Original Article
Rictor is an independent prognostic factor for endometrial carcinoma

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Abstract: Early-stage endometrial carcinoma (EC) patients have a high cure rate; however, those with high-risk factors may have poor prognosis. Thus, there is an urgent need for searching for new prognostic molecules to more accurately predict survival of patients. We detected the Rictor mRNA expression level in 30 fresh EC tissue and 17 normal endometrial tissue samples with real-time quantitative RT-PCR and Rictor protein expression level in 134 (test cohort) and 115 (validation cohort) paraffin tissue samples by immunohistochemistry, analyzed the correlation between variables and overall survival (OS) using Cox proportional hazards regression, compared the prognostic accuracy of Rictor with other clinicopathological risk factors by logistic regression. The results showed that Rictor mRNA expression of EC is higher than that of normal endometrium; Rictor protein expression level was closely correlated with FIGO stage, grade and vascular invasion in both cohorts; a univariate analysis showed that the pathological type, stage, grade, vascular invasion, lymphatic metastasis and Rictor were predictors of OS in both cohorts; furthermore, multivariate Cox proportional hazards regression analysis indicated that vascular invasion and Rictor were independent prognostic factors for EC in both cohorts; an ROX curve comparison showed that the area under the curve (AUC) for Rictor combined with other clinicopathological prognostic factors was higher than any individual factor or other clinicopathological prognostic factors' combination. Based on the above data, we concluded that Rictor is an independent prognostic factor for EC. It combined with other clinicopathological risk factors was a stronger prognostic model than individual risk factor or their combination.

Keywords: Rictor, prognosis, endometrial carcinoma

Introduction

Endometrial carcinoma (EC) is the fourth most common cancer among women worldwide [1]. Early-stage patients have a high cure rate; however, those who are at old age or low differentiation, lymphatic metastasis, vascular and myometrial invasion of EC [2, 3], may have poor prognosis. Thus, there is an urgent need for searching for new prognostic molecules to more accurately predict survival of patients.

mTOR, a highly conserved regulator of cell proliferation and growth in all eukaryotes [4], includes more than two complex, mTORC1 and mTORC2 [5, 6]. The mTORC1 complex is sensitive to rapamycin and responds to multiple stimuli, such as energy status, growth factors, amino acids, and inflammation [7]. The mTORC2 complex affecting cell morphology and actin polymerization [6, 8] mainly promotes cell proliferation and survival through phosphorylation of Akt and SGK [9, 10]. Rictor which is insensitive to rapamycin binds with mLST8, mSin1 and Protor to form a stable structure of the mTORC2 complex [10, 11] and also other protein partners that include the unconventional myosin motor Myo1C, Cullin-1, the integrin linked
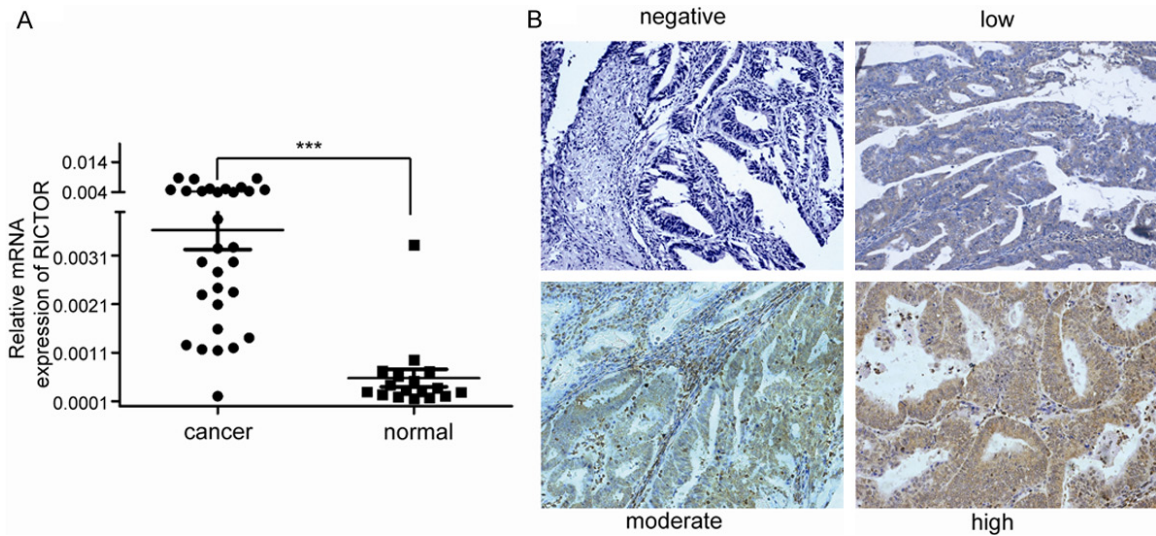
Figure 1. A. Rictor mRNA expression in 30 fresh EC tissue samples and 17 fresh normal endometrial tissues using real-time quantitative RT-PCR. Rictor mRNA expression of EC is higher than that of normal endometrium. The data were analyzed using the ΔΔCt approach and expressed as the Rictor/β-actin ratio [2-ΔCt (Rictor-β-actin)]. B. Representative image of Rictor expression in EC tissues was defined as negative, low, moderate and strong staining. Original magnification: ×200.

