Overexpression of the c-met Protooncogene in Human Gastric Carcinoma

Correlation to Clinical Features

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Overexpression of hepatocyte growth factor receptor (c-met) has been detected in many human tumors. To investigate the possible involvement of c-met in human gastric carcinogenesis, we examined c-met expression in 45 patients with gastric carcinoma using Northern blot analysis, reverse transcription-polymerase chain reaction (RT-PCR), and immunohistochemical staining. The c-met mRNA expression was increased twofold and sevenfold in gastric carcinoma tissues compared to the adjacent normal tissues by Northern blot analysis and RT-PCR, respectively. In the immunohistochemical study, c-met protein was detected in 32 of 45 (71.1%) patients, with marked overexpression in gastric carcinoma compared with matched normal gastric tissues. The c-met-positive immunoreactivities were more frequently encountered in serosa-exposed and serosa-infiltrating gastric cancer ($p = 0.003$). In addition, tumor stage was another statistically significant parameter that was observed between the two groups ($p = 0.02$). Multivariate analyses revealed that the tumor stage ($p = 0.014$) and c-met overexpression ($p = 0.031$) were independently correlated with survival. These data suggest that overexpression of c-met may play a part in gastric carcinogenesis and may represent a prognostic factor for gastric cancer.

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Gastric cancer is one of the most prevalent malignant tumors in Taiwan, and is also the third leading cause of cancer death (1). In Taiwan, the disease results in an annual mortality rate of 9.9 per 100 000 inhabitants. Despite the declining incidence of gastric cancer over the past several decades worldwide (2–5), the prognosis of gastric carcinoma is still dismal because of a high incidence of metastases and recurrence. Carcinogenesis is thought to be a multistep process resulting from a series of genetic alterations including activation of protooncogenes and inactivation of tumor suppressor genes. The protooncogene c-met encodes a transmembrane tyrosine kinase receptor for hepatocyte growth factor, a potent epithelial and endothelial mitogen. Overexpression of the hepatocyte growth factor receptor has also been implicated in neoplasia through constitutive activation of tyrosine kinase encoded in the receptors (6–8). However, the molecular events that lead to the development of gastric cancer remain poorly defined.

The c-met oncogene was originally identified in rearranged form by transfection of DNA from a human osteosarcoma cell line treated in vitro with a chemical carcinogen (9, 10). Recently, it has been argued that overexpression of the c-met gene encoding receptor for hepatocyte growth factor (HGF) may contribute to the development and progression of human tumors, both in vitro and in vivo (11–15). Overexpression and amplification of the met/HGF receptor was demonstrated in gastric carcinoma tissues and cell lines using immunohistochemistry and Southern blot hybridization (16–18). Furthermore, Kaji et al. indicated that c-met gene products were perhaps closely related to the proliferation or invasion of gastric cancer cells (19). However, there has been little information on c-met expression at both mRNA and protein levels in gastric cancer tissues at various stages of disease. In the present study, the c-met mRNA expression between gastric cancer tissues and their matched normal tissues was detected by Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). In addition, we examined the c-met protein expression in human gastric carcinoma using the immunohistochemical method, and then compared this with the clinicopathological parameters of gastric cancer.

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MATERIAL AND METHODS

Patients and tumor specimens

Cancer specimens were obtained surgically from 45 patients with pathologically proven gastric carcinoma at the Department of Surgery of Kaohsiung Medical University Hospital from January 1997 to May 1998. The patient group included 33 males and 12 females (average age, 65 years; ranging from 32 to 84 years). The clinicopathological records of these patients included sex ratio, age, depth of invasion, location, maximum tumor size, lymph node metastases, peritoneal metastases, liver metastases, microscopic tumor stage and histological features. Clinicopathological descriptions were carried out according to the General Rules for the Gastric Cancer Study in Surgery and Pathology from the Japanese Research Society for Gastric Cancer (JRSGC) (20). All patients were carefully followed-up until December 2000. All samples were collected immediately after surgical resection, frozen instantly in liquid nitrogen, and then stored in a freezer at $-80°C$ until analyzed.

