ORIGINAL PAPER

# The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma

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**Abstract** *Objective* The mammalian target of rapamycin (mTOR) pathway, an important regulator of multiple cellular functions including proliferation, differentiation, tumorigenesis, and apoptosis, is up-regulated in many cancers. It has achieved considerable importance. This study was conducted to determine the status of the mTOR pathway in human hepatocellular carcinoma (HCC) and to investigate its relationship with the prognosis of HCC. *Methods* PTEN, pAkt, p27, and pS6 expression in cryo-sections gathered from 528 cases with HCC by the method of immunohistochemistry. Kaplan–Meier survival and Cox regression analyses were performed to evaluate the prognosis of HCC. *Results* The mTOR pathway was more significantly altered in high-grade tumors, and tumors with poor prognostic features. Especially, pAkt and cytoplasmic p27 expression showed the strongest associations with pathological parameters of HCC. Statistical analysis showed that HCC patients expressing pAkt, PTEN, cytoplasmic p27, and pS6 have different overall survival rates relative to those not expressing these proteins. Cox multi-factor analysis showed that tumor differentiation ($P = 0.006$), vascular invasion ($P = 0.028$), TNM stage ($P = 0.005$), pAkt ($P = 0.021$), PTEN ($P = 0.003$), p27 ($P = 0.018$) and pS6 ($P = 0.002$) were independent prognosis factors for HCC. *Conclusion*: Expression of the mTOR pathway components, which are related with the transferability and invasive capacity of HCC cells, may be used as prognostic indicators in HCC.

**Keywords** Hepatocellular carcinoma · mTOR · S6 · Akt · PTEN · p27 · Prognosis

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## Abbreviation

mTOR    Mammalian target of rapamycin
HCC     Hepatocellular carcinoma

## Introduction

Human hepatocellular carcinoma (HCC) is one of the most common solid tumor entities worldwide and a leading cause of cancer death in China [1]. As with other cancers, HCC are characterized by an obvious multistage process of tumor progression. To date, HCC is refractory to conventional chemotherapeutics, and thus surgical resection, or liver transplantation, remain the only potential curative treatment options [2, 3]. Therefore, sensitive and specific biomarkers that can provide valuable information for the diagnosis and treatment of HCC are needed.

Recently, significant achievements in the basic research have led to a greater knowledge of the underlying signaling pathways in HCC, including the mammalian target of rapamycin (mTOR) pathway. mTOR, also known as FRAP, RAFT1, or RAPT1, has been shown to regulate mitogen-stimulated protein synthesis and cell cycle progression [4, 5]. Receiving input from multiple signals, including growth factors, hormones, nutrients, and other stimulants or mitogens, the mTOR pathway activates protein synthesis by phosphorylating key translation regulators such as ribosomal S6 kinase [6]. The mTOR pathway also contributes to many other critical cellular functions, including protein degradation and angiogenesis [7]. Inhibition of mTOR leads to G1 arrest of many malignant cell lines, and currently analogs of rapamycin are being investigated as cancer therapeutic agents [8]. Some studies also suggest that mTOR may be a cellular context-dependent, pleiotropic regulator of apoptosis [9, 10]. Hence, use

of inhibitors of this pathway represents a new strategy for the targeted treatment of HCC.

Unfortunately, with mTOR inhibitors, it is unclear what clinical parameters and/or molecular pathways will predict which patients will derive the greatest benefit. These agents might have clinical activity only in selected patients in whose diseases this pathway drives their biology. An enhanced ability to predict survival would allow patients most likely to benefit from mTOR targeting therapies to be selected. For patient selection, a wide spectrum of molecular biomarkers is currently available including upstream and downstream targets of mTOR. Therefore, the present study was undertaken to evaluate the prognostic value of this pathway in HCC and validate experimental data. This has important implications for designing effective clinical trials and selecting the right patients for molecular therapy. For these goals, we carried out an immunohistochemical study of the mTOR upstream and downstream targets phosphorylated Akt (pAkt), phosphorylated S6 ribosomal protein (pS6), and p27, as well as the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome), and correlated our findings with pathological parameters and survival.

