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ORIGINAL PAPER

mTOR/p70S6K Signal transduction pathway contributes to osteosarcoma progression and patients' prognosis

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Abstract The mTOR/p70S6K signal transduction pathway plays a key role in the regulation of cancer cells' survival and proliferation. However, its roles in osteosarcoma, which is one of the most rapidly growing sarcomas, remain unknown. This study investigated for the first time the correlation between the mTOR/p70S6K signal transduction pathway in human osteosarcoma and patients' prognosis. The expression patterns of mTOR and p70S6K in paraffin-embedded specimens gathered from 65 patients with primary osteosarcoma were detected by the method of immunohistochemistry using antibodies against mTOR and p70S6K. Kaplan–Meier survival and Cox regression analyses were performed to evaluate the prognosis of patients. Immunostaining revealed that the mTOR/p70S6K signal transduction pathway is activated in human osteosarcoma. Additionally, positive expression of mTOR and p70S6K proteins was significantly correlated with surgical stage, metastasis pattern and percentage of dead cells of osteosarcoma. Moreover, in univariate analysis, surgical stage, metastasis pattern and percentage of dead cells, mTOR and p70S6K expression showed significant influence on overall survival (OS) and disease-free survival (DFS). In multivariate analysis, surgical stage (IIA vs. IIB/III), metastasis pattern (without vs. with), percentage of dead cells ($\geq$90 vs. <90%), mTOR expression pattern (negative vs. positive) and p70S6K expression pattern (negative vs. positive) were significant for DFS and OS. Our results demonstrate the correlation of mTOR and p70S6K expression patterns with the oncological progression of osteosarcoma patients, suggesting the prognostic significance of the mTOR/p70S6K signal transduction pathway in osteosarcoma patients, which may lay a foundation for making further investigations on the mTOR/p70S6K signal transduction pathway as a potential target for osteosarcoma therapy.

Keywords Mammalian target of rapamycin · p70 S6 kinase · Clinical pathology · Osteosarcoma · Prognosis

Abbreviation

mTOR	Mammalian target of rapamycin
p70S6K	p70 S6 kinase

Introduction

Osteosarcoma, the most common primary malignancy of bone, predominantly afflicts young people in their second and third decades of life. It carries a 5-year survival rate near 60–70% in patients with no clinically detectable metastases at presentation [1]. Despite recent advances in multimodality treatments consisting of aggressive adjuvant chemotherapy and wide tumor excision, 40–50% patients with osteosarcoma may develop recurrent disease, with the

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lung being the most common site of distant recurrence [2]. Although, the histologic response of the tumor to pre-operative chemotherapy, surgical staging are the most powerful predictor of outcome, but one must wait until surgical resection to assess the histologic response of the tumor to preoperative chemotherapy, which often takes 2–3 months [3]. Therefore, it is of great significance to search for sensitive and specific markers that can stratify patients early into low- and high-risk groups, according to their potential for disease progression. With intensive efforts, several molecular markers, such as up-regulated gene 4 [4], vascular endothelial growth factor [5], extracellular matrix metalloproteinase inducer [5] and surviving [6] have been investigated as prognostic indicators in this disease. However, molecular prognostic indicators for disease progression are still few in number.

Mammalian target of rapamycin (mTOR), an evolutionarily conserved serine-threonine protein kinase, mainly controls protein synthesis via phosphorylation of its downstream targets [7]. It has been demonstrated that the components of the mTOR signal transduction pathway are deregulated in several kinds of human tumors with gain-or loss-of-function mutants leading to neoplastic transformation [8]. In mammals, the ribosomal S6 kinases S6K1 and S6K2, and the eukaryotic initiation factor (eIF4E)-binding protein 1 (4E-BP1) are the two best-characterized targets of mTOR [9]. mTOR activation leads to phosphorylations of S6K1/2 and 4E-BP1. The latter releases from the cap-dependent translation initiation factor eIF4E, ultimately resulting in enhanced translation from subset of genes required for cell growth [10]. The p70S6 kinase (p70S6K), a mitogen-activated serine/threonine kinase, plays an important role in the regulation of cell cycle, growth and survival. p70S6K, encoded by RPS6KB1, is located downstream of mTOR signal transduction pathway and is activated by 3-phosphoinositide-dependent protein kinase 1 and mTOR kinase [11]. p70S6K regulates protein synthesis by activating 40S ribosomal protein S6, leading to an increased rate of translation of the class of 5′ terminal oligopyrimide mRNA transcripts. These transcripts encode critical components of the cellular translational machinery, thus promoting protein synthesis [12]. Heinonen et al. [13] have reported that p70S6K is over-expressed in primary breast cancer tissues, and its upregulation is associated with aggressive disease and poor prognosis of patients with breast cancer. Moreover, p70S6K also has a crucial role in cell growth by regulating cell size and progression of cell cycle [14]. However, the roles of mTOR/p70S6K signal transduction pathway in osteosarcoma, which is one of the most rapidly growing sarcomas, remain unknown. This study investigated for the first time the correlation between this pathway in human osteosarcoma and patients' prognosis.

