RESEARCH COMMUNICATION

Clinical Significance of mTOR and p-mTOR Protein Expression in Human Colorectal Carcinomas

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Abstract

Aim: To investigate the significance of mammalian target of rapamycin (mTOR) and its active form, p-mTOR in colorectal carcinomas. Methods: Immunohistochemistry was used to detect the expression of mTOR and p-mTOR proteins in 108, 40 and 40 tissue samples from colorectal carcinoma, normal colonic mucosa and adenomatous polyps samples, respectively. The correlation of mTOR and p-mTOR expression with clinicopathological characteristics of colorectal carcinoma was analyzed. Results: The positive rates of mTOR and p-mTOR were significantly higher in colorectal carcinoma (61.1% and 61.1%, respectively, $p<0.05$) than in normal colonic mucosa (7.5% and 2.5%) and adenomatous polyps (27.5% and 20%). Overexpression of total mTOR protein was significantly associated with T1/T2 stage tumors, lymph node metastasis, distal metastasis) and degree of differentiation. p-mTOR overexpression was additionaly linked with degree of differentiation and TNM stage. Conclusion: The overexpression of mTOR and p-mTOR may play important roles in colorectal carcinogenesis with relations to the degree of differentiation, invasiveness and metastasis.

Keywords: Colorectal cancer - mTOR - p-mTOR - immunohistochemistry - overexpession

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Introduction

Colorectal carcinoma is the third most common type of malignant tumor in the world. Despite great advances in modern therapeutic strategies, overall survival of patients undergoing complete resection of carcinomas is short (Otake et al., 2010). Knowledge of molecular biomarkers associated with the prognosis of colorectal carcinomas may help to devise new treatment strategies and improve clinical outcome.

A potential candidate of biomarkers for colorectal carcinomas is the mammalian target of rapamycin (mTOR), a Ser/Thr protein kinase which plays a key role in regulating important cellular functions, including proliferation (Buck et al., 2006), growth (Shaw and Cantley, 2006), survival (Foster, 2009), mobility and angiogenesis (Jiang and Liu, 2008). In several non-colorectal tumors, activation of the mTOR pathway and overexpression correlate with more aggressive clinical courses, and has been reported to be useful target therapy (Raymond et al., 2004; O'Donnell et al., 2008; Rizell et al., 2008). The aim of the present study was to examine the expression of mTOR and p-mTOR in colon carcinomas and its correlation to clinicopathological characteristics.

Materials and Methods

Patients and specimens

A total of 108 patients with colorectal carcinoma who underwent curative surgery without prior treatments at the First Hospital of Changsha from 2002 to 2005 were enrolled in this study. These patients included 57 men and 51 women with ages ranging from 28 to 80 years (median, 62 years; mean, 58.9 years). The carcinoma were located in the colorectal (n=78) and rectum (n=30). Of these patients, 12 were grade I, 78 were grade II and 28 grade III, according to histological grading, and 24 were stage I, 37 were stage II, 45 were stage III and 12 were stage IV, according to Tumor, Node, Metastasis (TNM) stage system revised by International Union Against Cancer in 2003. Forty normal colonic mucosa samples and forty adenomatous polyps samples were obtained from the same hospital.

Immunohistochemistry

The resected specimens were fixed in 10% formalin, cut into 4m slices and mounted onto adhesive-coated slides. Slides were deparaffinized in xylene twice for 10 min and rehydrated through descending concentration of ethanol. Antigen retrieval was performed in 0.01 mol/L citrate buffer (pH 6.0) by microwave oven for 2 min and 30s at 100°C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase for 10 min. After washing with phosphate-buffered saline (PBS), the sections were incubated with blocking serum for one hour. Total mTOR and p-mTOR proteins were detected with primary polyclonal rabbit antibodies against total mTOR (P2476, 1:250, Bioworld Technology Inc. USA)

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Table 1. Expression of mTOR and p-mTOR in Normal Mucosa, Adenomatous Polyps and Adenocarcinomas