## Materials and methods

### Patients and tissue samples

Fresh HCC samples and their adjacent non-tumorous samples were obtained from 528 consecutive patients, who underwent curative liver resection between January 2000 and April 2005 at Xiangya Hospital, Changsha, China. HCC diagnosis was based on World Health Organization (WHO) criteria [11]. Tumor differentiation was defined according to the Edmondson grading system [12]. Liver function was assessed using the Child-Pugh scoring system [13]. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer [14]. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. Portions of the tissues were embedded in OCT compound (Miles, Elkhard, IN), frozen in liquid nitrogen, cut into cryostat sections and stored at −40°C for immunostaining. Standard light microscopic evaluation of each section stained with hematoxylin-eosin was performed for histological diagnosis. 20 normal liver tissues were collected from healthy living donors in Xiangya Hospital, Changsha, China.

The median age of the HCC patients was 65.8 years (range 35–81 years), and the male:female ratio was 6.65:1 (459:69). Among these patients, 162 were hepatitis B surface antigen (HBs-Ag) positive, 215 were hepatitis C antibody (HCV-Ab) positive, 30 were positive for both HBs-Ag and HCV-Ab, 115 were negative for both HBs-Ag and HCV-Ab in the presence of chronic liver disease, and the remaining 6 without chronic liver disease were negative for both HBs-Ag and HCV-Ab. With regard to background liver disease, 273 and 142 patients had chronic hepatitis and liver cirrhosis, respectively, and 113 patients had a normal liver.

For the analysis of survival and follow-up, the date of surgery was used to represent the beginning of the follow-up period. All the patients who died from diseases other than HCC or from unexpected events were excluded from the case collection. Follow-ups were terminated until April 11, 2008. The median follow-up was 42 months (range, 3–98 months). Treatment modalities after relapse were given according to a uniform guideline as described.

The study was approved by the Research Ethics Committee of Xiangya Hospital, Changsha, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

### Reagents

Rabbit monoclonal antibody phospho-Akt Ser473 (Cell Signaling, Danvers, Mass) at a concentration of 1.5 lg/ml was used to stain for pAkt. Immunostaining for PTEN was performed using rabbit polyclonal antibody PN37 (Zymed, San Francisco, California) at 2 lg/ml. Mouse monoclonal antibody SX53G8 (Dako) was used at a concentration of 8 lg/ml to stain for p27. Staining for pS6 was performed with polyclonal rabbit antibody phospho-S6 ribosomal protein Ser 235/236 (Cell Signaling) at a concentration of 0.125 lg/ml. A commercially available immunohistochemical staining kit (Vectastain Elite ABC kit) was obtained from Vector Laboratories, Burlingame, CA.

### Immunohistochemistry analysis

Peroxidase (DAB) immunohistochemistry staining was performed by DAKO EnVision System (Dako Diagnostics, Zug, Switzerland). Briefly, following a brief proteolytic digestion and a peroxidase blocking of tissue slides, the slides were incubated overnight with the primary antibody against respective target proteins at 4°C. After washing, peroxidase-labeled polymer and substrate-chromogen were then employed in order to visualize the staining of the interested proteins. Non-neoplastic liver tissues were used as control tissues and non-immune IgG was also used as negative control antibody for immunohistochemical staining.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological

parameters and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells showing immunoreactivity on the cell membranes and cytoplasm in ten representative microscopic fields was counted and the percentage of positive cells was calculated. Given the homogenicity of the staining of the target proteins, tumor specimens were scored in a semi-quantitative manner based on the percentage of tumor cells that showed immunoreactivity. The evaluation of expression involved subcellular localization and degree of reactivity (staining intensity: 0 = negative, 1 = weak, 2 = moderate, 3 = strong, and staining frequency: percentage of positive cells).
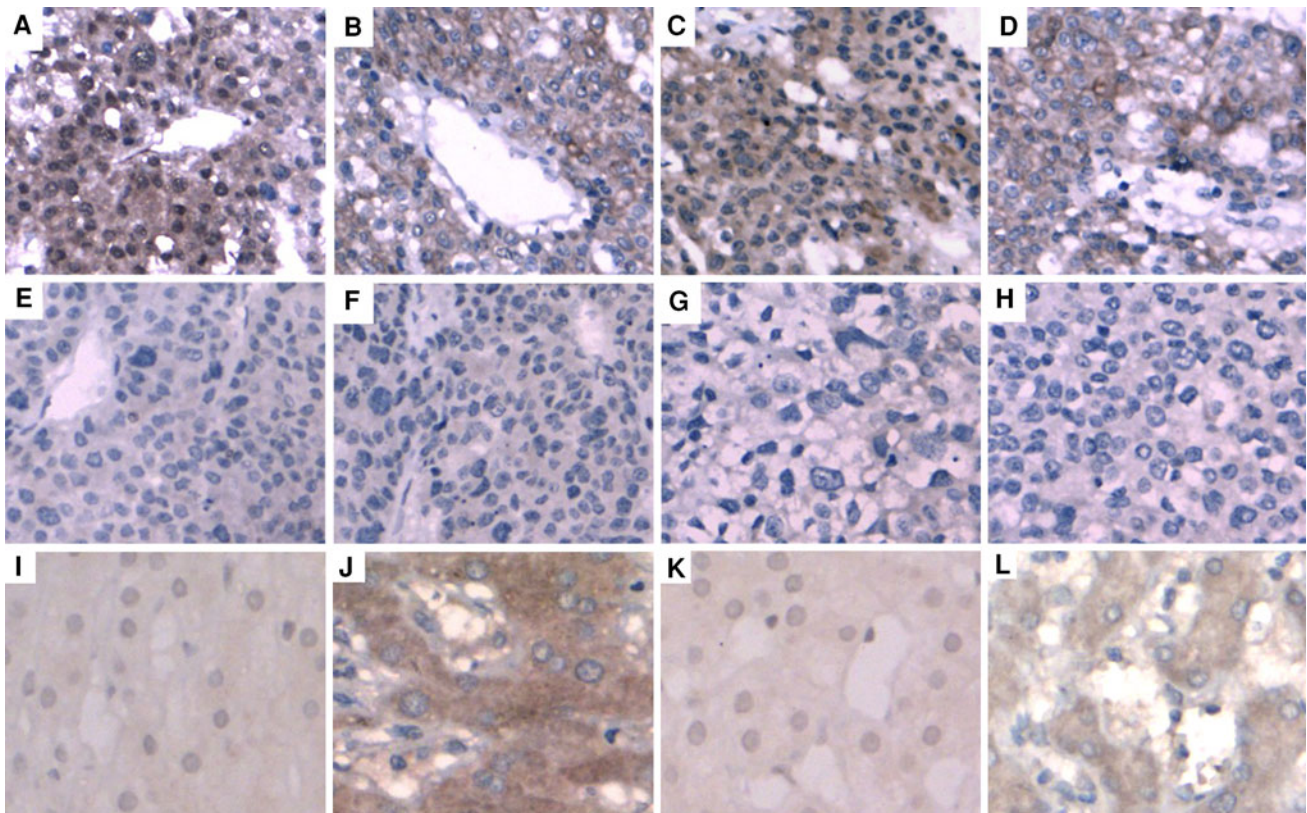
## Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. The associations between protein expression and different clinical parameters were evaluated using the nonparametric Mann–Whitney U-test (when two independent groups were compared) or the Kruskal–Wallis test (when more than two independent groups were compared). In the analysis of HCC morbidity for all patients, we used the Kaplan–Meier estimator and univariate Cox regression analysis to assess the marginal effect of each factor. The differences between groups were tested by log-rank analyses. The joint effect of different factors was assessed using multivariate Cox regression. Differences were considered statistically significant when $P$ was less than 0.05.

## Results

### Immunohistochemical expression and localization of pAkt, PTEN, p27, and pS6

The expression and localization of pAkt, PTEN, p27, and pS6 in the 528 HCC and 20 normal liver tissues were examined using immunohistochemical analysis. They expressed in all the tissues with varying malignancy. Anti-pAkt staining was seen in both cytoplasmic and nuclear cellular staining compartments in 92.99% (491/528) of the tumors (Fig. 1a). The anti-PTEN antibody stained in the