Materials and methods

Patients and tissue samples

The study was approved by the Research Ethics Committee of Ministry of Public Health of China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards. Sixty-five patients (36 males and 29 females, aging 5–58 years, mean ± SD = 18.5 ± 1.8 years) with primary osteosarcoma were obtained from the Department of Orthopaedics, Xiangya Hospital, Changsha, Hunan, People's Republic of China.

All patients recruited in this study underwent systemic neoadjuvant chemotherapy (methotrexate, adriamycin, cisplatin, isofosfamide). Closed biopsies of all patients were performed by fine-needle aspiration or trephine for diagnosis and then surgical treatment. The biopsy samples with bone tissue were decalcified. Non-cancerous adjacent tissues were available in 36 of 65 patients. The pathological diagnosis was performed by the same group of two senior pathologists experienced in osteosarcoma diagnosis. Tumor size data were available based on a review of the imaging studies and the pathology reports. Tumor volume was calculated on the basis of an ellipsoid formula, using the measurements: height × width × depth × 0.52. Surgical staging was done according to the method of Enneking et al. [15] with differentiation among highly malignant intra-compartmental osteogenic sarcomas (IIA), extra-compartmental lesions (IIB) and osteogenic sarcomas with manifestation of metastases present on recognition of the disease (III). The effect of pre-operative chemotherapy as well as the degree of histologic regression of the tumor, which was defined as percentage of dead cells, was studied by means of semi-quantitative methods obtained from the literature [16]. According to the percentage of dead cells, all cases were divided into two groups: tumors showing good response to pre-operative chemotherapy (dead cells $\geq$ 90%, surviving cells $<$ 10%), tumors showing poor response (surviving cells $\geq$ 10%). The mean follow-up time was 32 months (range 12–60 months).

Immunohistochemistry analysis

We carried out immunohistochemical detection of mTOR and p70S6K using the avidin–biotin complex method as described previously. The specimens were fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 3 μm and then deparaffinized with xylene and rehydrated for further H&E or peroxidase (DAB) immunohistochemistry staining employing DAKO EnVision System (Dako Diagnostics, Zug, Switzerland). Following a brief proteolytic

digestion and a peroxidase blocking of tissue slides, the slides were incubated with the primary antibody [rabbit antihuman mTOR polyclonal antibody (#sc-101956, 100 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100, rabbit antihuman p70S6K polyclonal antibody (#sc-230, 200 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100] against respective target proteins overnight at 4°C, as well as PBS as a control. After washing, peroxidase-labeled polymer and substrate-chromogen were then employed in order to visualize the staining of the interested proteins.

The quality (number, intensity and pattern) of every staining procedure for mTOR and p70S6K has been comparatively evaluated using consecutive control sections, and the immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained by re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells 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 staining results of mTOR staining were classified into three patterns: negative (0, less than 10% of cells stained), moderate (1, 10–50% of cells stained) and strong (2, more than 50% of cells stained); the staining results of p70S6K were classified into negative-low positive (0, staining of $\leq$70% of cells) or high positive (1, staining of >70% of cells) [17].

Statistical analysis

The software of SPSS version13.0 for Windows (SPSS Inc., IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\bar{X} \pm s$. Association between mTOR and p70S6K expression and various clinicopathological characteristics were analyzed using the χ^2 test. Two endpoints were examined for survival analyses: disease-free survival (DFS) and overall survival (OS). DFS and OS curves were plotted according to the Kaplan–Meier method, the Log-rank test being used to determine the significance of differences between clinicopathological parameters. The Cox proportional hazards model was used for multivariate analysis. Differences were considered statistically significant when P was less than 0.05.

