in patients with early recurrence. Further, there were higher rictor (Fig. 5a) and lower raptor (Fig. 5b) immunoreactivities in the same section of tumor tissue (rictor/raptor ≥0.3/ ‰Actin) in patients with early recurrence, and in contrast, lower rictor (Fig. 5c) and higher raptor (Fig. 5d) immunoreactivities in the same tumor section (rictor/raptor <0.3/ ‰Actin) in patients with long-term recurrence-free survival.

In support of these observations, Western blotting analysis of mTOR, rictor and raptor revealed that the patients with early recurrence had significantly higher levels of
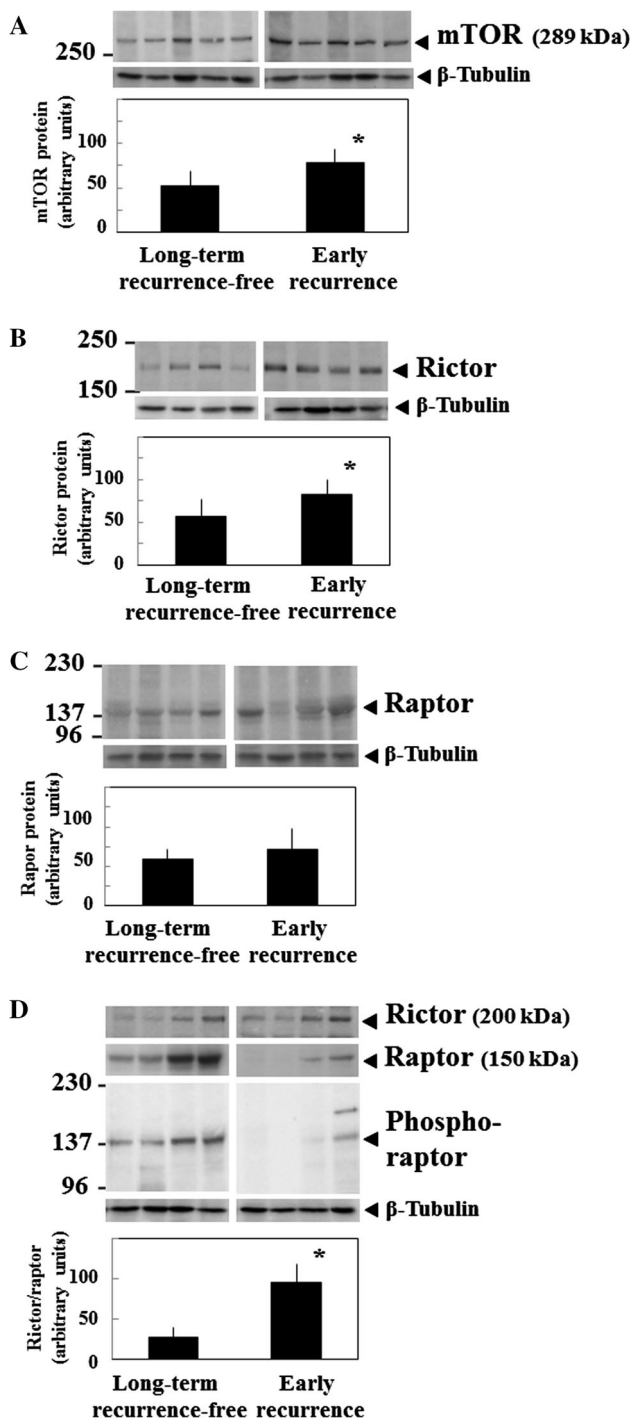
Fig. 6 Comparison of protein levels of mTOR, rictor, raptor and rictor/raptor in HCC patients. Representative results of the patients (7–9/group) are shown. The bands corresponding to mTOR, rictor or raptor were quantitated by densitometry (mean ± SD). Patients with early recurrence expressed significantly higher levels of **a** mTOR (*$P = 0.03$, $n = 9$/group), **b** rictor (*$P = 0.017$, $n = 8$/group) and (**d**) rictor/raptor (*$P = 0.001$, $n = 7$/group), but not of **c** raptor ($P = 0.275$, $n = 7$/group), in cancer tissue compared to those with long-term recurrence-free survival

mTOR, rictor and higher rictor/raptor ratios in cancer tissue than those with long-term recurrence-free survival (Fig. 6a, b, d). There was a tendency that the tumor tissues in patients with early recurrence increased the levels of raptor as compared to those with long-term recurrence-free survival, but not significantly (Fig. 6c). We detected the levels of phospho-raptor, which paralleled to the levels of raptor (Fig. 6d). However, we could not detect the phosphorylated form of rictor under conditions used.