Table 1. Relationship between Rictor expression and clinicopathologic features of endometrial cancer patients in test and validation cohorts

Variable	Test cohort (n=134)			Validation cohort (n=115)		
	RICTOR		P value	RICTOR		P value
	negative	positive		negative	positive	
Pregnancy						
No	4	3	0.949	3	3	0.741
Yes	71	56		62	47	
Pathological type						
Adenocarcinoma	70	51	0.181	60	42	0.163
Squamous carcinoma, Papillay serous carcinoma, clear cell carcinoma	5	8		5	8	
Stage						
I	71	44	0.001**	61	39	0.011*
II	3	8		3	6	
III, IV	1	7		1	5	
Grade						
G1	62	15	< 0.001**	55	14	< 0.001**
G2	12	30		9	23	
G3	1	15		1	13	
Vascular invasion						
No	74	52	0.011*	64	45	0.043*
Yes	1	7		1	5	
Lymphatic metastasis						
No	73	54	0.134	63	47	0.446
Yes	2	5		2	3	

*, P < 0.05; **, P < 0.001.

kinase (ILK), PKCz and Hsp70 [11]. Rictor mediates the activities of integrin-linked kinase [12] and controls neutrophil chemotaxis by regulating the activity of Rac/Cdc42 and the actin cytoskeleton [13].

Currently, only a limited number of reports exist regarding the roles of Rictor in benign diseases. Rictor expression has been shown to be up-regulated in tuberous sclerosis complex [14], pituitary adenomas [15] and hypertrophying
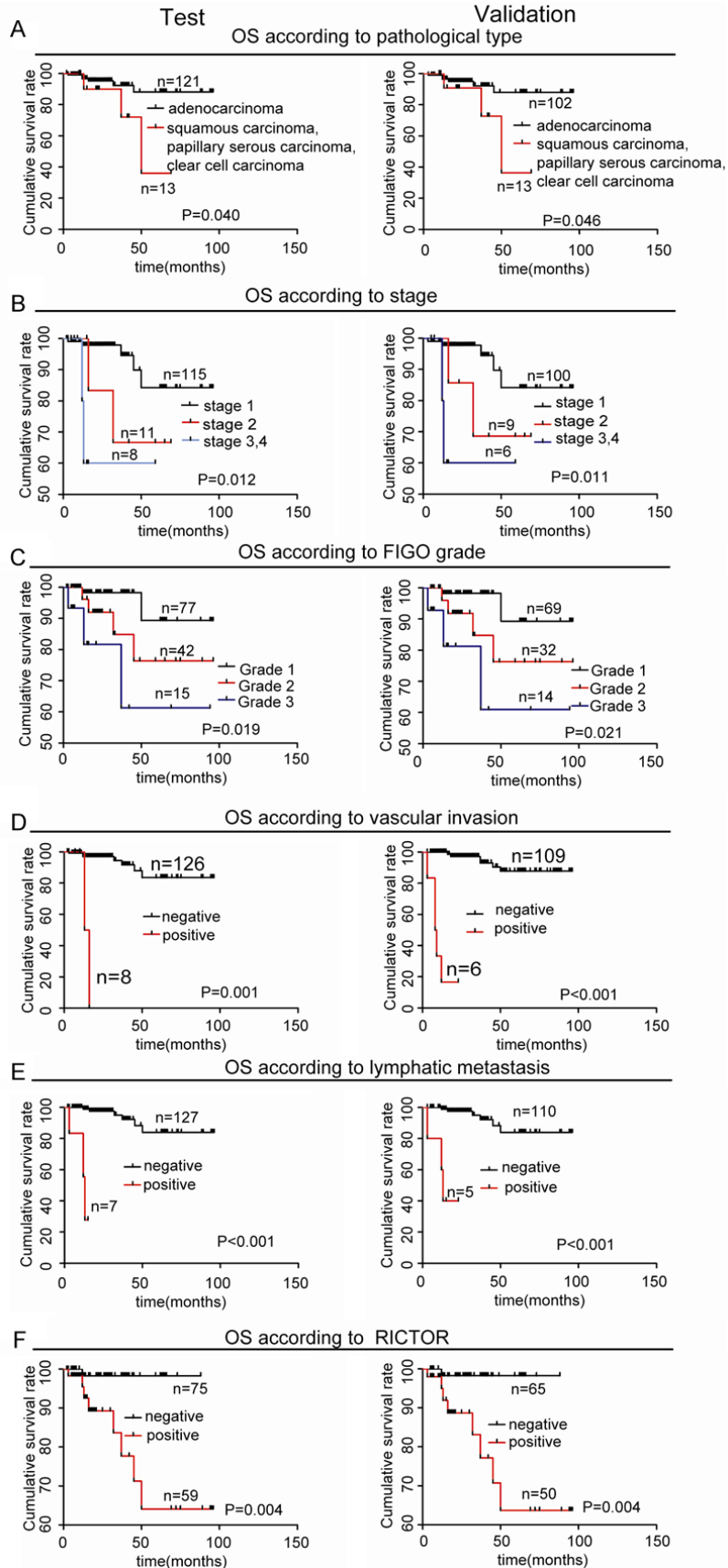
Figure 2. Univariate analyses of factors were associated with OS in test and validation cohorts by Kaplan-Meier method and log-rank test. (*, P < 0.05; **, P < 0.01). A. Comparisons of OS between adenocarcinoma and squamous carcinoma or papillary serous carcinoma or clear cell carcinoma groups in the test and validation cohorts. B. Comparisons of OS between stage I, stage II and stage III/IV groups in the test and validation cohorts. C. Comparisons of OS between G1, G2 and G3 groups in the test and validation cohorts. D. Comparisons of OS with and without vascular invasion groups in the test and validation cohorts. E. Comparisons of OS with and without lymphatic metastasis groups in the test and validation cohorts. F. Comparisons of OS between Rictor negative and positive groups in the test and validation cohorts.