Groups	Total n	Expression of mTOR[a] positive(%)	negative(%)	χ2	Expression of p-mTOR[a] positive(%)	negative(%)	χ2
Normal mucosa	40	3(7.5)	37(92.5)		1(2.5)	39(97.5)	
Adenomatous polyps	40	12(30.0)	29(70.0)		8(20.0)	32(80.0)	
Adenocarcinomas	108	66 (61.1)	42 (38.9)	86.0	66 (61.1)	42 (38.9)	101.8

[a]p<0.001 compared to normal mucosa and adenomatous polps

and p-mTOR (ser-2448, 1:250, Bioworld Technology Inc., USA), respectively. Specimens were incubated with the primary antibody overnight at 4°C. Total mTOR and p-mTOR protein expression was evaluated by two pathologists without knowledge of the patients' clinical data and the values were averaged, using Olympus CX42 microscope (Olympus Optical).

Evaluation of score

The intensity of staining was scored as follows: 0, negative (no brown staining); 1, weak (light brown staining); 2, moderate (intermediate brown staining); 3, strong (dark brown staining. The extent of staining was scored as 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), 4 (>75%) of the cells in the respective lesions. The final score was determined by multiplying the intensity of staining and the extent of staining scores, yielding a range from 0 to 12. Scores 9-12 was defined as preserved or strong staining pattern (++), 5-8 was defined as weak staining pattern (+) and 0-4 was defined as markedly reduced or negative expression (-). Specifically, a under-expression was defined as no staining or positive staining in tumor tissue being less than matched normal tissue, a normal expression as positive staining being similar to matched normal tissue, an over-expression as positive staining being higher than matched normal tissue.

RT-PCR

RNA isolated from tissues was reverse-transcribed and amplified using the One-Step RT-PCR System (Fermentas, Vilnius, Lithuania). Primer sequences used were: sense 5'-TGCAATCCAGCTGTTTGG-3' and antisense 5'-CCATTCCAGCCAGTCATCTTT-3' for mTOR. A 587-bp GAPDH fragment was amplified as an internal control. For GAPDH, the forward primer was 5'-AATCCCATCACCATCTTCCA-3' and the reverse primer was 5'- CCTGCTTCACCACCTTCTTG-3'. After heating denaturation at 95°C for 1 min, samples were exposed to 30 cycles (GAPDH, 25cycles) at 95°C for 30 s, 60°C fo 30 s and 68°C for 90s with a final extension at 68°C fo 10 min.

Western blot analysis

Whole-cell lysates were prepared from humai colorectal cancer or normal colorectal tissue specimens Standard Western blotting was performed using rabbi polyclonal antibodies against human mTOR (P2476 1:1000, Bioworld Technology Inc., USA) and p-mTOF (ser-2448, 1:1000, Bioworld Technology Inc., USA) Anti-GAPDH antibody was used as a loading control.

Statistical Analysis

All statistical analyses were done using the SPSS 10.0 software package (SPSS Inc., Chicago, USA). Differences between groups were compared using χ2 t-test or Pearson's test. All tests were two-tailed and p<0.05 were considered significant different.

Results

Association of mTOR and p-mTOR expression with colorectal carcinoma

As shown in Table 1, only 3 of 40 normal colonic mucosa cases (7.5%) showed weak mTOR expression. In contrast, mTOR was weakly to moderately expressed in 12 (30.0%) of 40 adenomatous polyps and over-expressed in 66 (61.1%) of 108 colorectal adenocarcinomas (p<0.001). In normal colonic mucosa samples, only 1 (2.5%) of 40 cases showed weak p-mTOR expression. In contrast, p-mTOR was weakly to moderately expressed in 8 (20.0%) of 40 adenomatous polyps and over-expressed in 66 (61.1%) of 108 colorectal adenocarcinomas. Increased expression of total mTOR and p-mTOR was observed in the membrane and/or cytoplasm of tumor cells. χ2 test indicated that the expression of mTOR and p-mTOR were significantly associated with colorectal adenocarcinomas (p<0.001) (Table1 and Figure 1).