Fig. 1 Immunohistochemical staining for pAkt, PTEN, p27, and pS6 in the 528 HCC tissues (original magnification ×400). **a–d**, pAkt, PTEN, p27, and pS6 positive staining is in HCC tissues, respectively; **e–h**, pAkt, PTEN, p27, and pS6 negative staining with non-immune IgG in HCC tissues, respectively; **i–l**, pAkt, PTEN, p27, and pS6 positive staining is in normal liver tissues, respectively

cytoplasmic cellular compartment in 76.70% (405/528) of the HCCs (Fig. 1b). Cellular staining with anti-p27 antibody occurred in both nuclear and cytoplasmic compartments in 90.10% (480/528) of the tumors (Fig. 1c). Anti-pS6 staining was only seen in the cytoplasmic cellular compartment. Its staining in tumors was noted in 88.26% (466/528) of the HCCs (Fig. 1d). The antibodies to pAkt, p27, and pS6 generally increased positive staining in cancerous tissues compared with matched normal tissues (Fig. 1i, k, and l), but tumors showed a lower PTEN expression than normal liver tissues (Fig. 1j). They were not present in negative controls with non-immune IgG (Fig 1e–h).

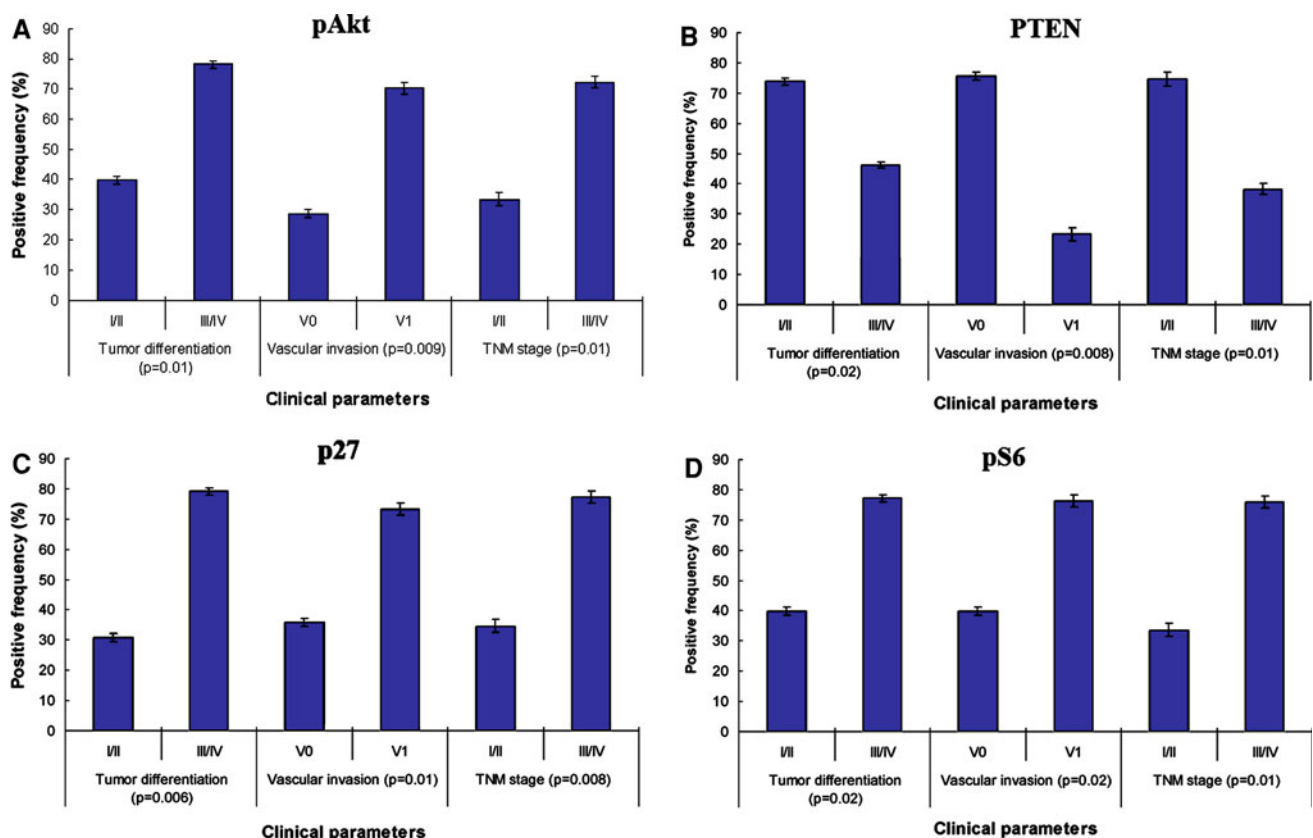## Association of pAkt, PTEN, p27, and pS6 expression with the clinicopathological characteristics of the HCCs

There are high intercorrelation between staining intensity and frequency (pAkt: R = 0.89, p27: R = 0.90, PTEN: R = 0.82, pS6: R = 0.91, each $P < 0.001$). Hence, the staining frequency was used for the subsequent analyses.