myocardium [16]. However, Rictor has diverse and complicated biological functions in malignant tumors. For example, Rictor enhances activity of the Rho proteins and cell migration by suppressing Rho-GDI2 [17] and induces the expression of c-Myc and cyclin E overexpressed in colorectal cancer by phosphorylation and activation of Akt to promote proliferation. The Rictor expression level is up-regulated in almost all of malignant tumors by now, such as colorectal cancer [18], urothelial carcinoma [19], Glioma [20], oral squamous cell carcinoma [21], prostate cancer [22], breast cancer [23], bladder cancer [24], hepatocellular carcinoma [25] and Oral Cancer [26]. In gynecological cancers, it is reported that miR-152 is a
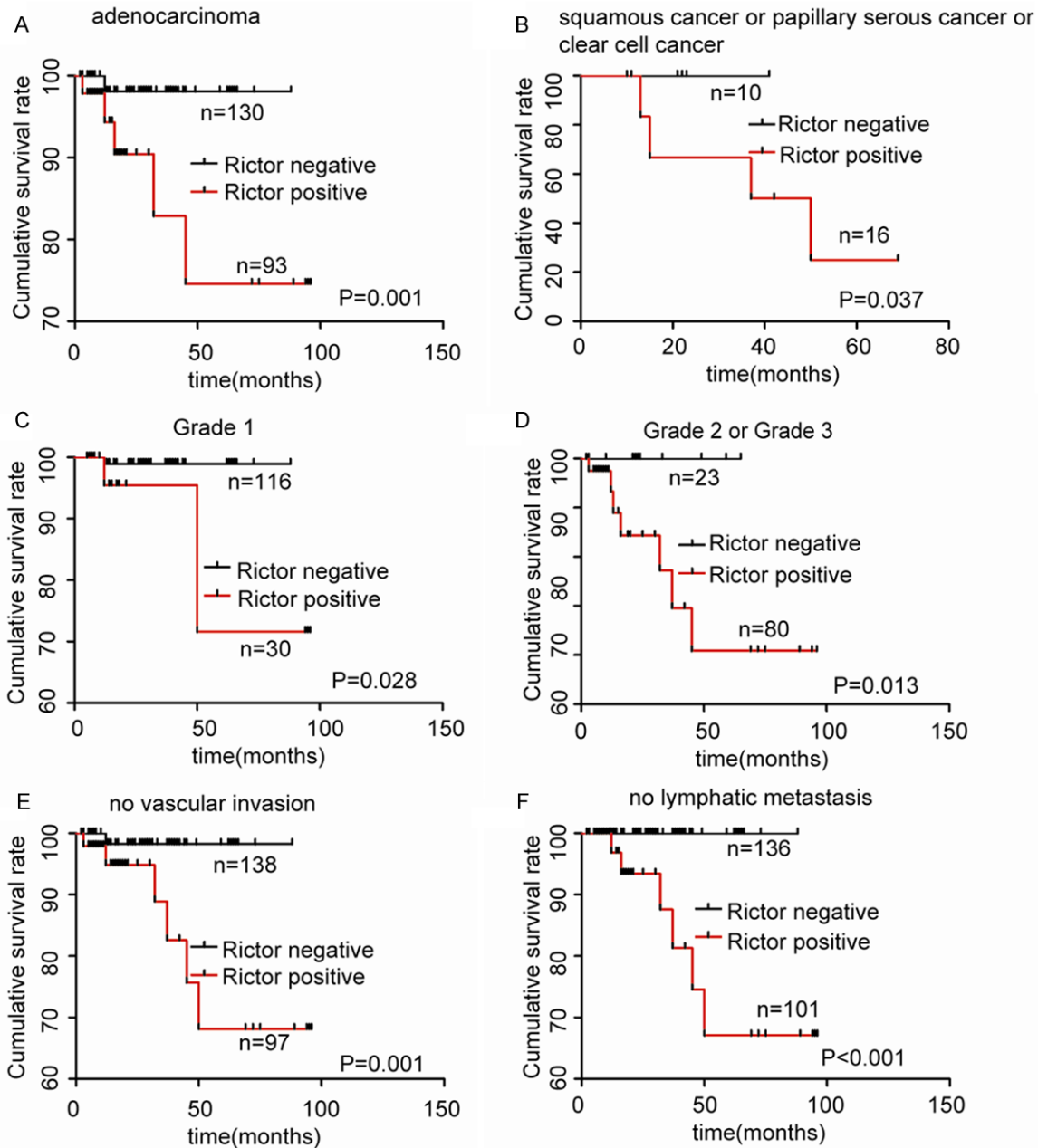
Figure 3. Prognostic significance of Rictor expression was assessed for all 249 samples separated according to pathological type, grade, vascular invasion and lymphatic metastasis by Kaplan-Meier method and log-rank test. A, B. Comparisons of OS between Rictor negative and positive groups in the adenocarcinoma cohort and in the squamous carcinoma or papillary serous carcinoma or clear cell carcinoma cohort. C, D. Comparisons of OS between Rictor negative and positive groups in G1 cohort and in G2 or G3 cohort. E. Comparisons of OS between Rictor negative and positive groups in no vascular invasion cohort. F. Comparisons of OS between Rictor negative and positive groups in no lymphatic metastasis cohort.

