Putative role of the mTOR/4E-BP1 signaling pathway in the carcinogenesis and progression of gastric cardiac adenocarcinoma

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Abstract. The mammalian target of rapamycin/eukaryotic translation inititiation factor 4E binding protein 1 (mTOR/4E-BP1) transduction pathway is activated in a range of malignant cancers, but its role in human gastric cardiac adenocarcinoma (GCA) has not been well defined. The present study used western blotting and reverse transcription polymerase chain reaction (RT-PCR) to assess the expression of mTOR, 4E-BP1 and eukaryotic translation initiation factor 4E (eIF4E) at the protein and mRNA levels in 33 cases of GCA and paired adjacent normal gastric mucosal tissues. The expression of mTOR at the protein level in GCA was significantly lower than that in the corresponding normal gastric mucosa (0.296±0.27 vs. 1.348±0.80, P<0.05), but the ratio of p-mTOR to mTOR was significantly increased in tumor tissues (1.425±1.07 vs. 0.450±0.24, P<0.05). The expression of 4E-BP1 was significantly decreased in GCA compared with normal tissues (P<0.05), while the levels of phosphorylated 4E-BP1 (p-4E-BP1) were markedly increased in tumor tissues (P<0.05). The levels of phosphorylated eIF4E (p-eIF4E) were significantly higher in the tumors in comparison to the corresponding normal tissues (1.822±0.63 vs. 0.997±0.38, P<0.05), and the levels of p-eIF4E were closely correlated with lymph node metastasis (P<0.05). The mTOR/4E-BP1 signaling pathway is activated in GCA, with mTOR activated mainly through increased mTOR phosphorylation rather than protein overexpression.

Introduction

Gastric cancer is one of the most common malignancies worldwide and ranks as the second leading cause of cancer-related mortality (1). During the past few decades, the incidence of distal gastric carcinomas has decreased markedly, while adenocarcinoma of the esophagogastric junction (AEG), including gastric cardiac adenocarcinoma (GCA) and adenocarcinoma of the distal esophagus, have shown a marked increase in western countries (2). Similar changes in the incidence of gastric carcinomas at varying subsites have also been identified in China (3), but the reason for these changes is not clear. As previous studies have identified differences in histological findings, phenotypic marker expression and genetic alterations between adenocarcinomas of the gastric cardia and distal stomach (4), the carcinogenesis and development of GCA may be different from that of adenocarcinomas of the distal stomach. These possible differences therefore require further study.

One of the most clinically significant molecular signaling networks that has been frequently studied over the past decade is the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway is a critical nutritional and cellular energy checkpoint sensor and regulator of cell growth in mammalian cells (5-7). mTOR was originally identified as the target of the macrolide antibiotic rapamycin. It is a Ser/Thr protein kinase that mediates nutrient-dependent intracellular signaling associated with cell growth, proliferation and differentiation (8). Eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) is the first downstream substrate of mTOR, which is a small molecular protein (9,10). Eukaryotic translation initiation factor 4E (eIF4E) is an oncogene encoding a cap-binding protein, and is also the main translation initiator. eIF4E is able to specifically identify the mRNA 5'-end cap structure (m7 GPPPN, where N is any nucleotide and m is a methyl group) and initiate translation, so it is an extremely significant regulatory site of translation in eukaryotes. 4E-BP1 suppresses eIF4E activity. The protein exerts an inhibitory effect by binding to eIF4E and preventing the assembly of the translation preinitiation complex. Once 4E-BP1 is phosphorylated, it dissociates from eIF4E, permitting cap-dependent translation to take place. Under the stimulation of hormones, mitogens and other related factors, mTOR modulates the activity of eIF4E by regulating the phosphorylation of 4E-BP1. Activation of mTOR leads to the phosphorylation of 4E-BP1, resulting in the dissociation of 4E-BP1 from the mRNA cap-binding protein eIF4E and promotion of protein synthesis. By contrast, hypophosphorylated 4E-BP1 inhibits cap-dependent translation (11).

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As activation of the mTOR growth pathway has been observed in numerous malignant tumors, mTOR has been suggested to be an attractive molecular target for cancer therapy. Several studies have shown that activation of the mTOR signaling pathway and overexpression of mTOR is common in gastric cancers (12). Treatment strategies using everolimus, the specific inhibitor of mTOR, have had a high efficacy and safety in gastric cancer in phase II studies, and therefore a global phase III study is now being prepared (13). The mTOR signaling pathway has become a new target for gastric cancer therapy, but the role of the mTOR signaling pathway in the varying subsites of gastric cancer has not yet been reported.

