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Immunohistochemical analysis of the mTOR pathway in intrahepatic cholangiocarcinoma

Z. WANG[1, 2, ‡], T. ZHENG[3, ‡], Q. WU[2], J. WANG[1], C. WU[1], J. WANG[1*]

[1]Department of Oncology, Changzheng Hospital, Second Military Medical University, Shanghai 200433, P.R. China; [2]Department of Medical Oncology, First Affiliated Hospital, Bengbu Medical College, Anhui 23300, P.R. China; [3]Cancer Center, Qilu Hospital, Shandong University, Jinan, 250012, P.R. China

**Correspondence: jiejunw2@163.com, jiejunw@csco.org.cn*
‡Contributed equally to this work.

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The aim of the study was to evaluate the expression of activated mammalian rapamycin (mTOR) and its downstream effectors, phosphorylated p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E–binding protein 1 (4EBP1), in intrahepatic cholangiocarcinomas (ICC), in order to strengthen the rationale for targeted therapy using mTOR inhibitors in patients with ICC. p-mTOR (Ser 2448), p-4EBP1 (Thr 70) and p-p70S6K (Thr 389) were detected in 77 primary ICC tumors by immunohistochemistry. High levels of p-mTOR, p-4EBP1 and p-p70S6K expression were defined in 48.1% (37/77), 50.6% (39/77) and 51.9% (40/77) of all tumors, respectively. No significant correlation was observed between mTOR pathway proteins overexpression with clinicopathological characteristics and patient's prognosis, except that high p-p70S6K expression correlated with the poorly differentiated subtype, and high expression of p-4EBP1 predicted poor prognosis in ICC patients and retained an independent prognostic factor in multivariate analysis. In conclusion, our results showed high prevalence of activation of mTOR pathway in ICC tumors, suggesting that a high proportion of ICC patients might benefit from mTOR pathway targeted therapies. In addition, p-4EBP1 phosphorylation at Thr 70 could be a useful prognostic biomarker for ICC patients.

Key words: intrahepatic cholangiocarcinoma, mTOR pathway, phosphorylation, targeted therapy, prognosis

Intrahepatic cholangiocarcinoma (ICC) is a less frequent disease, and ranks second in malignant liver tumors [1]. In the majority of ICC patients, a definitive diagnosis is established at their advanced stages; hence, the prognosis of these patients is still poor despite of extensive resection of carcinomas [2]. The lack of effective optional therapeutic approaches emphasizes the necessity of identifying novel prognostic factors and therapeutic targets for ICC patients.

The mammalian target of rapamycin (mTOR) pathway, which plays a key role in the cellular growth and homeostasis, is frequently altered in various cancers [3]. It has been proved that mTOR can promote protein synthesis and cell proliferation by phosphorylating p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E–binding protein 1 (4EBP1) [4]. Currently, mTOR pathway members have been evaluated as potential therapeutic anticancer targets in several types of cancer [5, 6]. Although some clinical trials using mTOR pathway small molecule inhibitors alone or in combination with other agents are on going, there are few evidences that these molecular-targeted treatments are effective in patients with ICC. The aim of the study was to evaluate the expression status and clinicopahological significance of activated mTOR and its downstream effectors in ICC tumors, in order to strengthen the rationale for targeted therapies using mTOR inhibitors in patients with ICC.

Materials and methods

Patients. Paraffin-embedded tissues from 77 patients with ICC who underwent resection of their tumors at the Changzheng Hospital, Second Military Medical University (Shanghai, China) and First Affiliated Hospital, Bengbu Medical College (Anhui, China), and Qilu hospital Shandong University (Shandong, China) from 1999 to 2006. Having com-

plete clinicopathological and followed-up data were criteria for patients inclusion in the study. None of the patients had received radio/chemotherapy prior to resection. Patients who had distant metastasis at the time of diagnosis were excluded. Overall survival time (OS) was defined as the time from surgical resection to cancer-related death only. Prior to the start, the study was evaluated and approved by the research ethics committees of our institutions, and the patients' informed consents were also obtained. The clinicopathological details were shown in Table 1.

Immumohistochemistry. 4 μm thick of formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated. After endogenous peroxidase activity blocking (3% hydrogen peroxide, 20 min) and antigen retrieval (boiling in 10 mM citrate buffer, pH 6.0), sections were incubated with the primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit mAb against p-mTOR (Ser 2448) (Cell signaling, 1:100 dilutions), rabbit mAb against p-4EBP1 (Thr 70) (Epitomics, 1:100 dilutions) and mouse mAb against phospho-p70S6K (Thr 389) (Cell signaling, 1:100 dilutions). Detection was performed with the DakoCytomation Envision Plus peroxidase system using diaminobenzidine (DAB) chromogen as a substrate followed by counterstaining with hematoxylin. Non-specific rabbit or mouse IgG substituting the primary antibodies served as the negative control.