Discussion

mTOR is a central regulator of cell growth in response to nutrients and growth factors [20]. mTORC1 and mTORC2 are the two structurally and functionally distinct mTOR complexes. mTORC1 comprises raptor and acts as a central regulator of cell growth and proliferation by activating S6 kinase, which in turn regulates protein synthesis and allows progression from G1 to the S phase of the cell cycle in a rapamycin-sensitive manner. mTORC2 comprises rictor, phosphorylates AKT Ser473 and is insensitive to rapamycin, although long-term rapamycin treatment inhibits mTORC2 activity in certain cell types [20]. The PI3 K/AKT/mTOR pathway is a major oncogenic cascade for targeting molecular therapies to various tumors, including HCC [23, 25, 35]. Villanueva et al. [25] reported that activation of the mTOR pathway contributes to the pathogenesis of HCC and showed for the first time that rictor may act as an oncogene that drives human hepatocarcinogenesis.

However, there are no published studies on the kinetics of mTORC2 expression and its relationship between mTORC1 and mTORC2 expression in HCC. In the present study, we found that higher rictor/raptor mRNA ratios and high levels of mTOR mRNA in tumors are independent prognostic indicators for disease-free and overall survival, respectively. Furthermore, the patients with early recurrence had higher levels of mRNA encoding mTOR and rictor, and higher rictor/raptor mRNA ratios than those with long-term recurrence-free survival. In support with this observation, analyses with immunohistochemistry and Western blotting revealed that there were differences in protein levels of mTOR, rictor and rictor/raptor between two groups. These findings imply that the expression of the mRNA encoding rictor as well as the rictor/raptor ratio correlates significantly with recurrence of HCC after curative treatment.

mTORC1 and mTORC2 are components of a negative feedback loop in which mTORC1 stimulation inhibits mTORC2 activity through the phosphorylation of the mTORC2

component Sin1 [36]. Further, mTORC1 inhibits the activation of mTORC2 by phosphorylating and stabilizing Grb10 [37, 38] and inhibits the phosphorylation of IRS-1 by S6K1 [39], which inhibits growth-factor signaling. These studies support the present findings that a high level of rictor/raptor exerts a more significant influence on disease-free survival compared with the expression of the mRNA encoding rictor mRNA.

The type I transmembrane glycoprotein EpCAM is over-expressed in certain epithelial cell malignancies [40] and serves as a cancer-specific antigen 17-1A [41]. Murakata et al. [42] reported that EpCAM expression is associated with overall survival and recurrence-free survival in patients with confluent multinodular (CM) type according to the characteristics of Eggel's nodular-type HCC. Moreover, their findings suggest that EpCAM plays a critical role in the aggressiveness of CM-type HCC. Because there were few cases of CM-type HCC among the 62 patients studied here, EpCAM expression did not predict poor prognosis for recurrence-free and overall survival of our HCC patients (Table 2).

Treatment using the mTOR inhibitor everolimus (RAD001) provides no survival benefit for patients with advanced HCC after progression or intolerance to sorafenib [43]. We found that protein ratios of rictor/raptor showed significant difference between the patients with early recurrence and long-term recurrence-free survival. Although we could not get the phospho-rictor/phospho-raptor ratios, our present results demonstrate that the activation of rictor and inactivation of raptor is essential to the development of HCC. These findings should guide efforts to develop new drugs that regulate the expression of rictor and raptor to levels that will enhance long-term recurrence-free survival of HCC patients after surgery.

In conclusion, HCC is more likely to recur early after curative hepatic resection, because the levels of mTOR and rictor expression and the rictor/raptor ratio are high in cancer tissues. For such patients, we recommend the administration of adjunct therapy using targeted drugs immediately after surgery.

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