Further analysis indicated that both mTOR and p-mTOR proteins were over-expressed in primary tumor tissue compared with that in normal colorectal tissue. The scores of both mTOR and p-mTOR in adenocarcinomas or adenomatous polps were significantly higher than that in normal mucosa (p<0.05) although there was no significant difference between adenomatous polyps and adenocarcinomas (p>0.05) (Figure 2). RT-PCR results indicated that the mRNAs for mTOR and p-mTOR in colorectal adenocarcinomas were also significantly higher than that in normal tissues (Figure 3A). In addition, Western blot indicated that mTOR and p-mTOR proteins in adenocarcinomas were significantly higher than that in

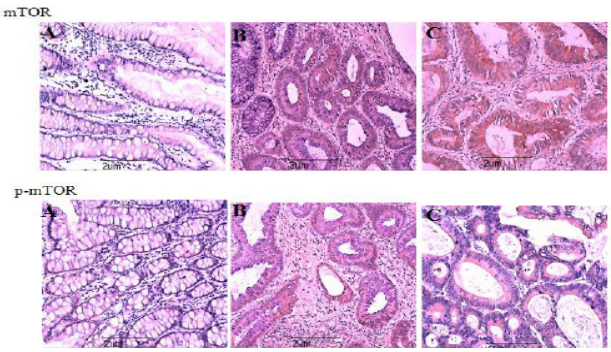

Figure 1. Expression of mTOR (upper panel) and p-mTOR (lower panel) in Normal Colonic Mucosa (A), Adenomatous Polyp (B) and Colorectal Adenocarcinoma (C) (SABC× 200). Scale bar, 2microM.

Table 2. Differences in the Overexpression of Mtor and P-Mtor in Relation to Clinicopathology Parameters of Colorectal Cancers Patients

Variable	Total n	Positive n	mTOR* p value	Positive p- n	mTOR* p value
Gender					
Male	57	39		38	
Female	51	27	0.099	28	0.211
Age(y)					
<60	41	23		25	
≥60	67	43	0.403	41	0.982
Location					
Colon	78	46		47	
Rectum	30	20	0.463	19	0.769
T stage					
T1/T2	26	10		8	
T3/T4	82	56	0.007	58	<0.001
TNM stage					
I/II	51	26		21	
III/IV	57	40	0.099	45	<0.001
Lymph node metastasis					
pN(-)	58	30		26	
pN(+)	50	36	0.031	40	<0.001
Distant metastasis					
Negative	97	56		56	
Positive	11	10	0.032	10	0.032
Differentiation					
Well	10	4		5	
Moderate	56	27		25	
Poor	42	35	0.004	36	0.001

*overexpression

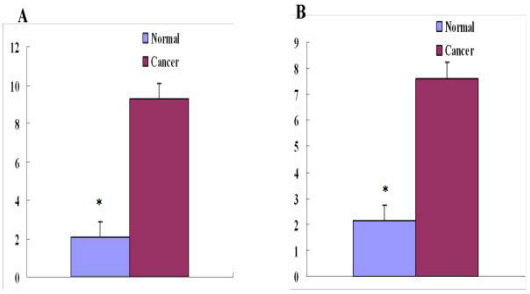

Figure 2. Immunohistochemistry Scores of the Expression of mTOR (A) and p-mTOR (B) Proteins in Human Colorectal Cancer and Matched Normal Colorectal Tissues. *p<0.05 compared to cancer tissue

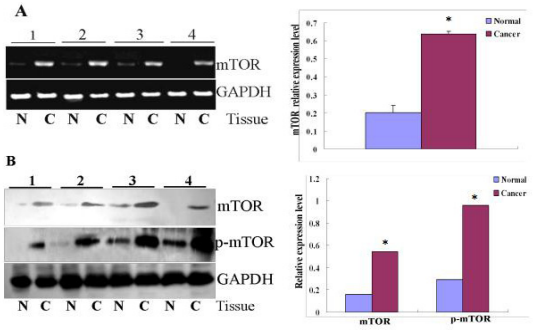

Figure 3. RT-PCR (A) and Western Blot (B) Analysis of mTOR and p-mTOR from Four Paired Colorectal Cancer and Normal Colorectal Tissue Specimens. (N, normal tissues; C, tumor tissues). *p<0.05 compared to control tissue

normal tissues RT-PCR (Figure 3B).