The aim of this study was to evaluate the activation of the mTOR/4E-BP1 signal transduction pathway in GCA, as it had not often been studied in the past, and to explore the putative role of the mTOR/4E-BP1 signal transduction pathway in the carcinogenesis and progression of cardiac adenocarcinomas.

Patients and methods

Patients. A total of 33 patients with GCA who underwent curative surgery at The Fouth Hospital of Hebei Medical University and Cixian County Hospital between 2008 and 2009 were included in this study. Fresh samples from pathologically representative tumor regions and paired adjacent normal gastric mucosal tissues were obtained. These tissues were stored at -80˚C until the proteins and RNA were extracted. The pathological nature of each specimen was confirmed by examination with hematoxylin and eosin staining. Prior treatments, including radiotherapy or chemotherapy, were not used before surgery in any of the cases. The tumors were selected carefully according to the definition of the Siewert II AEG, the epicenters of which were at the gastroesophageal junction, i.e., from 1 cm above to 2 cm below the junction between the end of the tubular esophagus and the beginning of the saccular stomach (14). This study was approved by the ethics committee of Hebei Medical University, Shijiazhuang, China. Informed consent was obtained from all patients.

Western blot analysis. Whole-cell lysates were prepared from human GCA and corresponding adjacent normal gastric mucosal tissues. Balanced amounts of protein from each sample (100 μg) were fractionated by SDS-PAGE. Subsequent to transferring the proteins onto Immobilon-PVDF, the membranes were blocked with blocking buffer containing 5% skimmed, dry milk and then incubated with the primary antibody overnight at 4˚C. The monoclonal antibody mTOR, phosphorylated mTOR (p-mTOR; Ser2448), 4E-BP1, phosphorylated 4E-BP1 (p-4E-BP1; Ser65) and phosphorylated eIF4E (p-eIF4E) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA) and the monoclonal antibodies eIF4E and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Primary antibodies included mTOR (1:2,000), p-mTOR Ser2448 (1:1,000), 4E-BP1 (1:1,000), p-4E-BP1 Ser65 (1:1,000), eIF4E (1:200), p-eIF4E (1:1,000) and β-actin (1:200) which were diluted in blocking buffer. Depending on the primary antibodies used, either anti-mouse or anti-rabbit horseradish peroxidase was used as a secondary antibody. β-actin expression was used as a loading control and to assess the protein extract quality.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted by a single-step method from the tumors and corresponding benign tissues using guanidinium isothiocyanate. The integrity of the total RNA was identified by 1% agarose gel electrophoresis. Quantitation was achieved using an ultraviolet spectrophotometer. The expression of mTOR, 4E-BP1 and eIF4E in GCA and normal gastric mucosa at the mRNA level was determined using the RT-PCR method. Relative expression was calculated as the ratio between the density of the target gene and that of GAPDH by a BIO-LD densitometric image analyzer. The primers were as follows: mTOR, forward 5'-AGAGAGGAC ACAAGCAC-3' and reverse 5'-CACAGATAATGGCAA TG-3'; eIF4E, forward 5'-TAATCAGGAGGTTGCT-3' and reverse 5'-TTCTCACTTCCCACA-3'; 4E-BP1, forward 5'-GGGGACTACAGCACGAC-3' and reverse 5'-CGCCCG CTTATCTTCT-3'; GAPDH, forward 5'-GGAAGGTGA AGGTCGGAGT-3' and reverse 5'-CCTGGAAGATGGTGA TGGG-3'. PCR products were visualized by ethidium bromide staining of 1.5% agarose gel.