Figure 2 summarizes the association of pAkt, PTEN, p27, and pS6 immunostaining with the clinicopathological parameters of the HCCs. Significantly higher expressions of pAkt, p27, and pS6 were observed in tumors with poor tumor differentiation ($P = 0.01$, 0.006, and 0.02, respectively), higher TNM stage ($P = 0.01$, 0.008, and 0.01, respectively), and vascular invasion ($P = 0.009$, 0.01, 0.02). However, PTEN expression was higher in tumors with well tumor differentiation ($P = 0.02$), lower TNM stage ($P = 0.01$), and non-vascular invasion ($P = 0.008$).
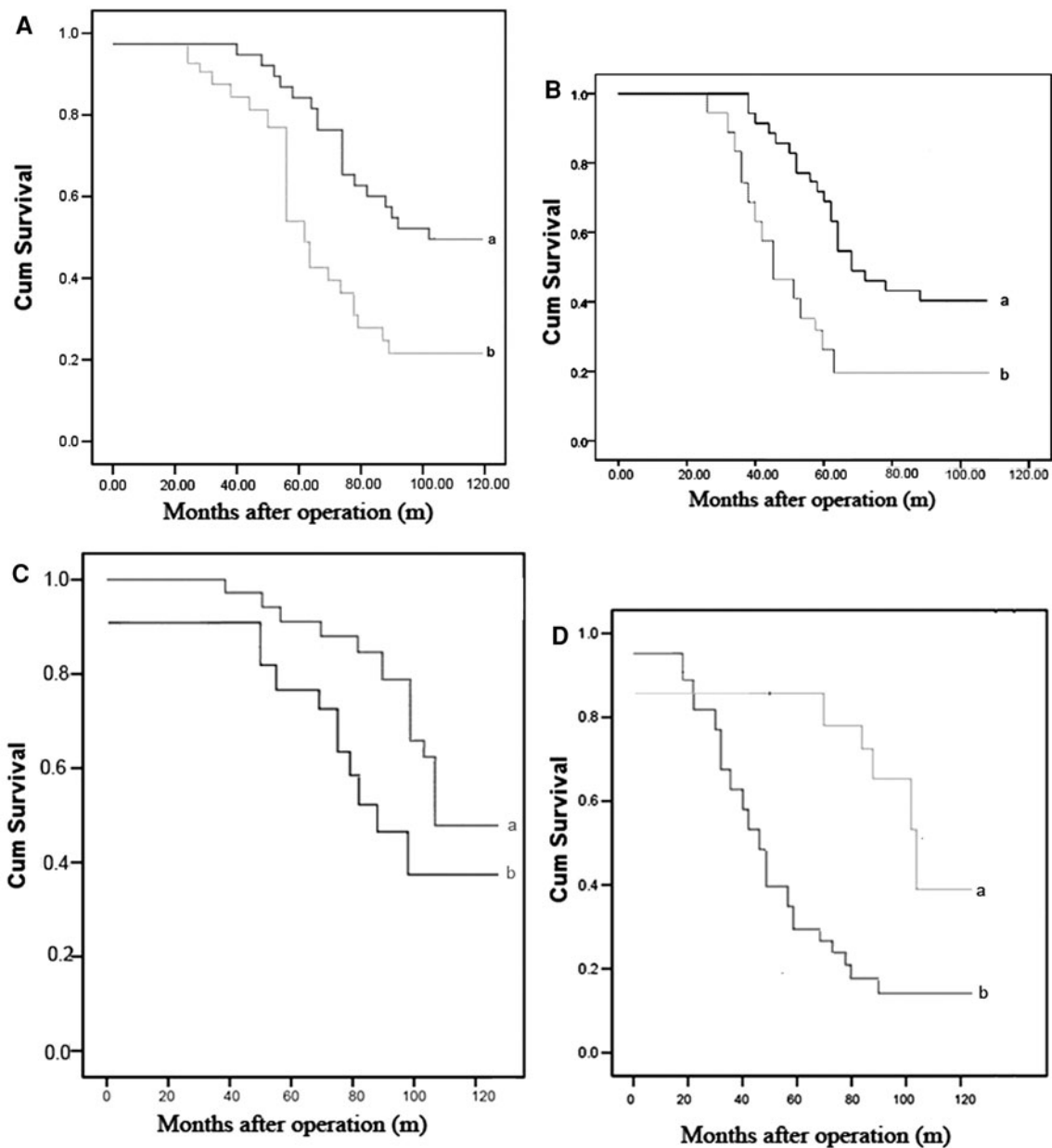
## Prognostic values of pAkt, PTEN, p27, and pS6 expression in HCCs