Total RNA isolation and first-strand cDNA synthesis

Total RNA from gastric tissues was extracted using the acid guanidinium thiocyanate-phenol/chloroform method (21). Its concentration was determined spectrophotometrically on the basis of its absorbance at 260 nm. First-strand cDNA was synthesized from total RNA (1.2 μg) using 5 × buffer solution (50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$), 10 mmol/L dithiothreitol, 0.5 mmol/L dNTP (deoxynucleotides), RNase inhibitor (50 U/μL), 0.1 mg/mL bovine serum albumin (BSA), Moloney murine leukemia virus reverse transcriptase (2000 U/μL; Gibco BRL, Life Technologies Inc., Grand Island, NY, USA). The reaction was performed at 42°C for 2 h and stopped by adding 2 μl of 0.2 M EDTA.

Northern hybridization

Using electrophoresis, 20 μg total RNA was fractionated on 1.2% agarose formaldehyde gels and then transferred to a nylon membrane (Schleicher and Schuell, Dassel, Germany). The membrane was hybridized with a random primed, ^{32}P-labeled, 655 bp-c-met cDNA for 16 h at 65°C, according to the standard technique. After hybridization, the blot was washed twice in a solution containing 0.5% sodium dodecyl sulfate and 3 × SSC for 15 min at 65°C, then washed in 0.1% sodium dodecyl sulfate and 0.5 × SSC 65°C. Blots were exposed to Kodak XAR (Eastman Kodak, Rochester, NY, USA) film at $-70°C$. We rehybridized each blot with a ^{32}P-labeled β-actin probe to standardize the amount of RNA in each lane.

Multiplex PCR

In order to further compare the relative c-met mRNA level in the gastric cancer tissues and the corresponding adjacent normal tissues, multiplex PCR was performed by using β-actin primers as internal control to correct the differences in total RNA amounts between the tumor tissues and normal tissues. PCR was carried out at a final concentration of 1 × PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2 mmol/L $MgCl_2$), 50 μmol/L dNTP, 0.1 μmol/L sense and antisense primers for c-met and 0.01 μmol/L sense and antisense primers for β-actin (Table 1), as well as 2.5 U *Tag* DNA polymerase (Promega Corp., Madison, WI, USA) in a total volume of 50 μl. The amplification cycles were 35 s at 94°C, 10 s at 66°C and 30 s at 74°C in a programmable thermal cycler (PTC-100, MJ Research, Watertown, MA). The cycle was repeated 35 times, and then the PCR products were analyzed in 3% agarose gel. The signals on the UV transilluminator were scanned with a computing laser densitometer (Alpha Inotech, San-Leandro, CA, USA) to calculate the relative mRNA density ratio between the two groups.

Immunohistochemistry

The frozen tissue samples were mounted in Tissue-Tek OCT compound (Ames Division, Miles Laboratory, Elkhart, IN, USA), and 8-μm sections of each specimen were cut, air dried, and fixed in acetone for 10 min. The antigen enhancement was performed by immersing the slides in 10 mM citric acid buffer (pH 6.0) for 15 min at 800 W in a microwave. By incubating the slides with 3% hydrogen peroxide for 5 min, we blocked endogenous peroxidase activity. After washing in TBS, the slides were incubated with diluted rabbit polyclonal antibody against a C-terminus of c-met peptide (C-28) (Santa Cruz Biotechnlogy, Inc., Santa Cruz, CA, USA) at a dilution of 1 : 200. The primary antibody was detected using the avidin-biotin-complex-peroxidase technique (Vectastain Elite kit, Vector Laboratories, Inc., Burlingame, CA, USA) with biotinylated anti-rabbit IgG as second antibody. The reaction was developed with diaminobenzidine-hydrogen peroxide (Sigma Chemical Company, St Louis, MO, USA) as chromogen and the sections were then counterstained with light hematoxylin. To examine the

Table 1

Nucleotide sequence of primers used for RT-PCR analysis

	Forward primer	Reverse primer
c-met	5′-ACAGTGGCATGTCAACATCGCTCTAATTCAGAG-3′	5′-GCTTCCACTCTATATTTAGCTCGCTGTTC-3′
β-actin	5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′	5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′