Immunohistochemistry evaluation. Staining results were assessed in 10 fields (× 200 magnifications). To score a tumor cell as positive, cytoplasmic staining was required for p-mTOR (Ser2448), p-4EBP1 (Thr70) and p-p70S6K (Thr389). The immunostaining was evaluated independently by a specialized pathologist who was blinded towards the patient's clinical features. For the analysis of the correlation between biomarkers, H scores for each staining were calculated by multiplying the intensity of the staining (0 to 3) and the fraction of positively stained tissue score (0 to 100 percent). For each biomarker, the cut-off point was determined by the median score of all cases, and a tumor was considered high expression when H scores were more than the median scores, as described previously [7].

Statistical analysis. Chi-square test and Fisher's exact test were used to test relationships between the expression of biomarkers and clinical/pathological factors. Kaplan–Meier curves and log-rank test were used to assess the differences in overall survival between positive and negative groups. Prognostic factors identified were further analysized in the multivariate analysis by a Cox proportional hazard model. Two-sided p values of 0.05 or less were considered statistically significant. SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistic analysis.

Results

High levels of p-mTOR, p-4EBP1 and p-p70S6K expression were detected in 48.1% (37/77), 50.6% (39/77) and 51.9% (40/77) of all ICC tumors, respectively. Immunostaining of p-mTOR was cytoplasmatic and partly membranous, while p-p70S6K and p-4EBP1 was positive in the cytoplasm of cancer cells. And p-mTOR, p-4EBP1 and p-p70S6K showed no immunohistochemical staining in the surrounding stroma (Figure 1). In quantitative immunohistochemistry (by H score)

Table 1. Clinicopathological characteristics of the patients with ICC according to the activiation of mTOR pathway.

	Total	p-mTOR expression		P	p-4EBP1 expression		P	p-p70S6K expression		P
		High n=37	Low N=40		High n=39	Low n=38		High n=40	low n=37	
Age (y)										
<65	60	28	32	0.648	27	33	0.063	31	29	0.926
≥65	17	9	8		12	5		9	8	
Gender										
Female	24	10	14	0.451	12	12	0.94	11	13	0.470
Male	53	27	26		27	26		29	24	
Tumor stage										
T1-T2	23	12	11	0.637	11	12	0.746	10	13	0.332
T3	54	25	29		28	26		30	24	
Lymph node status										
Negative	40	21	19	0.417	21	19	0.736	22	18	0.577
Positive	37	16	21		18	19		18	19	
Tumor grade										
G1-2	38	15	23	0.137	17	21	0.306	15	23	0.031
G3	39	22	17		22	17		25	14	
Recurrence										
No	47	20	27	0.227	23	14	0.466	26	21	0.459
Yes	30	17	13		16	14		14	16	