Correlation between mTOR and p-mTOR overexpression and clinicopathological characteristics

By evaluating the clinical significance of mTOR and p-mTOR overexpression, we found that the overexpression of total mTOR protein was significantly associated with T1/T2 stage tumors (p=0.007), lymph node metastasis (p=0.031), distant metastasis (p=0.031), degree of differentiation (p=0.004). Moreover, p-mTOR overexpression was associated with T1/T2 stage tumors (p<0.001) lymph node metastasis (p<0.001), distant metastasis (p=0.032) and degree of differentiation (p=0.004). However, our result showed that p-mTOR, not total mTOR, significantly correlated with TNM stage (p<0.001) (Table2).

Discussion

mTOR is a serine/threonine kinase involved in multiple intracellular signaling pathways promoting tumor growth. The phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR signaling pathway in particular is frequently altered in non-colorecteral cancers, including gastric cancer (Yu et al., 2009), biliary tract adenocarcinoma (Herberger et al., 2007), pancreatic ductal adenocarcinoma (Pham et al., 2008), lung carcinoma (Dobashi et al., 2009), prostate cancer (Kremer et al., 2006), cervical carcinoma (Feng et al., 2009), renal cell carcinoma (Pantuck et al., 2007). Activated mTOR (p-MTOR) has been shown to be tumor-associated in many cancer tissues. It has been found that expression of mTOR and p-mTOR were elevated in extrahepatic cholangiocarcinoma, respectively (Chung et al., 2009), high-grade squamous intraepithelial lesions/cervical squamous cell carcinoma compared to normal cervical epithelium (Feng et al., 2009). In our study, we detected that the positive rate of mTOR and p-mTOR were both significantly higher in colorectal cancer tissues than in normal tissues. Furthermore, the mTOR and p-mTOR expression level were higher in colorectal adenocarcinoma tissue samples than in normal colonic mucosa and adenomatous polyp tissue samples, which suggests that activated mTOR is highly associated with colorectal cancer and plays a key role in tumor carcinogenesis.

It has been suggested that mTOR and p-mTOR expression are associated with different clinicopathological variables in some non-colorecteral tumors. For example, Yu et al reported that the overexpression of total mTOR protein was significantly correlated with tumor differentiation, T1/T2 tumors and stage I/II/III disease, whereas the p-mTOR overexpression was significantly correlated with lymph node metastasis and all stage disease (Yu et al., 2009). Jae et al found that the mTOR expression was highly correlated with old age, menopausal status, but not other clinicopathologic characteristics (No et al., 2009). Dobashi et al found that the p-mTOR expression in lung adenocarcinoma specimens was correlated with the grade of histologic differentiation, whereas the p-mTOR expression was correlated with lymph node metastasis in squamous cell carcinoma specimens (Dobashi et al., 2009). Although Herberger et al found that positive

p-mTOR in 56 of 88 billiary tract carcinomas showed no association with any clinicopathologic variables of patients but predicted overall survival of the patients (Herberger et al., 2007). In our study, we found that the overexpression of total mTOR protein was significantly associated with T1/T2 stage tumors, lymph node metastasis, distant metastasis, degree of differentiation. Moreover the p-mTOR overexpression was associated with T1/T2 stage tumors lymph node metastasis, distant metastasis, degree of differentiation. However, our result showed that p-mTOR, but not total mTOR, significantly correlated with TNM stage. This finding suggested that mTOR overexpression, especially p-mTOR, is an important event in colorectal tumorigenesis. To date, this is the first report on mTOR and p-mTOR overexpression and its clinicopathological characteristics in colorectal carcinoma. These studies also indicated that mTOR was frequently activated and its overexpression may be an important step in carcinogenesis and progression in human colorectal cancer.

Recently, it is postulated that targeting mTOR with siRNA may inhibit proliferation of tumor cell growth. Ji et al. observed that targeting mTOR with a specific siRNA reduced Hela cervical cancer cell proliferation and survival in vitro (Ji and Zheng, 2010). Our findings also suggest that deregulated mTOR could be a promising new molecular target for designing novel therapeutic strategies to control colorectal cancinoma. This hypothesis is supported by one recent study suggesting that targeting mTOR2 inhibits colon cancer cell proliferation in vitro and tumor formation in vivo (Roulin et al., 2010).