Results

Expression and location of mTOR and p70S6K in osteosarcoma

The expression of mTOR and p70S6K was detected in 52/65 (80.00%) and 46/65 (70.77%) of patients with osteosarcoma, respectively. Their signals concentrated primarily within the cytoplasm (Fig. 1a for mTOR and Fig. 1b for p70S6K) of the tumor cells. In contrast, non-cancerous adjacent tissues showed no mTOR and p70S6K immunoreactivity.

Correlation of mTOR and p70S6K expression with the clinicopathological features of osteosarcoma

The correlation of mTOR and p70S6K expression with the clinicopathological features of patients with osteosarcoma is summarized in Table 1. mTOR and p70S6K expression were significantly associated with surgical stage ($P = 0.006$ and 0.01, respectively), metastasis pattern ($P = 0.009$ and 0.01, respectively) and percentage of dead cells ($P = 0.007$ and 0.02, respectively). However, other clinicopathological features, including gender, age, anatomic location, tumor

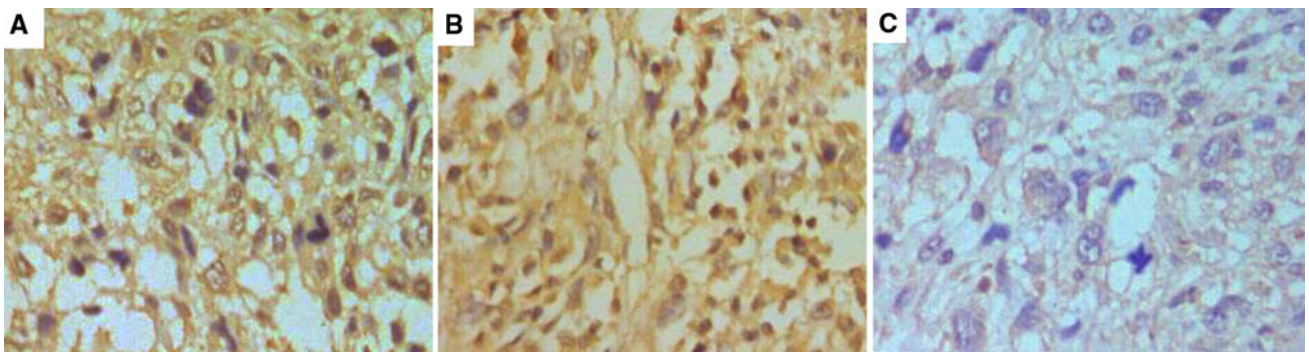

Fig. 1 Immunohistochemical staining for mTOR and p70S6K expression in osteosarcoma (original magnification ×400). **a** mTOR and **b** p70S6K strong positive expression were both found in cell cytoplasm at various levels in tumor cells, respectively; **c** negative control for **a** and **b** with normal rabbit serum

Table 1 Correlation of mTOR and p70S6K expression with clinical features in osteosarcoma

Clinical features	No.	mTOR (*n*, %)			p70S6K (*n*, %)	
		0	1	2	0	1
Gender						
Male	36	8 (22.22)	14 (38.89)	14 (38.89)	10 (27.78)	26 (72.22)
Female	29	5 (17.24)	13 (44.83)	11 (37.93)	9 (31.03)	20 (68.97)
Age (years)						
≤20	42	8 (19.05)	18 (42.85)	16 (38.10)	12 (28.57)	30 (71.43)
>20	23	5 (21.74)	9 (39.13)	9 (39.13)	7 (30.43)	16 (69.57)
Tumor size (cm^3)						
≤50	30	6 (20.00)	13 (43.33)	11 (36.67)	9 (30.00)	21 (70.00)
>50	35	7 (20.00)	14 (40.00)	14 (40.00)	10 (28.57)	25 (71.43)
Anatomic location						
Tibia/femur	40	9 (22.50)	16 (40.00)	15 (37.50)	11 (27.5)	29 (72.5)
Elsewhere	25	4 (16.00)	11 (44.00)	10 (40.00)	8 (32.00)	17 (68/00)
Surgical stage*						
IIA	30	12 (40.00)	10 (33.33)	8 (26.67)	14 (46.67)	16 (53.33)
IIB–III	35	1 (2.86)	17 (48.57)	17 (48.57)	5 (14.29)	30 (85.71)
Metastasis*						
Without	40	11 (27.50)	16 (40.00)	13 (32.50)	16 (40.00)	24 (60.00)
With	25	2 (8.00)	11 (44.00)	12 (48.00)	3 (12.12)	22 (88.88)
Treatment						
Limb salvage	43	8 (18.60)	19 (44.17)	16 (37.21)	12(27.91)	31(72.09)
Amputation	22	5 (22.73)	8 (36.36)	9 (40.91)	7 (31.82)	15(68.18)
Percentage of dead cells (%)*						
≥90	37	12 (32.43)	15 (40.54)	10 (27.03)	14 (37.94)	23 (62.16)
<90	28	1 (3.57)	12 (42.86)	15 (53.57)	5 (17.86)	23 (82.14)