According to the criterion of the previous literature [15] and our staining results, we identified expression frequencies of pAkt (42%), PTEN (10%), p27 (70%), and pS6 (70%), respectively, as ideal cutoff values for further patient stratification. The corresponding $P$-values for the dichotomized patients, calculated with the log-rank test, were 0.005, 0.01, 0.001, and 0.02, respectively (Fig. 3). Notably, high pAkt, p27, and pS6 expression was associated with poor prognosis, whereas high PTEN expression was associated with favorite prognosis. In univariate Cox regression analysis, tumor differentiation, vascular invasion, TNM stage, pAkt, PTEN, p27 and pS6 were all predictors of overall survival of the HCCs. In multivariate Cox regression analysis, tumor



Fig. 2 Association of tumor differentiation, vascular invasion, TNM stage and mean expression frequencies of pAkt, PTEN, p27, and pS6. The Kruskal–Wallis test was used to compare expression among tumor differentiation and TNM stage. Mann–Whitney U-test was applied to compare expression between the HCCs with vascular invasion and those without vascular invasion

**Fig. 3** Kaplan–Meier survival curves for pAkt (**A**; 'a' refers to positive expression rate <42%, 'b' refers to positive expression rate >42%), PTEN (**B**; 'a' refers to positive expression rate >10%, 'b' refers to positive expression rate <10%), p27 (**C**; 'a' refers to positive expression rate <70%, 'b' refers to positive expression rate >70%), and pS6 (**D**; 'a' refers to positive expression rate <70%, 'b' refers to positive expression rate >70%) expression in the HCC patients. Survival was significantly better for patients with EMMPRIN-/MMP2-expression than those with positive expression ($P < 0.05$)

differentiation ($P = 0.006$), vascular invasion ($P = 0.028$), TNM stage ($P = 0.005$), pAkt ($P = 0.021$), PTEN ($P = 0.003$), p27 ($P = 0.018$) and pS6 ($P = 0.002$) were independent prognostic factors (Table 1).

## Discussion

Although the mTOR pathway has been linked to various cellular functions, including activation, proliferation, pathological angiogenesis, and metastasis, to the best of our knowledge, this is the first report to investigate the association of this pathway with clinical parameters and prognosis of HCC. In the present study, we confirmed that expression of pAkt, p27, and pS6 was markedly increased in HCC tissues compared with that in adjacent non-tumor and normal liver tissues, which express higher PTEN than tumors. Moreover, we showed that over-expression of pAkt, p27, and pS6 was involved in poor differentiation, vascular invasion and high TNM stage of HCC, which was

**Table 1** Multivariate Cox regression analysis for HCC patients

| | Wald[a] | df | P | Exp (B) | 95.0% CI for exp(B) | |
|---|---|---|---|---|---|---|
| | | | | | Lower | Upper |
| Tumor differentiation | 21.11 | 1 | 0.006 | 2.15 | 1.32 | 1.51 |
| Vascular invasion | 10.94 | 1 | 0.028 | 4.98 | 1.46 | 12.01 |
| TNM stage | 21.03 | 1 | 0.005 | 2.32 | 1.11 | 3.09 |
| pAkt | 12.19 | 1 | 0.021 | 2.96 | 1.18 | 10.79 |
| PTEN | 28.69 | 1 | 0.003 | 2.61 | 1.69 | 3.98 |
| p27 | 16.33 | 1 | 0.018 | 1.69 | 1.12 | 2.55 |
| pS6 | 29.81 | 1 | 0.002 | 3.86 | 1.71 | 8.76 |

[a] Wald refers to Chi-square value

df Degree of freedom

TMN stage Tumor-node-metastasis stage

also contrary to PTEN. In line with recent studies, our results revealed that HCC cells in which pAkt, p27, and pS6 was down-regulated showed decreased invasion and metastasis. Moreover, high PTEN HCC cells had less serious clinical parameters. The above results lend support to the notion that the mTOR pathway does make a substantial contribution to tumor cell invasion and metastasis.