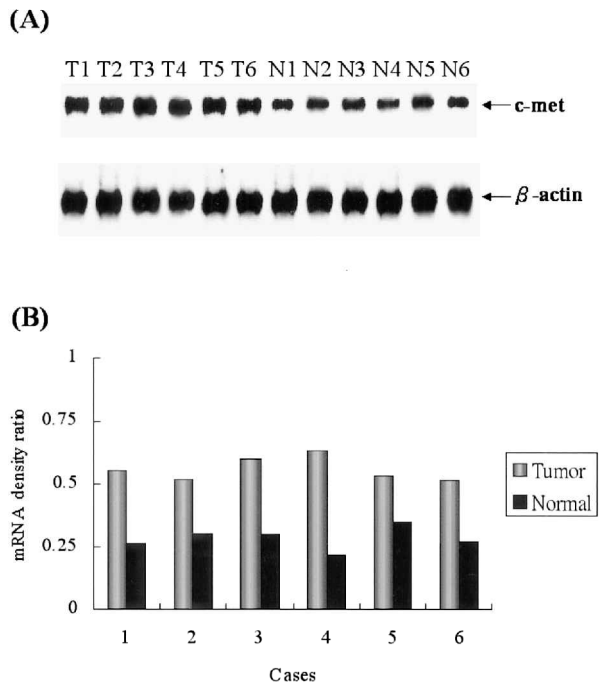

Fig. 1. Results of the Northern blot analysis. The RNA was electrophoresed, blotted, hybridized to a ^{32}P-labeled 655-bp c-met probe and rehybridized with an 807-bp *Pst*I fragment of the β-actin probe to correct for differences in loading (A). The density ratio of mRNA was considerably higher in the gastric cancer tissues than the normal gastric tissues (B; $p = 0.004$). N = normal, T = tumor.

false-positive tumors, we used a non-immune antiserum instead of the primary antibody as negative control. The percentage of positively stained tumor cells was evaluated for each tumor section. And the tumors were considered as positive immunreactivities if $\geq 5\%$ of neoplastic cells showed distinct plasma membrane staining.

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences Ver. 8.0 software (SPSS Inc., Chicago, IL, USA). The association between the expression of c-met protein and histopathological features was analyzed by the χ^2 test. Student's t-test was utilized for comparing age between the c-met positive and negative groups. In the multivariate analysis, independent prognostic factors for survival were determined using the Cox proportional hazards model. The c-met mRNA expression of the gastric cancer tissues and adjacent normal tissues was determined by the non-parametric Mann–Whitney U-test. A probability of less than 0.05 was accepted as significant.

RESULTS

Expression of the c-met protooncogene

To determine whether there was any difference in level of c-met mRNA between gastric carcinoma tissues and adjacent normal tissues, a Northern hybridization was carried out. Northern blot analysis with a 655 bp c-met cDNA fragment provided evidence of quantitative c-met mRNA (9 kb) overexpression in gastric cancer tissues (Fig. 1A). There was a twofold increment in c-met mRNA compared to that in the paired remnant normal gastric tissues, and the density ratios of mRNA between both groups were statistically significant ($p = 0.004$, Fig. 1B). With respect to the expression of c-met mRNA by RT-PCR, an amplified product for c-met of 655 bp was visible on the ethidium bromide-stained agarose gel of both the gastric cancer tissues and the adjacent normal tissues (Fig. 2A). An 807-bp DNA fragment was amplified from specific β-actin primers. The density ratios of mRNA were markedly increased in gastric cancer tissues compared with the normal tissues ($p = 0.009$, Fig. 2B). The c-met mRNA level was shown to be increased sevenfold in gastric carcinoma tissues compared with the matched normal tissues.