* $P < 0.05$

size and surgical treatment were not associated with mTOR and p70S6K expression.

Prognostic implications of mTOR and p70S6K expression in osteosarcoma

According to the follow-up analysis, 37 patients (56.92%) developed recurrent disease (10 showed regional recurrence, 20 showed distant metastases and 7 showed regional recurrence and distant metastases) and 48 patients (73.85%) died from their osteosarcoma, during the observation period. The surgical stage and metastasis pattern of the patient, percentage of dead cells, mTOR and p70S6K expression patterns significantly influenced DFS ($P = 0.01$, 0.008, <0.001, <0.001 and 0.001, respectively) and OS ($P = 0.009$, 0.006, 0.001, <0.001 and 0.005, respectively) in univariate analysis (Log-rank test, Table 2).

A multivariate analysis (Cox regression model, Table 3) including surgical stage, metastasis pattern, percentage of dead cells, mTOR and p70S6K expression patterns was performed for OS and DFS. The surgical stage (IIA vs. IIB/III), metastasis pattern (without vs. with), percentage of dead cells (≥90 vs. <90%), mTOR expression pattern (negative vs. positive) and p70S6K expression pattern (negative vs. positive) were significant for DFS ($P = 0.02$, 0.02, 0.02, 0.01 and 0.01) and OS ($P = 0.03$, 0.02, 0.02, 0.01 and 0.02), respectively. The Kaplan–Meier curves for OS and DFS are shown in Fig. 2 for all patients.

Discussion

The data presented here provide evidence that mTOR and p70S6K proteins are differentially expressed in osteosarcoma and that there is a statistically significant association of high levels of mTOR and p70S6K proteins with worse prognosis in human osteosarcoma.

Osteosarcoma is a highly aggressive neoplasm typically composed of spindle cells producing osteoids [18]. Although its precise origin is unknown, it is thought that osteosarcoma arises from primitive mesenchymal bone-forming cells and results from alterations in their differentiation program. The large majority of conventional,

Table 2 Univariate survival analysis (Log-rank tests) of clinicopathological features

Clinicopathological features	Disease-free survival	*P*	Overall survival	*P*
Surgical stage				
IIA	1265 ± 68	0.01	1362 ± 85	0.009
IIB–III	522 ± 106		587 ± 71	
Metastasis				
Without	1332 ± 102	0.008	1565 ± 66	0.006
With	557 ± 62		603 ± 75	
Percentage of dead cells (%)				
≥90	1688 ± 103	<0.001	2329 ± 89	0.001
<90	501 ± 75		1013 ± 171	
mTOR expression pattern				
0	1592 ± 108	<0.001	1605 ± 97	<0.001
1	438 ± 93		537 ± 81	
p70S6K expression pattern				
0	1530 ± 105	0.001	1598 ± 92	0.005
1	476 ± 89		599 ± 83	

Table 3 Prognostic value of clinicopathological features in multivariate analysis by Cox regression

Clinicopathological features	Disease-free survival		Overall survival	
	P	Relative risk 95% confidence interval	*P*	Relative risk 95% confidence interval
Surgical stage				
IIA versus IIB/III	0.02	1.362–16.385	0.03	1.601–13.359
Metastasis				
Without versus with	0.02	1.082–12.339	0.02	1.365–13.008
Percentage of dead cells (%)				
≤90 versus <90	0.02	0.056–0.698	0.02	0.041–0.876
mTOR expression				
Negative versus positive	0.01	1.196–17.038	0.01	1.683–17.905
p70S6K expression				
Negative versus positive	0.01	1.028–16.397	0.02	1.319–14.258