tumor suppressor by targeting Rictor in endometrial cancer [27]. These reports suggested complex roles for Rictor in cancer progression.

Although Rictor has been implicated in cancer progression, its prognostic value in EC remains unclear. By analysis of EC microarray datasets from the GEO database (GSE21882, GSE-17025), we found that Rictor was up-regulated in deceased patients or those with low differentiation compared to surviving patients or those with high differentiation, which suggested that

Table 2. Multivariate analyses of factors associated with OS in test and validation cohorts with the Cox proportional hazard regression model with stepwise manner (forward: condition, entry α=0.05, stay α=0.1)

Variable	Test cohort (n=133)		Validation cohort (n=109)	
	OS hazard ratio (95% CI)	P value	OS hazard ratio (95% CI)	P value
Vascular invasion	11.961	0.009**	12.034	0.008**
(Yes vs No)	(1.858-76.980)		(1.895-76.433)	
Rictor	8.612	0.046*	8.736	0.045*
(Yes vs No)	(1.034-71.702)		(1.050-72.707)	

*, $P < 0.05$; **, $P < 0.01$.

Rictor might be involved in cancer progression of EC. However, the prognostic value of Rictor in EC remains obscure.

In the study, we detected the Rictor mRNA expression level in 30 fresh EC tissue samples and 17 normal endometrial tissue samples with real-time quantitative RT-PCR and Rictor protein expression level in 134 paraffin tissue samples (test cohort) and 115 paraffin tissue samples (validation cohort) by immunohisto-chemistry, analyzed the correlation between variables and overall survival using Cox propor-tional hazards regression, compared the prog-nostic accuracy of Rictor with other clinicopath-ological risk factors by logistic regression and assessed the prognostic efficiency of Rictor in EC patients.

