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

HAI YAN YANG1, LI YING XUE2, LING XIAO XING2, JUAN WANG1, JUN LING WANG1, XIA YAN2 and XIANG HONG ZHANG1,2

1Department of Pathology, The Second Hospital, Hebei Medical University, Shijiazhuang 050000; 2Department of Pathology, Hebei Medical University, Shijiazhuang 050017, P.R. China

Received August 11, 2012; Accepted November 12, 2012

DOI: 10.3892/mmr.2012.1208

Abstract. The mammalian target of rapamycin/eukaryotic translation initiation 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 (1.822±0.63 vs. 0.997±0.38, P<0.05). The expression of p-4E-BP1 was closely correlated with lymph node metastasis (r=0.38, P<0.05), and the levels of p-eIF4E were closely correlated with lymph node metastasis (r=0.38, 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 GPPP, 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).

Correspondence to: Dr Xiang Hong Zhang, Department of Pathology, The Second Hospital, Hebei Medical University, Shijiazhuang, Hebei 050000, P.R. China
E-mail: zhangxianghong2008@163.com

Key words: mTOR, 4E-BP1, eIF4E, gastric cardiac adenocarcinoma
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 antibodies 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.

**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. 1.84±0.45, P<0.05; Fig. 3). There was no significant difference in eIF4E expression between GCA and the corresponding normal gastric mucosa (2.02±0.63 vs. 0.99±0.80, P<0.05), while the level of p-eIF4E was significantly higher in GCA in comparison to the corresponding normal gastric mucosa (1.82±0.63 vs. 0.99±0.80, 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 correlated with age, differentiation, tumor size, infiltration depth or lymph node metastasis (P>0.05), while the level of p-eIF4E was closely
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 correlated 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 correlated 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.

<table>
<thead>
<tr>
<th>Clinicopathological factors</th>
<th>All N (%)</th>
<th>H</th>
<th>L</th>
<th>P-value</th>
<th>H</th>
<th>L</th>
<th>P-value</th>
<th>H</th>
<th>L</th>
<th>P-value</th>
<th>H</th>
<th>L</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>17</td>
<td>13</td>
<td>4</td>
<td>0.465</td>
<td>12</td>
<td>5</td>
<td>0.721</td>
<td>10</td>
<td>7</td>
<td>1.000</td>
<td>10</td>
<td>7</td>
<td>1.000</td>
</tr>
<tr>
<td>≥60</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td></td>
<td>10</td>
<td>6</td>
<td></td>
<td>9</td>
<td>7</td>
<td></td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>15</td>
<td>8</td>
<td>7</td>
<td>0.126</td>
<td>11</td>
<td>4</td>
<td>0.712</td>
<td>9</td>
<td>6</td>
<td>1.000</td>
<td>8</td>
<td>7</td>
<td>0.493</td>
</tr>
<tr>
<td>Well/moderate</td>
<td>18</td>
<td>15</td>
<td>3</td>
<td></td>
<td>11</td>
<td>7</td>
<td></td>
<td>10</td>
<td>8</td>
<td></td>
<td>12</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Length of tumor (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>17</td>
<td>14</td>
<td>3</td>
<td>0.141</td>
<td>12</td>
<td>5</td>
<td>0.721</td>
<td>9</td>
<td>8</td>
<td>0.728</td>
<td>12</td>
<td>5</td>
<td>0.296</td>
</tr>
<tr>
<td>≥5</td>
<td>16</td>
<td>9</td>
<td>7</td>
<td></td>
<td>10</td>
<td>6</td>
<td></td>
<td>10</td>
<td>6</td>
<td></td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Depth of tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td>0.397</td>
<td>5</td>
<td>2</td>
<td>1.000</td>
<td>5</td>
<td>2</td>
<td>0.670</td>
<td>6</td>
<td>1</td>
<td>0.202</td>
</tr>
<tr>
<td>T2/3/4</td>
<td>26</td>
<td>17</td>
<td>9</td>
<td></td>
<td>17</td>
<td>9</td>
<td></td>
<td>14</td>
<td>12</td>
<td></td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (N1/2/3)</td>
<td>19</td>
<td>13</td>
<td>6</td>
<td>1.000</td>
<td>14</td>
<td>5</td>
<td>0.459</td>
<td>10</td>
<td>9</td>
<td>0.723</td>
<td>11</td>
<td>8</td>
<td>1.000</td>
</tr>
<tr>
<td>Negative (N0)</td>
<td>14</td>
<td>10</td>
<td>4</td>
<td></td>
<td>8</td>
<td>6</td>
<td></td>
<td>9</td>
<td>5</td>
<td></td>
<td>9</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

H, higher expression in tumor tissue than in normal; L, lower expression in tumor tissue than in normal. *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.
and the subsequent beginning of protein translation (25). eIF4E and allows its binding to the cap structure of the mRNA. 4E-BP1 binds to and thereby inactivates the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E), which serves as a molecular marker for the invasion and metastasis of GCA. The signals are not sustained activity in 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 overexpression 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 correlated 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.

Acknowledgements

This work was supported by Key R&D Project of Hebei Province (No. 07276102D), the Natural Science Foundation of Hebei Province (No. H2012206134), the project of Educational Commission of Hebei Province (No. 20111105) and the Department of Health founded project of Hebei Province (No. 20120269).

References