high-grade osteosarcomas are either poorly differentiated or undifferentiated. As the result, the prognosis for patients with osteosarcoma has typically been very poor. The responses to pre-operative chemotherapy, surgical stage and tumor size have long been used as the only prognostic factors for osteosarcoma patients [19]. However, the clinical course of patients with locally advanced and/or metastatic osteosarcoma varies, and patients with the same surgical stage have different outcomes from the same therapy, so the conventional prognostic factors do not precisely predict which tumors will undergo rapid malignant progression and are difficult to give an accurate prognosis to patients. For this reason, a growing research on the molecular biology of osteosarcoma has been performed, and the presence or absence of some molecules in osteosarcoma cells has also been shown to be prognostic factors.

In mammals, several upstream growth signals, such as insulin, various G-protein-coupled receptors and nutrients, have been identified to activate mTOR, which can be regulated by a kinase cascade consisting of pI3K, pI3K-dependent kinase l and Akt [20]. The downstream effectors of mTOR include the S6 kinases (S6K1 and S6K2) and phosphorylate 4E-BP1 in parallel [21]. S6K1 is primarily, but not exclusively, cytosolic, phosphorylating the ribosomal S6 protein and thereby modifying translational dynamics [22]. The S6K1 homologue S6K2, on the other hand, localizes mostly in the nucleus, with functions that have yet to be clarified. Phosphorylation of 4EBP1 relieves inhibition of the initiation factor eIF4E, a component of the large translation initiation complex, thereby modulating translation. mTOR can promote the phosphorylation of p70S6K and 4E-BP1, thus stimulating the translation of proteins required for the cell cycle progression from the G1 to S phase [23]. It has been demonstrated that rapamycin can inhibit mTOR and the phosphorylation of p70S6K and 4E-BP1 in many cancers [24]. Previous reports have identified that the activated mTOR has many functions not