Materials and methods

Samples and clinical database

In this study, 30 fresh EC tissue samples, 17 fresh normal endometrial tissue samples and 249 formalin-fixed paraffin-embedded (FFPE) tissue samples were collected during April 2002 and March 2013 from Shanghai Jiao Tong University Affiliated Sixth People's Hospital, China, the First People's Hospital of Huai'an city, Jiangsu, China and the Shanghai Changning District Central Hospital, China. We excluded patients with the history of chemora-diotherapy, other solid tumors, or other anti-cancer therapies before surgery, without com-plete follow-up and clinicopathologic data, and without completed the ethics committee's con-sent and approval of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, or the Shanghai Changning District Central Hospital, or the first People's hospital of Huai'an city, Jiangsu, China, for the use of samples. Two

pathologists reassessed all the samples. We assigned 134 samples for test cohort and 115 samples for validation cohort using computer-generated random numbers (Supplementary Figure 1, Supplementary Table 1). The FFPE tis-sues samples comprised at least 80% tumor cells. Overall survival (OS) was defined as the time from the date of surgery to the date of the last follow-up examination or death by us.

Tissue microarray construction

Shanghai Zuoli Biotechnology Co., Ltd (Zuoli Biotechnology Co, Shanghai, China) construct-ed tissue microarrays. Pathologists stained paraffin tissue blocks of EC samples for test and validation cohorts using hematoxylin-eosin to affirm the diagnoses and marked at fixed points including the most typical histological characteristics. We diverted cores of 1.1 mm diameter from per donor block into a recipient block microarray, cut four-micron-thick sections from the recipient block and used with an adhe-sive tape transfer system to divert them to glass slides so as to ultraviolet cross linkage.

Immunohistochemistry

We de-paraffinized the slides in xylene for 10 min per time for three times, rehydrated with a graded series of ethanol concentrations (in 100%, 95%, 85%, 75% ethanol for 10 min respectively) and performed antigen retrieval in 100°C water with 0.01 M citrate buffer for 30 minutes. The sections were incubated in 37°C with 0.3% hydrogen peroxide/phosphate-buff-ered saline for 30 minutes and blocked at room temperature with 10% BSA for 1 hour. Rictor antibody (1:100, Abcam Biotechnology, Cam-bridge, UK) and a labeled polymer-HRP anti-mouse secondary antibody (1:1000, Dako, Carpentaria, CA, USA) were incubated at room temperature for 1 hour. We visualized the slides using DAB substrate liquid (Thermo Scientific,

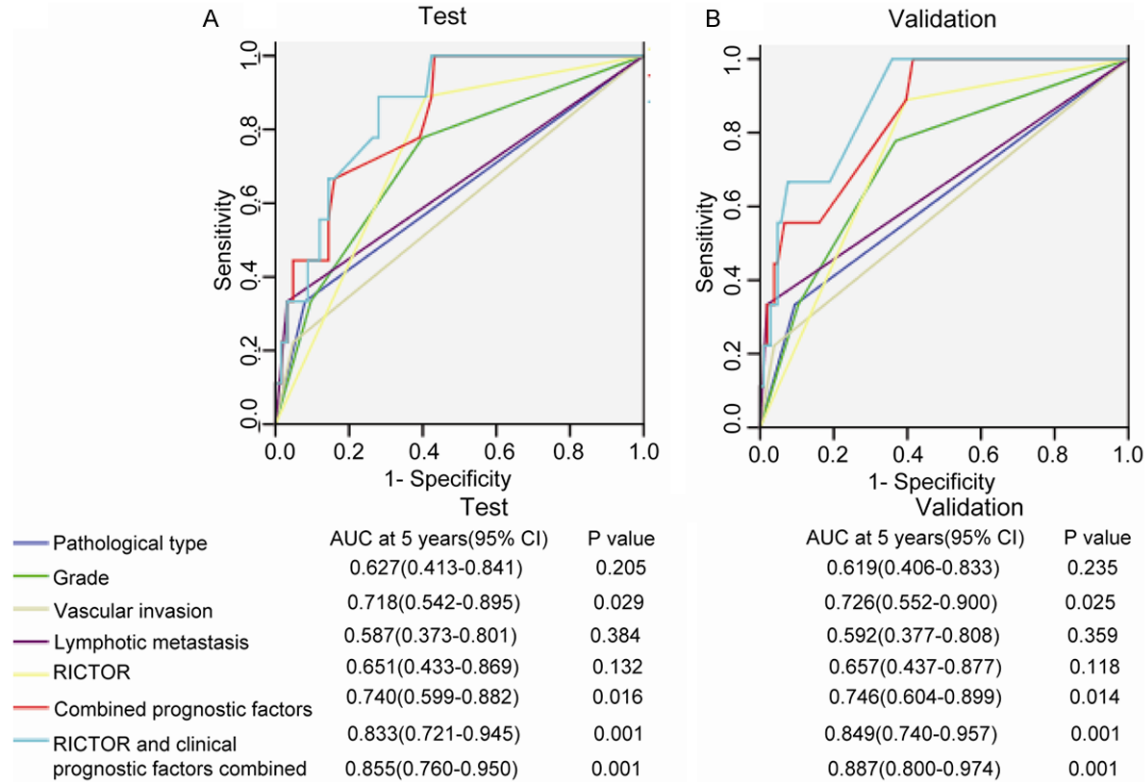|	Test			Validation	
	AUC at 5 years(95% CI)	P value		AUC at 5 years(95% CI)	P value
Pathological type	0.627(0.413-0.841)	0.205		0.619(0.406-0.833)	0.235
Grade	0.718(0.542-0.895)	0.029		0.726(0.552-0.900)	0.025
Vascular invasion	0.587(0.373-0.801)	0.384		0.592(0.377-0.808)	0.359
Lymphotic metastasis	0.651(0.433-0.869)	0.132		0.657(0.437-0.877)	0.118
RICTOR	0.740(0.599-0.882)	0.016		0.746(0.604-0.899)	0.014
Combined prognostic factors	0.833(0.721-0.945)	0.001		0.849(0.740-0.957)	0.001
RICTOR and clinical prognostic factors combined	0.855(0.760-0.950)	0.001		0.887(0.800-0.974)	0.001