Expression of c-met protein

Of the 45 samples of gastric cancer tissue analyzed, the c-met protein was detected in 32 (71.1%) of them, with marked overexpression in gastric carcinoma compared to

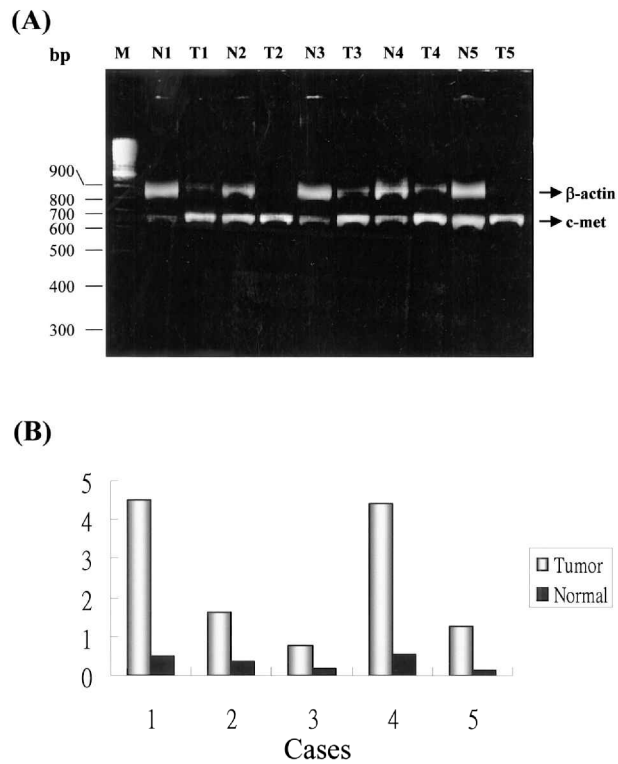

Fig. 2. Comparison of c-met mRNA expression in the gastric cancer tissues and their matched normal tissues by RT-PCR. A 655-bp DNA fragment representing the c-met gene and an 807-bp band representing the β-actin gene were amplified (A). The density ratio of mRNA was significantly higher in gastric cancers than in the normal tissues (B; $p = 0.009$). M = marker; N = normal; T = tumor.