Statistical analysis. SPSS 13.0 software was employed to analyze all data. The significance of differences between two groups was determined using the paired-samples t-test. The Fisher's exact test was used to test possible associations between the expression levels of members of the mTOR/4E-BP1 pathway and clinicopathological factors. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Western blot analysis. Western blot analysis results showed that the expression of mTOR was significantly decreased in GCA compared with that in the corresponding normal gastric mucosa (0.296±0.27 vs. 1.348±0.80, $P<0.05$). There was no difference identified in the level of p-mTOR between the tumor and the corresponding normal gastric tissues (0.348±0.27 vs. 0.475±0.23, $P>0.05$; Fig. 1). Further analysis showed that the ratio of p-mTOR to mTOR in GCA (1.425±1.07) was significantly higher than that in the corresponding normal gastric mucosa (0.450±0.24, $P<0.05$; Fig. 2), suggesting that mTOR was activated to a greater extent in GCA. In comparison with expression in the corresponding normal gastric mucosa, the expression of 4E-BP1 in the tumor tissues was significantly decreased (2.210±0.87 vs. 4.498±1.78, $P<0.05$), while western blot analysis detected an increased level of p-4E-BP1 in tumor tissues compared with the normal tissues (2.165±0.86 vs. 1.184±0.45, $P<0.05$; Fig. 3). There was no significant difference in eIF4E expression between GCA and the corresponding normal gastric mucosa (2.194±0.80 vs. 2.033±0.73, $P>0.05$), while the level of p-eIF4E was significantly higher in GCA in comparison to the corresponding normal gastric mucosa (1.822±0.63 vs. 0.997±0.38, $P<0.05$; Fig. 3).

The expression of mTOR, p-mTOR, mTOR/p-mTOR, 4E-BP1, p-4E-BP1 and eIF4E in GCA was not corelated with age, differentiation, tumor size, infiltration depth or lymph node metastasis ($P>0.05$), while the level of p-eIF4E was closely

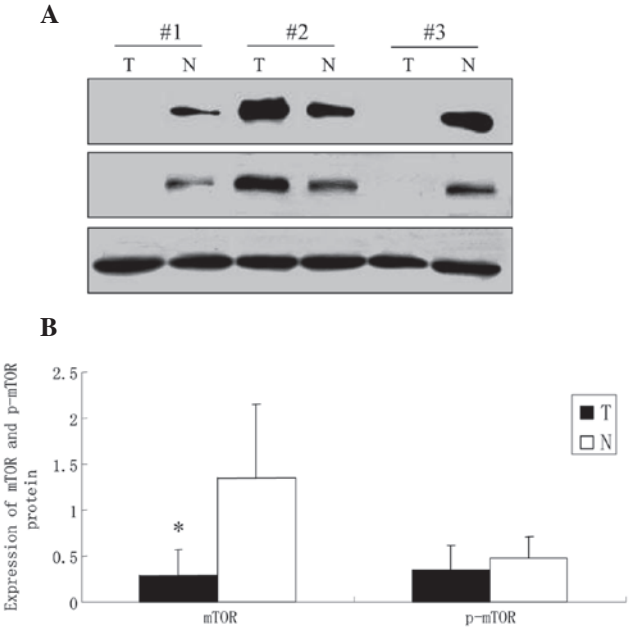

Figure 1. Western blot analysis of mTOR and p-mTOR protein expression in GCA and paired adjacent normal gastric mucosal tissues. (A) Representative immunoblots showed the expression of mTOR and p-mTOR in cases 1, 2 and 3. β-actin served as a loading control. (B) Intensities of the immunoreactive bands were quantified by densitometric scanning. T, GCA tumor tissues; N, normal gastric mucosa. Values are mean ± SD. *P<0.05 compared with normal. GCA, gastric cardiac adenocarcinoma; mTOR, mammalian target of rapamycin; p-mTOR, phosphorylated mTOR.

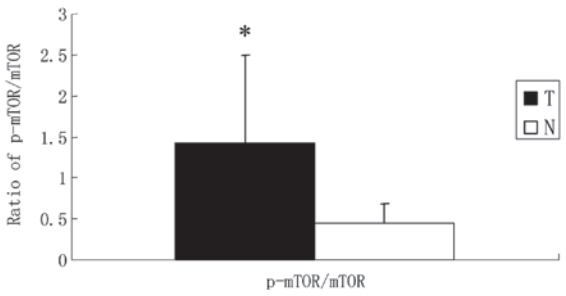

Figure 2. Ratios of p-mTOR to mTOR in GCA and corresponding adjacent normal gastric mucosa tissues as determined by western blot densitometry analysis. T, GCA tumor tissues; N, normal gastric mucosa. *P<0.05 compared with normal. mTOR, mammalian target of rapamycin; p-mTOR, phosphorylated mTOR; GCA, gastric cardiac adenocarcinoma.