A credible result identified in this study is the relationship between high pAkt, p27, and pS6 expression and poor prognosis of HCC patients, which was opposite to PTEN. This finding may be supported by the fact that aggressive histopathological characteristics, such as poor differentiation, vascular invasion, and high TNM staging of HCC were significantly more frequent in patients with high pAkt, p27, and pS6 expression and low PTEN expression than in those with low pAkt, p27, and pS6 expression and high PTEN expression. This indicates that pAkt, PTEN, p27, and pS6 protein expression may be powerful prognostic indicators for HCC.

HCC is a primary cancer of the liver that is predominant in developing countries, with nearly 600,000 deaths each year worldwide [16, 17]. A variety of risk factors have been associated with HCC. Such factors include exposure to hepatitis viruses, vinyl chloride, tobacco, foodstuffs contaminated with aflatoxin B1, heavy alcohol intake, nonalcoholic fatty liver disease, diabetes, obesity, diet, coffee, oral contraceptives, and hemochromatosis [18]. In general, these factors vary according to the geographical region, adding complexity to the extrapolation of data obtained from one region and applying it to others [19, 20]. The growing incidence of HCC has generated intense research to understand the physiological, cellular, and molecular mechanisms of the disease with the hope of developing new treatment strategies [21, 22]. The growing incidence of HCC has generated intense research to

understand the physiological, cellular, and molecular mechanisms of the disease with the hope of developing new treatment strategies.

An understanding of the central role of mTOR and its relevance for tumors requires an appreciation for multiple related pathways, including the mTOR pathway. mTOR can be activated in response to a diverse set of stimuli, including various growth factors as well as certain nutrients and amino acids [23]. One of the first pieces of evidence that the PI3K/Akt pathway might play a role in mTOR activation came from studies using pharmacologic inhibitors of PI3 K to block mTOR activation in response to serum challenge [24]. While mTOR has several functions, it appears that regulation of translation is the best studied in relation to oncogenesis. To this end, phosphorylation of two mTOR substrates regulates protein synthesis, i.e, ribosomal S6 kinase (S6K). S6K plays an important role in the regulation of cell growth, cell cycle progression, and cell proliferation, by enhancing the translation of components required for protein synthesis, a process known as "ribosome biogenesis" [25]. It is felt that S6K does this by enhancing the translational efficiency of mRNA transcripts containing terminal oligopyrimidine tracts at their 5′ end [26]. PTEN is a tumor suppressor protein that is encoded by the tumor suppressor gene PTEN. It has been shown that PTEN loss occurs during carcinogenesis and is associated with adverse prognosis in RCC [27]. In addition, Neshat et al. [28] were able to demonstrate that PTEN-deficient tumors have an increased sensitivity to temsirolimus. p27 a member of the CIP/KIP family, causes G1 arrest by inhibiting the activities of G1 cyclin–cyclin-dependent kinases [29]. As a negative regulator of the cell cycle, p27 is a new class of tumor suppressor controlling cell cycle progression. Recent findings indicate that the decreased expression of p27 protein is associated with high aggressiveness and a poor prognosis in a large variety of cancers, including classical HCC. Because the growth restraining activity of p27 depends on its nuclear localization, the aberrant localization may impair its tumor suppressor functions [30]. Thus, the mTOR pathway is increasingly activated in tumors with cytoplasmic mislocalized p27.

In conclusion, the mTOR pathway appears to be very important in patients with HCC. We were able to demonstrate highly significant associations with pathological parameters and, more important, with survival. The results also suggest that the mTOR pathway is more significantly altered in poor differentiation, vascular invasion, and high TNM stage tumors, and tumors with poor prognostic features. Thus, these patients with a highly activated mTOR pathway may benefit most from therapy specifically targeting this pathway. This study is hypothesis generating, and that further prospective analysis would be worth doing.

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