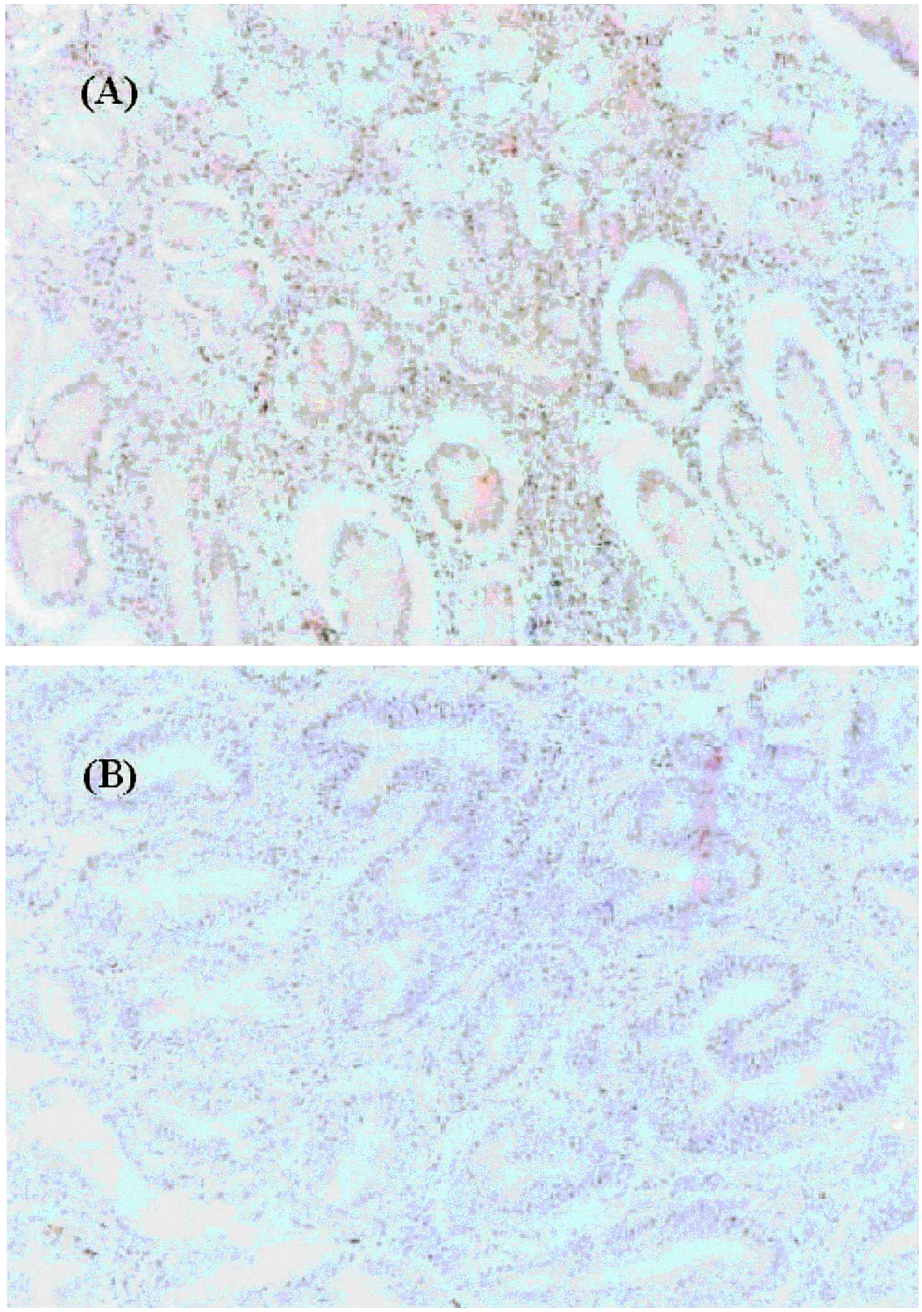

Fig. 3. Immunohistochemical studies of c-met protein in gastric cancer. Cryostat sections were fixed in acetone and stained with rabbit polyclonal antibody against a C-terminus of c-met peptide (C-28) and detected by the peroxidase–antiperoxidase method. The slides were counterstained with hematoxylin. Positive staining was found over the plasma membrane of gastric cancer cells (A). The staining was absent over the control section when the primary antibody was replaced with non-immune antiserum (B).

corresponding normal gastric tissues. All cases considered as positive showed brownish cytoplasmic staining but not nuclear staining (Fig. 3A). The absence of staining was found over the negative control when the primary antibody was replaced with non-immune antiserum (Fig. 3B). The clinical and histological features between patients with or without c-met protein expression in gastric cancer tissues are summarized in Table 2. The mean age of patients with c-met expression was 64.0 ± 2.3 years and that of patients without c-met expression was 67.2 ± 3.2 years. With regard to the histological features of primary tumor, signet ring cells and mucinous carcinomas were detected in 4 (12.5%) patients and 3 (9.4%) patients in the c-met-positive group, whereas no signet ring cell and mucinous carcinomas were observed in patients in the c-met-negative group. However, the statistical analysis was not significant ($p = 0.188$). There were no statistical differences between status of c-met protein and the clinicopathological features studied, with the exception of depth of tumor invasion ($p = 0.003$) and tumor stage. ($p = 0.02$). The incidence of

Table 2

Relationship between c-met protein expression in gastric cancer and clinicopathological variables

	c-met stain		
	Positive (n = 32)	Negative (n = 13)	p-value
Age (year)	64.0 ± 2.3 [a]	67.2 ± 3.2 [a]	0.451
Sex			
Male	22	11	0.275
Female	10	2	
Location of tumor			
Upper third	3	3	0.383
Middle third	9	2	
Lower third	20	8	
Maximum tumor diameter			
<5 cm	15	9	0.173
≥5 cm	17	4	
Depth of tumor invasion (JRSGC)			
t1 and t2	7	9	**0.003**
t3 and t4	25	4	
Lymph node metastasis			
Positive	23	7	0.245
Negative	9	6	
Liver metastasis			
Positive	3	0	0.253
Negative	29	13	
Peritoneal dissemination			
Positive	5	1	0.478
Negative	27	12	
Stage (JRSGC) [b]			
I, II	8	8	**0.02**
III, IV	24	5	
Histological features			
Well differentiated	3	4	0.188
Moderately differentiated	8	2	
Poorly differentiated	14	7	
Signet ring cell	4	0	
Mucinous	3	0	