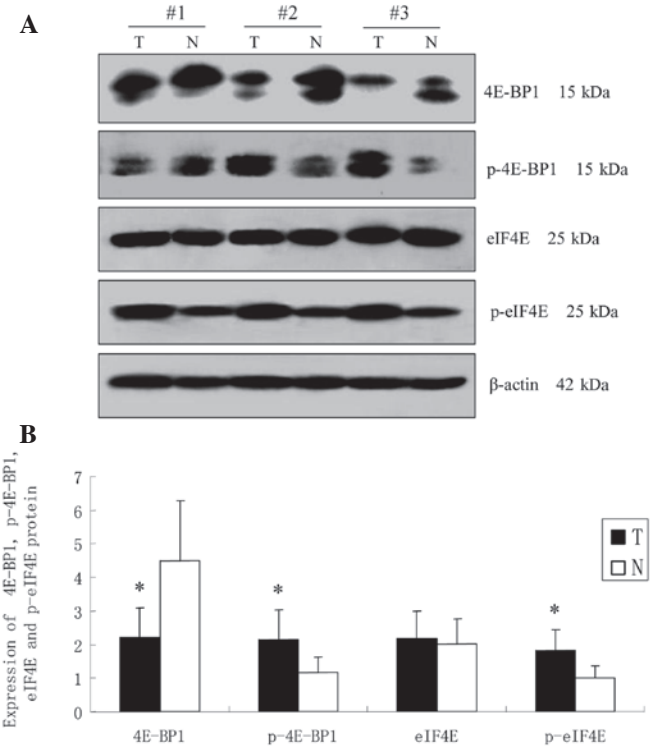

Figure 3. Expression of 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E at the protein level in GCA and paired normal gastric mucosa investigated by western blot analysis. (A) Representative immunoblots showing the expression of 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E in cases 1, 2 and 3. β-actin served as a loading control. (B) Intensities of the immunoreactive bands were quantified by densitometric scanning. T, GCA tumor tissues; N, normal gastric mucosa. Data are the mean ± SD. *P<0.05 compared with normal. 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; p-4E-BP1, phosphorylated 4E-BP1; eIF4E, eukaryotic translation initiation factor 4E; p-eIF4E, phosphorylated eIF4E; GCA, gastric cardiac adenocarcinoma.

correlated with lymph node metastasis (P<0.05; Table I). The level of p-eIF4E in the lymph node metastasis group was markedly higher compared with that of the non-lymph node metastasis group. There was no correlation identified between the level of p-eIF4E in GCA and the age, gender, differentiation, tumor size or infiltration depth (P>0.05).

RT-PCR analysis. The relative expression of mTOR, 4E-BP1 and eIF4E in GCA and corresponding normal tissues at the mRNA level were detected by semi-quantitative RT-PCR (Fig. 4). The optical density of the mTOR mRNA was 3.305±0.78 in tumor tissues and 7.564±1.60 in normal tissues. The statistical results revealed that the mTOR mRNA expression in tumor tissues was markedly lower than that in the normal tissues (P<0.05). mRNA expression of 4E-BP1 in GCA tumor tissues was reduced significantly in comparison to the corresponding normal tissues (4.388±1.34 compared with 7.117±1.77, P<0.05). The optical density of the eIF4E mRNA was 4.912±1.54 in tumor tissues, which was markedly higher than that of the normal control group (1.095±0.32, P<0.05).

Discussion

The mTOR/4E-BP1 signaling pathway, which is activated in a variety of malignances, is closely corelated with tumor occurrence and progression. Aberrant activation of the mTOR signaling pathway has been identified in numerous cancers, including colorectal cancer (15), lung cancer, renal cell carcinoma (16), breast cancer (17) and cervical carcinoma (18). Several previous studies using other cancer models have identified mTOR signaling as a potential target for anticancer therapy (19). mTOR is a Ser/Thr protein kinase that mediates nutrient-dependent intracellular signaling corelated with cell growth, proliferation and differentiation. Osaki *et al* (20) revealed that mTOR plays a significant role in the resistance to Fas-mediated apoptosis in the human gastric carcinoma cell line MKN-45. p-mTOR is the activated form of mTOR, and the level of this form may better reflect the activation status of the pathway. mTOR phosphorylation is frequently detected in ovarian cancer and may be targeted to disrupt ovarian tumor cell growth (21). Overexpression of p-mTOR predicted

Table I. Correlations between p-mTOR/mTOR, 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E and clinical pathological characteristics in GCA.

