Clinical value of CagA, c-Met, PI3K and Beclin-1 expressed in gastric cancer and their association with prognosis

XIAOJUN HUANG[1,2*], CHAOQUN WANG[1,3*], JINMIN SUN[3], JUN LUO[1], JIANGZHOU YOU[1], LINCHUAN LIAO[1] and MINGYUAN LI[1]

[1]Department of Microbiology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041; [2]Department of Pathogenic Biology and Immunology, School of Medicine, Hubei University for Nationalities, Enshi, Hubei 445000; [3]Department of Pathology, Xuzhou Medical College, Xuzhou, Jiangsu 221004, P.R. China

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Abstract. Gastric cancer (GC) is the fourth most common type of malignant tumor worldwide, and causes the second highest number of cancer-associated mortalities in 2012. Gastric tumorigenesis is a multistep and multifactorial process. In the present study, tissue microarray and immunohistochemistry analysis were used to detect cytotoxin-associated gene A (CagA), c-Met, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and Beclin-1 expression in 121 GC tumors and 120 normal gastric tissues. The clinical relevance and prognostic implications of CagA, c-Met, PI3K and Beclin-1 expression in GC patients were analyzed. Furthermore, the Cox proportional hazards model was performed to indicate the independent prognostic factors for GC patients, including various clinicopathological parameters and CagA, c-Met, PI3K and Beclin-1 expression. The results indicated that CagA-positive *H. pylori* infection, c-Met, PI3K and Beclin-1 may have major roles in the oncogenesis, invasion and lymph node metastasis of GC. The disease-free survival rate was negatively associated with the expression of c-Met and CagA in tissues, and was positively associated with Beclin-1 expression. Overall survival was also negatively associated with the expression of c-Met and PI3K, and was positively associated with Beclin-1 expression. This indicated that c-Met and Beclin-1 may be independent and efficient biomarkers for predicting the DFS of patients with GC. Furthermore, in CagA-positive *H. pylori* infection-associated GC, c-Met expression was significantly upregulated and Beclin-1 expression was significantly downregulated. CagA-positive *H. pylori* infection therefore associated with the c-Met signaling pathway and the suppression of autophagy in the neoplasia, invasion and metastasis of GC.

Correspondence to: Professor Mingyuan Li, Department of Microbiology, West China School of Preclinical and Forensic Medicine, Sichuan University, 17 People's South Road, Chengdu, Sichuan 610041, P.R. China
E-mail: lmy3985@sina.com

*Contributed equally

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Introduction

In February 2014, the World Health Organization (WHO) released the 2014 World Cancer Report (1). The report included the estimation that in 2012, there were 14 million new cases of cancer and 8.2 million cancer-associated mortalities worldwide, of which 21.9 and 26.8%, respectively, occurred in China. Gastric cancer (GC) is the fourth most common type of malignant tumor worldwide and causes the second largest number of mortalities (2). Every year, ~1 million individuals are diagnosed with GC, including ~42% of patients with cancer in China (3). According to the WHO data, there were 404,996 newly diagnosed patients with GC in China in 2012. The number of relatively young patients with GC is gradually increasing (3). According to clinical and epidemiological studies, the incidence of GC is as high as 6-11% among 35-year-olds, as much as three times higher than rates reported in other countries (3). GC-associated mortality rates appear to be trending downwards; however, the GC 5-year survival rate is 30-57.1% in China, causing ~300,000 lives to be lost every year (2,4,5).

Gastric tumorigenesis is a multistep and multifactorial process involving genetic, regional, environmental and dietary factors. The International Agency for Research on Cancer confirmed that *Helicobacter pylori* infection was the most significant risk factor for GC in 2012 (6). *H. pylori*-mediated GC progression is dependent on the bacterial components expressed, including urease enzymes, adhesins, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A. CagA-positive *H. pylori* strains contain a functional type IV secretion system (TFSS) to facilitate the transfer of bacterial products into host cells (7). Inside the host cell, CagA localizes to the inner surface of the plasma membrane, disrupts cell-cell junctions and undergoes tyrosine phosphorylation interactions from a number of host proteins (8,9). CagA is the only identified protein to be delivered through the TFSS into epithelial cells (5). Previous studies have demonstrated that individuals infected with CagA-positive *H. pylori* strains present increased inflammation and have a higher risk of developing peptic ulcer disease and GC (10-12).

The receptor for hepatocyte growth factor/scatter factor, c-Met, is a receptor tyrosine kinase with a well-documented participation in cell invasion (13-16). *H. pylori* induces an invasive phenotype in gastric epithelial cells through a mechanism that requires the TFSS and the phosphorylation of c-Met. Churin *et al* (17) reported that c-Met was phosphorylated in response to co-culture with CagA-positive *H. pylori*; however, the study did not elucidate the mechanism for c-Met activation. The phosphorylation of c-Met can activate the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt serine/threonine kinase signaling pathway, which suppress autophagy, and promotes tumor cell invasion and the expression of oncogenes. Akt-mediated regulation of autophagy and tumorigenesis is achieved through Beclin-1 phosphorylation (18). Previous studies have suggested that the c-Met/PI3K signaling pathway is particularly active in tumors of the salivary glands, osteosarcoma, ovarian cancer and cervical cancer (19-22). Whether the c-Met/PI3K signaling pathway and the associated suppression of autophagy are involved in CagA-positive *H. pylori* infection-associated GC has yet to be established.