Figure 4. ROC curve compares the prognostic accuracy of Rictor with clinicopathological risk factors in all 249 endometrial carcinoma patients by logistic regression. ROC=receiver operator characteristic. AUC=area under curve. A. Comparisons of the prognostic accuracy by Rictor (positive vs. negative), poor prognostic features (with vs without any poor prognostic feature), pathological type (adenocarcinoma vs squamous carcinoma, papillary serous carcinoma and clear cell carcinoma), stage (I vs II vs III, IV), grade (G1 vs G2 vs G3), vascular invasion (yes vs no), lymphatic metastasis (yes vs no), combined clinicopathological prognostic factors alone, or Rictor and clinicopathological prognostic factors combined. *P* values show the AUC at 5 years for Rictor vs the AUC at 5 years for other features.

USA), washed them with deionized water before hematoxylin counterstaining and conducted scoring in the light of the staining intensity and percentage of positive-staining cells. 0-5% scored 0; 6-35% scored 1; 36-70% scored 2; more than 70% scored 3. We designated the final score as negative or positive expression group as follows: score 0, negative expression, score 1-3, positive expression. Two senior pathologists determined the scores independently.

Statistical analysis

We conducted statistical analyses with SPSS 17.0 software (Chicago, IL, USA), compared test and validation cohorts using Fisher exact or χ^2 test for enumeration data, Mann-Whitney U test for ranked data, analyzed the correlation between OS and variables using the Kaplan-Meier method and compared survival curves

using the log-rank test for survival analyses. Univariate analyses were based on a Cox proportional hazard regression. Multivariate analyses were used with the Cox proportional hazard regression model with stepwise manner (forward: condition, entry α=0.05, stay α=0.1). ROC curves were used to compare the prognostic accuracy of Rictor with clinicopathological risk factors in test and validation cohorts. Statistical significance was set at 0.05.

Results

The mRNA expression of Rictor is upregulated in EC

To evaluate the mRNA expression of Rictor, we first detected Rictor mRNA expression in 30 fresh EC tissue samples and 17 fresh normal endometrial tissues using real-time quantitative RT-PCR. Rictor mRNA expression of EC is higher than that of normal endometrium (**Figure**

1A), which suggested that Rictor may be involved in cancer progression in EC.

The Rictor protein expression level is closely correlated with FIGO stage, grade and vascular invasion

To further investigate the expression and significance of Rictor protein in EC, we first selected 134 paraffin-embedded EC (test cohort) tissue samples and 115 (validation cohort) samples to detect Rictor protein expression with immunochemical methods. The rate of Rictor -positive expression was observed in 59 cases (44%) in the test cohort and in 50 cases (43.5%) in the validation cohort (Supplementary Table 1). As shown in **Table 1**, we compared the relationship between Rictor expression and the clinicopathological features. Statistical analyses displayed that the expression level of Rictor protein was closely related with the International Federation of Gynecology and Obstetrics (FIGO) stage, grade and vascular invasion but not with pregnancy, pathological type or lymphatic metastasis in the test and validation cohorts. Moreover, the Rictor positive rate was lower in Grade 1 than Grade 2 or Grade 3 in both cohorts. These results suggest that Rictor might be associated with differentiation in EC. However, the prognostic value of Rictor in human EC remains obscure.