Clinicopathological factors	All N (%)	p-mTOR/ mTOR			4E-BP1			p-4E-BP1			eIF4E			p-eIF4E		
		H	L	P-value	H	L	P-value	H	L	P-value	H	L	P-value	H	L	P-value
Age (years)																
<60	17	13	4	0.465	12	5	0.721	10	7	1.000	10	7	1.000	13	4	1.000
≥60	16	10	6		10	6		9	7		10	6		12	4	
Differentiation																
Poor	15	8	7	0.126	11	4	0.712	9	6	1.000	8	7	0.493	13	2	0.242
Well/moderate	18	15	3		11	7		10	8		12	6		12	6	
Lengh of tumor (cm)																
<5	17	14	3	0.141	12	5	0.721	9	8	0.728	12	5	0.296	12	5	0.688
≥5	16	9	7		10	6		10	6		8	8		13	3	
Depth of tumor																
T1	7	6	1	0.397	5	2	1.000	5	2	0.670	6	1	0.202	6	1	0.652
T2/3/4	26	17	9		17	9		14	12		14	12		19	7	
Lymph node metastasis																
Positive (N1/2/3)	19	13	6	1.000	14	5	0.459	10	9	0.723	11	8	1.000	17	2	0.047[a]
Negative (N0)	14	10	4		8	6		9	5		9	5		8	6	

H, higher expresssion in tumor tissue than in normal; L, lower expresssion in tumor tissue than in normal. [a]P<0.05. GCA, gastric cardiac adenocarcinoma; mTOR, mammalian target of rapamycin; p-mTOR, phosphorylated mTOR; 4E-BP1, eukaryotic translation initiation factor 4E binding protein; p-4E-BP1, phosphorylated 4E-BP1; eIF4E, eukaryotic translation initiation factor 4E; p-eIF4E, phosphorylated eIF4E.

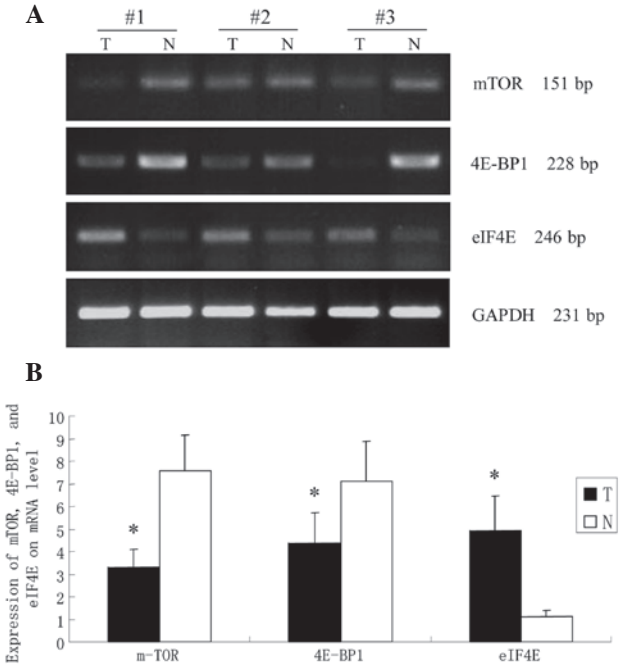

Figure 4. RT-PCR analysis of mTOR, 4E-BP1 and eIF4E mRNA expression in GCA and paired normal gastric mucosa. (A) Representative expression of 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E at the mRNA level. GAPDH was used to normalize any differences in mRNA loading between lanes. (B) Intensities of the electrophoresis bands were quantified by densitometric scanning. T, GCA tumor tissues; N, normal gastric mucosa. Values are mean ± SD. *P<0.05 vs. normal. mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; eIF4E, eukaryotic translation initiation factor 4E; GCA, gastric cardiac adenocarcinoma; p-4E-BP1, phosphorylated 4E-BP1; p-eIF4E, phosphorylated eIF4E.

the angiogenic phenotype of human gastric cancer (22) and was significantly corelated with tumor progression and outcome (23).