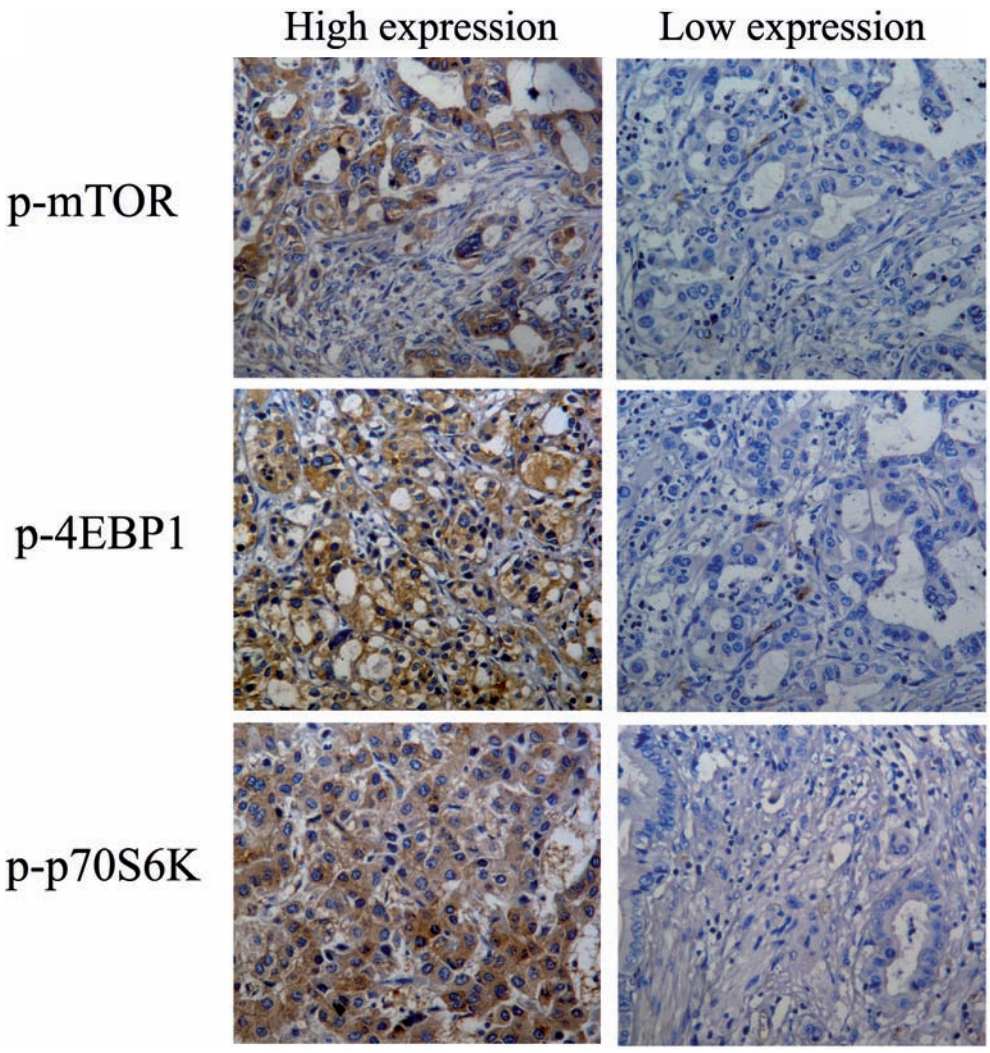

Figure1. Immunohistochemical staining of p-mTOR (Ser2448), p-4EBP1 (Thr70) and p-p70S6K (Thr389) in intrahepatic cholangiocarcinoma tissues.

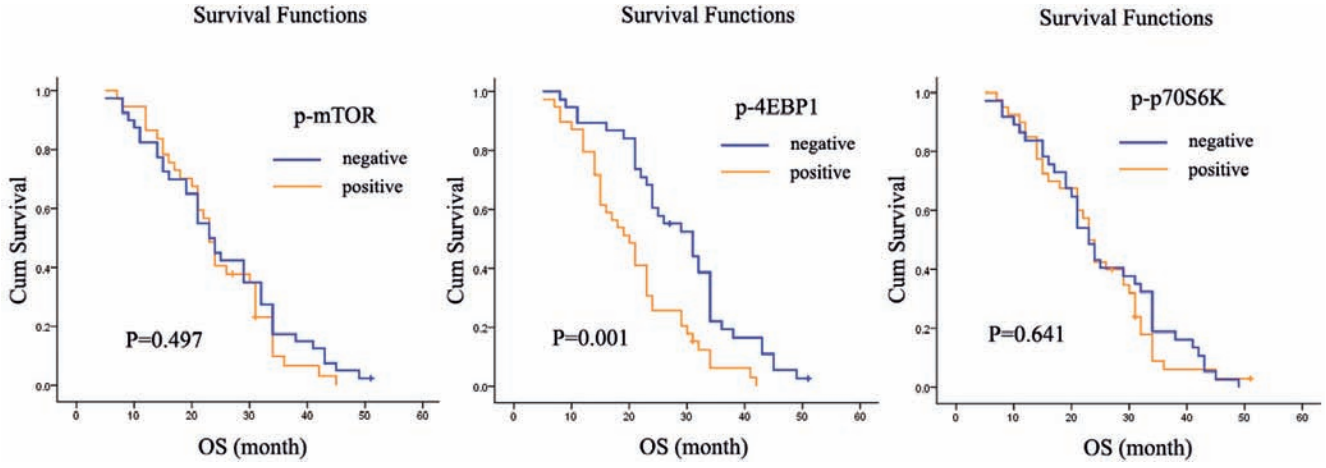

Figure 2. Kaplan-Meier survival analysis of intrahepatic cholangiocarcinoma according to p-mTOR (Ser2448), p-4EBP1 (Thr70) and p-p70S6K (Thr389) expression status.

analysis, we found no association among the mTOR pathway proteins.

As seen in Table 1. No significant correlation was observed between p-mTOR and p-4EBP1 overexpression with clinicopathological factors, including age, gender, tumor stage, lymph node status and grade. Tumors with high p-p70S6K expression had a trend to higher grade than those with low expression (62.5% vs. 37.8%), a statistical significance was reached (P = 0.031). However, high p-p70S6K expression was also not associated with the other clinicopathological factors.