Rictor is an independent prognostic factor for OS of EC patients

Because the GEO database showed that Rictor was associated with patients survival status in EC, we next analyzed the correlation between Rictor expression status and overall survival (OS) using the Kaplan-Meier method and compared the survival curves using the log-rank test for EC in the test and validation cohorts. A univariate analysis demonstrated that the pathological type, FIGO stage, grade, vascular invasion, lymphatic metastasis and Rictor were predictors of OS in the test and validation cohorts (**Figure 2**). In addition, The OS of Rictor-negative group was distinctly better than that of the Rictor-positive one for all 249 samples separated according to pathological type, grade, vascular invasion and lymphatic metastasis (**Figure 3**). Furthermore, multivariate Cox proportional hazards regression analyses showed that vascular invasion and Rictor were independent prognostic factors for EC in the

both cohorts (**Table 2**). These results suggested that Rictor was a risk prognostic factor for the OS of EC patients.

The sensitivity and specificity of Rictor for EC prognosis

To further confirm the prognostic efficiency of Rictor, we compared the sensitivity and specificity of Rictor for EC prognosis by logistic regression. We constructed seven models including Rictor, individual clinicopathological risk factor, combination of clinicopathological risk factors and Rictor combined with clinicopathological risk factors in both cohorts. We performed an ROC curve to compare the prognostic accuracy of Rictor with clinicopathological risk factors. As shown in **Figure 4**, the area under the curve (AUC) was 0.627 in test cohort and 0.619 in validation cohort for pathological type, 0.718 in test cohort and 0.726 in validation cohort for grade, 0.587 in test cohort and 0.592 in validation cohort for vascular invasion, 0.651 in test cohort and 0.657 in validation cohort for lymphatic metastasis, 0.740 in test cohort and 0.746 in validation cohort for Rictor, 0.833 in test cohort and 0.849 in validation cohort for clinicopathological prognostic factors' combination and 0.855 in test cohort and 0.887 in validation cohort for Rictor combined with clinicopathological prognostic factors. Statistical analyses displayed that AUC for Rictor combined with other clinicopathological prognostic factors was higher than any individual factor or the combination of clinicopathological prognostic factors. The results showed that Rictor combined with other clinicopathological prognostic factors had more sensitivity and specificity and was a stronger prognostic model than the single risk factor or their combination.

Discussion

Many studies show that multiple genetic molecules characterize the initiation, development, and poor prognosis of EC [28]. Thus, it is urgent to find new prognostic factors involved in EC to improve the survival of patients.

Rictor, a component of the mTORC2 complex, is closely associated with cancer proliferation, migration, invasion, metastasis, EMT and poor prognosis [17, 22, 23, 29]. For example, Integrin-linked kinase (ILK), a focal adhesion

adaptor, is a serine/threonine protein kinase that regulates cell proliferation, survival and EMT. The ILK/Rictor complex acts as a potential molecular target for preventing/reversing EMT causing fibrosis, cancer progression and metastasis [29]. Although Rictor mostly carries its role as the key component of mTORC2, the recent studies show that Rictor also exerts the mTORC2-independent functions in cell migration. Rictor alone without other components of mTORC2 interacts with the regulators of cell morphology and migration such as actin based molecular motor myosin 1c and the integrin-linked kinase [30, 31]. These data suggested complex roles for Rictor in cancer progression.

Although Rictor is correlated with cancer cell invasion, proliferation, EMT, migration and metastasis, its prognostic value in EC remains unclear. In the study, we found that the expression of Rictor mRNA and protein was higher in EC tissue than in normal endometrium, suggesting that Rictor may be involved in EC progression. Through analysis of the relationship between Rictor and clinicopathologic features of EC patients, we further confirmed Rictor is associated with FIGO stage, grade and vascular invasion; moreover, the Rictor positive rate was higher in the samples of higher stage, low differentiation and vascular invasion. It has been reported that the upregulation of Rictor or EpCAM mRNA expression links with a higher rate of relapse in hepatocellular carcinoma [32]. Taking together, we speculate that Rictor might be associated with cancer proliferation, migration and invasion in EC. It has been reported that the repression of miR-152 is attributed to the upregulation of Rictor in EC [21]. In other words, it is possible that miRNA-mediated Rictor downregulation represses tumor progression. Thus, Rictor evaluated in EC expression potentially offers clinical value in directing personal treatment by targeting miRNAs.