In the present study, we investigated the expression of the mTOR pathway in GCA and normal gastric tissues. The results showed that mTOR and p-mTOR were expressed in GCA and normal gastric mucosa. The expression of mTOR in cancer tissues was significantly lower than that in the normal gastric mucosa, while the level of p-mTOR in cancer and normal gastric mucosa demonstrated no significant difference. Therefore, simple analysis of the protein expression status of mTOR and p-mTOR may be insufficient to reveal the correlation between mTOR and GCA. When comparing the ratio of p-mTOR to mTOR between GCA and corresponding normal gastric mucosa, the data showed a considerably higher ratio of p-mTOR to mTOR in the tumor tissue in comparison to the benign gastric tissue. The present study suggested that the phosphorylation of mTOR may be a significant step in the progression of GCA. The activation of mTOR is mainly through increased mTOR phosphorylation rather than protein overexpression.

4E-BP1 is a translation initiation inhibitory factor and the first downstream substrate of mTOR (24). Hypophosphorylated 4E-BP1 binds to and thereby inactivates the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E), then following phosphorylation by mTOR 4E-BP1 releases eIF4E and allows its binding to the cap structure of the mRNA and the subsequent beginning of protein translation (25). Study results, particularly PTEN and mTOR expression data, suggest that 4E-BP1 overexpression is strongly associated with prostate cancer (26). Certain investigators have suggested that p4E-BP1 may play a central role in determining the growth self-sufficiency capacity of tumors (27). The p-4E-BP1 level was identified to be significantly increased in poorly differentiated groups of endometrial carcinoma, and is closely correlated with the tumor stage (28). High levels of p-4E-BP1 indicated a poor prognosis in human melanomas (29). Overexpression of p-4E-BP1 in cervical carcinoma was strongly correlated with shortened disease-free and overall survival (30).

In the present study, RT-PCR and western blot analysis results indicated that compared with normal gastric mucosa, the expression of 4E-BP1 was markedly decreased in GCA, while the level of p-4E-BP1 was significantly elevated. These results indicated that GCA cells grow faster than normal, so they are required to synthesize more protein to support active cell proliferation and division. 4E-BP1 directly blocks translation by binding to eIF4E and preventing translation. Hyperphosphorylation of 4E-BP1 releases it from eIF4E enabling assembly of the eIF4F complex, which permits translation to proceed. Translation initiation is the rate-limiting step of protein synthesis.

eIF4E does not only play a central role in the regulation of protein translation (31,32) but is also a proto-oncogene (33). As eIF4E is the least abundant among the initiation factors and is considered to be the rate-limiting factor for cap-dependent translation initiation, changes in the levels of eIF4E profoundly affect the translation rates. High levels of eIF4E may lead to overexpresssion of a number of proto-oncogenes, growth factors and cell cycle-related proteins (34). eIF4E is dysregulated in a wide variety of human cancers. Numerous studies have shown that eIF4E is elevated in a number of solid tumors, including breast, bladder, colon, head and neck, prostate, cervical and lung cancers and lymphomas (35). High eIF4E expression was correlated with a decreased overall survival rate in lung adenocarcinoma patients and may be a better clinical marker for predicting the prognosis in these cases (36). The activity of eIF4E is not only regulated by the expression of protein, but also by the phosphorylation level. eIF4E phosphorylation enhances its mRNA transport functions and its transformation activity in cell culture (37).

The present study observed that compared with the normal gastric mucosa, the expression of eIF4E demonstrated no significant difference in the GCA, while the level of eIF4E phosphorylation was significantly increased. In addition, the level of p-eIF4E in GCA was closely corelated with lymph node metastasis. The phosphorylation of eIF4E in the lymph node metastasis group was markedly increased compared with that of the non-lymph node metastasis group. Thus, the level of eIF4E phosphorylation may be correlated with carcinogenesis and progression in GCA and therefore may serve as a molecular marker for the invasion and metastasis of GCA.

Based on our results, we suggest that the mTOR/4E-BP1 signaling pathway is activated in GCA and that sustained activation of the mTOR signaling is able to induce gene expression and lead to excessive cell proliferation and tumor formation. mTOR/4E-BP1 signaling may play a significant role in the carcinogenesis and progression of GCA. The signals are not

only a molecular marker for malignant changes, but they also provide the rationale for targeting this pathway therapeutically in GCA patients.

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