In the present study, a systematic immunohistochemical analysis of CagA, c-Met, PI3K and Beclin-1 expression in GC tissues was performed. Subsequently, the association between c-Met, PI3K and Beclin-1 expression with clinicopathological factors and CagA-positive *H. pylori* infection-associated GC was assessed. Disease-free survival (DFS) and overall survival (OS) time data were analyzed to describe the prognostic implications of c-Met, PI3K, Beclin-1 and CagA expression.

Materials and methods

Tissue specimens. A total of 121 patients with GC were included in the study, including 71 males and 50 females; the patients were aged 34-79 years, with a mean age of 61 years. An additional 120 healthy individuals, to provide normal tissue controls acquired by gastroscopy, were enrolled in the present study. The healthy individuals included 60 male and 60 female volunteers aged between 30 and 66 years. The GC tissue specimens (≥5 cm from the tumor tissue) were collected from resective surgery in the Affiliated Hospital of Xuzhou Medical College (Xuzhou, China) from June 2008 to June 2010. Tissue specimens were formalin-fixed and paraffin-embedded. Patients who had received preoperative chemotherapy, radiotherapy or other treatments were excluded from inclusion in the study, as were those with a family history of GC, or associated inflammatory disease.

All specimens were pathologically verified by the Department of Pathology at Xuzhou Medical College. Tumor staging was classified according to the American Joint Committee on Cancer GC TNM staging system (7th edition) (23). DFS was defined as the period between surgery and the day that GC recurrence was first detected. When there was no detected recurrence, the date of patient mortality or last follow-up was used. OS was defined as the period between surgery and the date of patient mortality. The total follow-up period was 5 years after the surgery for the primary lesion. Data including gender, age, histological grade and stage were retrieved from surgical and pathological records.

Informed consent was obtained from all individuals enrolled in the study, and the Xuzhou Medical College Ethics Committee provided ethical approval for the study.

Tissue microarray and immunohistochemistry. The formalin-fixed, paraffin-embedded 4 μm-thick tissue samples were deparaffinized in xylene (twice for 10 min), rehydrated in a graded series of ethanol (100, 95, 90, 80 and 75% for 5 min each) and rinsed with tap water.

To produce a tissue microarray, hematoxylin and eosin staining was performed to differentiate carcinoma tissue from the surrounding tissue. A total of 3 sites, which were considered typical cancerous gland tissues, were selected by two pathologists of Xuzhou Medical College (Xuzhou, China) from each sample for inclusion in the tissue microarray. The tissue microarray preparation instrument was the MiniCore Tissue Arrayer (ALPHELYS, Plaisir, France), which was used with a 1.0 mm sampling needle diameter and 2.0 mm core spacing in a 10x7 organization of 70 sites, in each of 9 tissue chip wax blocks. Each tissue array block was incubated at 52˚C to integrate for 2 h, then stored at 4˚C until later use.

Immunohistochemical studies for CagA, c-Met, PI3K and Beclin-1 were performed on 4 μm-thick tissue sections by machine (Roche Benchmark XT) using the streptavidin-peroxidase method. Antigen retrieval was performed in a microwave at pH 6 for 3 min at 80˚C and 3 min at 50˚C. The activity of endogenous peroxidases was blocked through incubation with 3% hydrogen peroxidase at room temperature for 20 min. Primary antibodies, consisting of rabbit monoclonal antibodies against CagA (dilution, 1:50; cat. no., sc-25766; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), c-Met (dilution, 1:100; cat. no., ab51067; Abcam, Shanghai, China), PI3K (dilution, 1:25; cat. no., ab86714; Abcam) and Beclin-1 (dilution, 1:100; cat. no., ab55878; Abcam), were incubated with the tissue sections at 4˚C overnight. Incubation with the alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:500; cat. no. ZB-2308; ZSGB-BIO Technology, CO., Ltd., Beijing, China) was performed for 15 min at 37˚C. The sections were developed with 3,3'-diaminobenzidine solution (Maxim Biotech, Inc., Rockville, MD, USA) for 2 min at room temperature and counterstained with hematoxylin. The tissues were then rinsed with PBS for 5 min and differentiated with 0.1% HCl in alcohol at room temperature for 10 sec. Finally, the sections were dehydrated, cleared and mounted.

Immunohistochemical scoring. Sections were observed with a light microscope at x200 magnification (DM2500; Leica Biosystems, Wetzlar, Germany) and representative images were captured. Immunostaining was classified according to its location (membrane, cytoplasm or nucleus). Immunoreactivity for CagA, c-Met, PI3K or Beclin-1 were defined by the presence of yellow cytoplasm staining. No evaluation of staining intensity was used in the present study; only the percentage of tumor cells was assessed (0-100%) for each sample, by counting the frequency of stained cells in five high-power fields (magnification, x400), each containing ~100 cells. The percentage of immunoreactive tumor cells was scored on a scale of 0-4: 0, no staining; 1+, 1-10%; 2+, 11-30%; 3+, 31-50%; 4+, ≥50%). The expression levels for each protein were divided into two groups, according to score: Low (1+, 2+) and high (3+, 4+).