By analysis of the correlation between different variables and overall survival we found that Rictor was an independent prognostic factor for EC. By correlating the expression levels of Rictor with clinicopathological risk factors, we hope to validate the utility of Rictor as a factor to evaluate the prognosis of patients. Some tissue samples did not express high Rictor levels, leading us to suggest that the examination of tissue Rictor levels in combination with the other risk factors would increase the specificity and sensitivity of the prognosis. Thus, we compared the prognostic accuracy of Rictor with other clinicopathological risk factors. Our results showed that Rictor combined with other clinicopathological risk factors was a stronger prognostic model than individual risk factor or their combination.

It has been reported that the myometrium of pregnancy women differently expresses mTOR signaling components including Rictor that could be regulated by progesterone [33]. The status of progesterone receptor (PR) in EC has been confirmed an independent prognostic factor [34]. PR includes two isoforms, PRA and PRB. The absence of one or both were correlated with shorter disease-free or overall survival of EC [35]. PR positive EC patients have more effective with progesterone treatment. Thus, our results provide enlightenment for indentifying whether Rictor combination with PR could exert evaluation of a more accurate prognosis of EC. Clinical research show that Rictor contributes to the resistance of cisplatin in ovarian cancer [36] and increased the sensitivity to vincristine and temozolomide in Glioblastoma [37]. Therefore, our data supplied reference for indentifying whether the enhancement of chemosensitivity by targeting Rictor in EC. Accumulating evidence show Rictor is associated with Glucose metabolism and senescence [38, 39], Metformin potentiates the effects of paclitaxel in EC by inhibition of cell proliferation and modulation the activity of the mTOR pathway [40]. The estrogen-dependent EC is often accompanied with diabetes, obesity and age. This could suggest that we should uncover the correlation which could be released into the circulation at the tumor onset stage associated with systematic cancer status.

However, our current study is limited because of its retrospection and all Chinese patient population. In addition, as an initial report of the potential role of Rictor in EC, further work, such as a prospective study and molecular mechanism, should be performed to clarify issues that were not explored in this study. Moreover, we should treat patients in a different way on the basis of Rictor expression. However, there were only qualitative data of Rictor and no quantitative data in our original source.

In conclusion, we clarified that Rictor expression is associated with FIGO stage, grade and vascular invasion; confirmed that Rictor is an independent prognostic factor in EC patients; moreover, we developed and validated that Rictor combined with other clinicopathological risk factors is a more novel prognostic tool than individual risk factor or clinicopathological risk factors' combination. Thus, Rictor potentially offers clinical value in directing personal treatment for EC patients.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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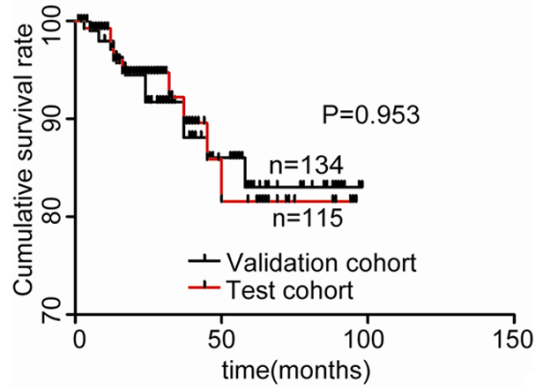
Supplementary Figure 1. Comparisons of OS between in the test and validation cohorts. (P=0.953).

Supplementary Table 1. Background disposition and clinical characteristics of endometrial cancer in test and validation cohort

	No. (%)		
	Test cohort (n=134)	Validation cohort (n=115)	P value
Pregnancy			
No	7 (5.2%)	6 (5.2%)	0.998
Yes	127 (94.8%)	109 (94.8%)	
Pathological type			
Adenocarcinoma	121 (90.3%)	102 (88.7%)	0.680
Squamous carcinoma, papillay serous carcinoma, clear cell carcinoma	13 (9.7%)	13 (11.3%)	
Stage			
I	115 (85.8%)	100 (87%)	0.789
II	11 (8.2%)	9 (7.8%)	
III, IV	8 (6%)	6 (5.2%)	
Grade			
G1	77 (57.5%)	69 (60%)	0.776
G2	42 (31.3%)	32 (27.8%)	
G3	15 (11.2%)	14 (12.2%)	
Vascular invasion			
No	126 (94%)	109 (94.8%)	0.797
Yes	8 (6%)	6 (5.2%)	
Lymphatic metastasis			
No	127 (94.8%)	110 (95.7%)	0.748
Yes	7 (5.2%)	5 (4.3%)	
RICTOR			
No	75 (56%)	65 (56.5%)	0.930
Yes	59 (44%)	50 (43.5%)	