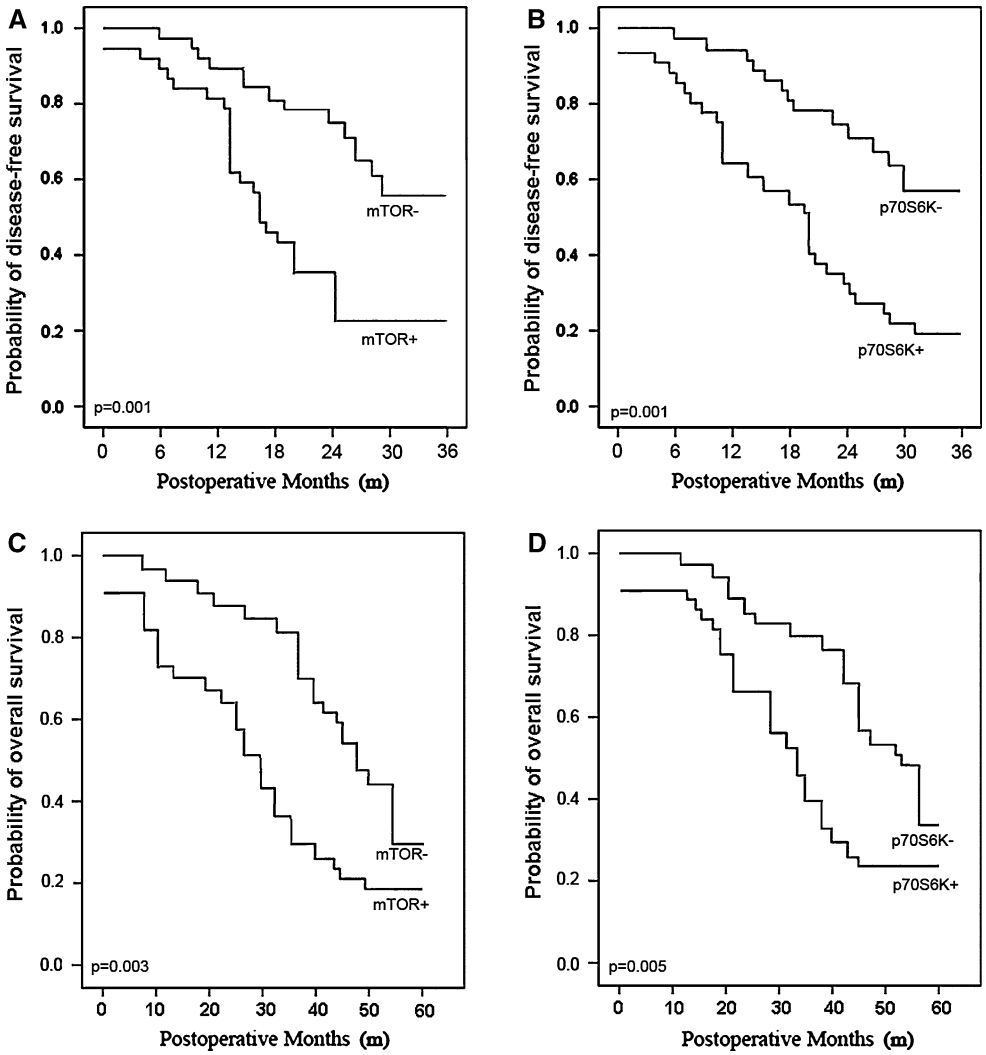

Fig. 2 Kaplan–Meier survival curves for mTOR and p70S6K expression in osteosarcoma. **a** Disease-free survival (DFS) of 65 patients with osteosarcoma, as a function of mTOR+ versus mTOR− expression ($P = 0.001$). **b** Disease-free survival (DFS) of 65 patients with osteosarcoma, as a function of p70S6K+ versus p70S6K− expression ($P = 0.001$). **c** Overall survival (OS) of 65 patients with osteosarcoma, as a function of mTOR+ versus mTOR− expression ($P = 0.003$). **d** Overall survival (OS) of 65 patients with osteosarcoma, as a function of p70S6K+ versus p70S6K− expression ($P = 0.005$)

only for growth but also for invasion and metastasis of tumors [25]. In the present study, we investigated mTOR expression at protein level in 65 osteosarcoma patients, and found that 52 osteosarcomas had positive mTOR staining and 25 osteosarcomas had an intensive mTOR expression, indicating that mTOR is also up-regulated in human osteosarcoma. We also found that mTOR was significantly over-expressed in osteosarcoma patients with higher surgical stage or metastasis. When regarding the relationship between mTOR and clinical outcome in all the follow-up patients, we found a statistically significant correlation between mTOR expression and disease-free survival or overall survival by univariate analysis. Cox multivariate analysis confirmed that mTOR over-expression was an important predictor of poor prognosis in both disease-free survival and overall survival.

p70S6K is one of downstream targets of mTOR, which regulates the translation initiation complex in a rapamycin-sensitive manner. p70S6K is a major regulator of translation under the control of multiple signal transduction pathways including phosphatidylinositol 3-kinase [26]. It increases translational capacity by promoting the expression of several members of the translational machinery whose mRNAs display oligopyrimidine tracts at their 5′ ends [27]. It has been reported that p70S6K is an important regulator of cell cycle progression through the G1/S point [28]. Our data suggested that p70S6K staining was positive in 46 osteosarcoma specimens (70.77%). Microscopic observations indicated that p70S6K was intensely expressed in the cytoplasm of tumor cells. The p70S6K protein was significantly over-expressed in osteosarcoma patients with higher surgical stage, metastasis and poor response to pre-operative chemotherapy, when compared to those without. Univariate analysis showed p70S6K expression was significantly correlated with disease-free survival and overall survival; Kaplan–Meier curve and log rank test also

indicated that patients with strong p70S6K expression had shorter disease-free survival and overall survival than those with low p70S6K expression. By Cox multivariate analysis, p70S6K strong expression was an independent prognosis factor for disease-free survival and overall survival of the osteosarcoma patients.

This is, to our knowledge, the first study determining the influence of mTOR/p70S6K signal transduction pathway on the prognosis of human osteosarcoma. We identify that this pathway is activated in human osteosarcoma tissues. The overexpression of mTOR and p70S6K is well correlated with tumor surgical stage and metastasis pattern, as well as with the response to pre-operative chemotherapy, which might be an important mechanism responsible for the survival and proliferation of osteosarcoma cells. Patients with high expression of mTOR and p70S6K had shorter survival time, suggesting the prognostic significance of the mTOR/p70S6K signal transduction pathway in osteosarcoma patients, which may lay a foundation for making further investigations on the mTOR/p70S6K signal transduction pathway as a potential target for osteosarcoma therapy.

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Conflict of interest statement The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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