Table 2 demonstrated the univariate analysis of the immunohistochemical results of the mTOR pathway proteins with regard to overall survival (OS). As seen in figure 2, a tendency towards shorter overall survival was observed in patients with high p-4EBP1 expression, but not with p-mTOR and p-p70S6K overexpression. In addition, multivariate analysis also showed that high p-4EBP1 expression was a significant independent prognostic factor for ICC patients (table 2).

Discussion

This study investigated three activated mTOR pathway proteins, p-mTOR, p-4EBP1 and p-p70S6K, in intrahepatic cholangiocarcinomas (ICC), in order to evaluate which subgroup of ICC patients might potentially benefit from mTOR pathway targeted inhibitors.

Our study found that high level of mTOR phosphorylated at Ser2448 in 48.1% of the ICC tumors. It is believed that mTOR phosphorylated at Ser2448 is induced via the PI3 kinase/Akt signaling pathway and autophosphorylation [8]. Previous studies have demonstrated that mTOR phosphorylation at Ser2448 would play a key role in cellular growth and homeostasis, and is frequently altered in several tumors [9, 10]. Phosphorylation of mTOR in hepatocellular carcinoma (HCC) ranged from 15 to 41% [11]. Chung et al. [12] and Herberger et al. [13] found that p-mTOR was positive in 83.7% of the extrahepatic cholangiocarcinomas and 64% of biliary tract adenocarcinomas. Consistent with biliary tract adenocarcinomas, our data also indicated no association between mTOR phosphorylation with any of the clinical or pathologic variables. However, p-mTOR failed to predict overall survival of the patients, which is inconsistent with that in biliary tract adenocarcinomas.

We further detected the expression of two downstream effectors of activated mTOR pathway, p-4EBP1 and p-p70S6K, in ICC tumors. Similar with p-mTOR, about half of the ICC tumors indicated high p-4EBP1 and p-p70S6K expression, and no association was observed between p-4EBP1 and p-p70S6K expression with any of the clinical or pathologic variables, except that high p-p70S6K expression correlated with poorly differentiated tumors.

Previous studies have found that phosphorylation of p-4EBP1 at the priming sites (Thr37/46) was not an independent prognostic factor for many cancers such as ovarian cancers [14]. In this study, we used an antibody against p-4EBP1 at the subsequent phosphorylation site (Thr 70), which is potentially involved in the role of p-4EBP1 during carcinogenesis, including release of the eIF-4E and stimulation of cap-dependent translation of mRNA. Our overall survival analysis indicated that overexpression of p-4EBP1 was closely linked to poor prognosis of the ICC patients, and retained as an independent prognostic factor after adjustment for other clinical and pathologic factors in multivariate analysis. These findings suggest that phosphorylation at Thr 70 may be an evidence for activation of p-4EBP1 which impacts the prognosis of cancer patients.

Several studies have also identified that p-mTOR was not associated with expression of its downstream effectors, which is consistent with our findings. Recently, No et al. [15] found that p-4EBP1 expression was correlated with p-p70S6K expression in ovarian cancer. In this study, analysis of correlations among mTOR pathway proteins using immunostaining scores revealed no significant association between p-mTOR, p-4EBP1 and p-p70S6K. The discrepancy may be due to the different antibodies used and existing other regulatory mechanisms of mTOR proteins phosphorylation [16, 17].

In conclusion, our results showed the high prevalence of activation of mTOR pathway in ICC tumors, which suggests ICC patients might benefit from the personalized therapeutic

Table 2. Univariate and multivariate analysis for the prognosis

Variable	Univariate survival analyses		Multivariate survival analysis	
	HR for death (95% CI)	P	Adjusted HR for death (95% CI)	P
Age	1.258 (0.710-2.215)	0.427	1.100 (0.588-2.055)	0.766
Gender	0.872 (0.531-1.433)	0.589	0.794 (0.463-1.361)	0.401
Tumor stage	1.355 (0.815-2.252)	0.241	1.100 (0.548-2.209)	0.788
Lymph node status	1.616 (1.019-2.563)	0.041	2.045 (1.087-3.845)	0.026
Tumor grade	1.106 (0.694-1.763)	0.670	0.967 (0.581-1.612)	0.899
Recurrence	1.096 (0.676-1.777)	0.709	1.400 (0.788-2.488)	0.252
High p-mTOR expression	1.176 (0.737-1.874)	0.497	1.306 (0.757-2.251)	0.337
High p-4EBP1 expression	2.285 (1.406-3.715)	0.001	3.105 (1.751-5.505)	<0.001
High p-p70S6K expression	1.126 (0.709-1.789)	0.641	0.842 (0.490-1.446)	0.532

strategies with inhibitors of the mTOR pathway. In addition, p-4EBP1 phosphorylation at Thr 70 could be a useful biomarker for the prognostic evaluation of ICC patients.

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