In summary, the present study indicated that mTOR and p-mTOR are overexpressed in human colorectal carcinoma and the overexpression of mTOR and p-mTOR are correlated with some clinical characteristics, implying mTOR and p-mTOR may play one key role of in colorectal carcinoma and be therapeutic target.

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References

Buck E, Eyzaguirre A, Brown E, et al (2006). Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors. *Mol Cancer Ther*, **5**, 2676-84.

Chung JY, Hong SM, Choi BY, et al(2009). The expression of phospho-AKT, phospho-mTOR, and PTEN in extrahepatic cholangiocarcinoma. *Clin Cancer Res*, **15**, 660-7.

DobashiY, Suzuki S, Matsubara H, et al (2009). Critical and diverse involvement of Akt/mammalian target of rapamycin signaling in human lung carcinomas. *Cancer*, **115**, 107-18.

Feng W, Duan X, Liu J, Xiao J, Brown RE (2009). Morphoproteomic evidence of constitutively activated and overexpressed mTOR pathway in cervical squamous carcinoma and high grade squamous intraepithelial lesions. *Int J Clin Exp Pathol*, **2**, 249-60.

Foster DA (2009). Phosphatidic acid signaling to mTOR: signals for the survival of human cancer cells. *Biochim Biophys Acta*, **1791**, 949-55.

Herberger B, Puhalla H, Lehnert M, et al (2007). Activated mammalian target of rapamycin is an adverse prognostic factor in patients with biliary tract adenocarcinoma. *Clin Cancer Res*, **13**, 4795-9.

Ji J, Zheng PS (2010). Activation of mTOR signaling pathway contributes to survival of cervical cancer cells. *Gynecol Oncol*, **117**, 103-8.

Jiang BH, Liu LZ (2008). Role of mTOR in anticancer drug resistance: perspectives for improved drug treatment. *Drug Resist Updat*, **11**, 63-76.

Kremer CL, Klein RR, Mendelson J, et al (2006). Expression of mTOR signaling pathway markers in prostate cancer progression. *Prostate*, **66**, 1203-12.

No JH, JeonYT, Park IA, et al (2009). Expression of mTOR protein and its clinical significance in endometrial cancer. *Med Sci Monit*, **15**, BR301-5.

O'Donnell A, Faivre S, Burris HA III, et al (2008). Phase I pharmacokinetic and pharmacodynamic study of the oral mammalian target of rapamycin inhibitor everolimus in patients with advanced solid tumors. *J Clin Oncol*, **26**, 1588-95.

Otake S, Takeda H, Fujishima S, et al (2010). Decreased levels of plasma adiponectin associated with increased risk of colorectal cancer. *World J Gastroenterol,* **16,** 1252-7.

Pantuck AJ, Seligson DB, Klatte T, et al (2007). Prognostic relevance of the mTOR pathway in renal cell carcinoma: implications for molecular patient selection for targeted therapy. *Cancer*, **109**, 2257-67.

Pham NA, Schwock J, Iakovlev V, et al (2008). Immunohistochemical analysis of changes in signaling pathway activation downstream of growth factor receptors in pancreatic duct cell carcinogenesis. *BMC Cancer*, **8**, 43.

Raymond E, Alexandre J, Faivre S, et al (2004). Safety and pharmacokinetics of escalated doses of weekly intravenous infusion of CCI-779, a novel mTOR inhibitor, in patients with cancer. *J Clin Oncol*, **22**, 2336-47.

Rizell M, Andersson M, Cahlin C, et al (2008). Effects of the mTOR inhibitor sirolimus in patients with hepatocellular and cholangiocellular cancer. *Int J Clin Oncol*, **13**, 66-70.

Roulin D, Cerantola Y, Dormond-Meuwly A, Demartines N, Dormond O (2010). Targeting mTORC2 inhibits colon cancer cell proliferation in vitro and tumor formation in vivo. *Mol Cancer*, **9**, 57.

Shaw RJ, Cantley LC (2006). Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*, **441**, 424-30.

Yu G, Wang J, ChenY, et al (2009). Overexpression of phosphorylated mammalian target of rapamycin predicts lymph node metastasis and prognosis of chinese patients with gastric cancer. *Clin Cancer Res*, **15**, 1821-9.