[a] Mean ± SE.
[b] JRSGC = Japanese Research Society for Gastric Cancer.

c-met protein expression was significantly higher in the t3 and t4 gastric cancers (78.1%) compared to the t1 and t2 gastric cancers (30.8%), and also higher in stages III and IV gastric cancers. Table 3 shows that the independent prognostic factors identified by multivariate analysis were tumor stage ($p = 0.014$) and c-met overexpression ($p = 0.031$). Furthermore, c-met expression in gastric cancer was identified as being a significant and powerful prognostic indicator.

DISCUSSION

In currently conducted studies it has been found that alterations in oncogenes and tumor suppressor genes make up the mutational network leading to the development of tumors. Oncogenes encoding tyrosine kinase are amplified and/or overexpressed in a variety of human cancers. The c-met protooncogene encodes a transmembrane protein with structural and functional features of a growth factor receptor, the HGF receptor. This 190-kDa protein is composed of two disulfide-linked chains: an extracellular 50-kDa α-subunit and a transmembrane 145-kDa β-subunit endowed with tyrosine kinase activity (22, 23). Overexpression of c-met is found in some cell lines and tumors. However, little information is available regarding c-met expression in gastric cancer.

In our study, the c-met expression in gastric cancer was examined at both the mRNA and the protein levels using Northern blot analysis and RT-PCR, as well as immunohistochemical staining. By analyzing the expression of c-met mRNA in human gastric carcinoma tissues and adjacent normal tissues with Northern hybridization and RT-PCR, we found a marked increase in c-met expression in carcinoma tissues. The immunohistochemical study revealed that positive staining of c-met was found in 71.1% of gastric carcinoma tissues, indicating that c-met protein expression was a frequent event in gastric cancer. Thus, it was further shown that c-met was overexpressed in gastric cancer tissues. The increased c-met protein in gastric carcinoma tissues might have resulted from the increased level of c-met mRNA.

In agreement with previous investigators (16–18), we observed an amplification or overexpression of c-met in human gastric cancer tissues and gastric cancer cell lines. Moreover, we found a significant correlation between c–met protein expression and tumor stage, as well as depth of tumor invasion, which is a similar finding to that from a previous report (24). In addition, no statistical differences were shown between c-met expression and other variables in the tumor stage including lymph node

Table 3

Factors significantly associated with survival after multivariate analyses using Cox stepwise proportional hazard model

Variable	β	SE	p-value	Relative risk	95% confidence interval
Positive c-met immunoreactivities	2.23	1.04	0.031	9.30	1.22–70.81
Tumor stage (I and II, III and IV)	1.85	0.75	0.014	6.38	1.46–27.92

β = coefficient; SE = standard error.

metastases, liver metastases, and peritoneal metastases according to the JRSGC classification. Therefore, we suggest that depth of tumor invasion was the most important variable related to c-met expression. Although c-met overexpression is not related to positive lymph node metastases, liver metastases or peritoneal metastases, this is not ruled out, because of the limited number of cases in our study. The higher incidence of c-met expression was observed in serosa-exposed (t3) and serosa-infiltrating (t4) gastric cancer; thus, c-met overexpression might be closely related to serosa invasion and tumor progression. Since serosa invasion was a significant prognostic factor in patients with gastric cancer (25), c-met could be a vital prognostic factor in these patients, a supposition that is in agreement with Nakajima et al. (24). Multivariate analyses further identified that c-met overexpression is correlated with survival; hence c-met may be a negative prognostic factor in patients with gastric cancer (17, 18, 26). Recently, it was indicated that c-met appears to affect the invasiveness of scirrhous gastric cancer cells (27). However, our data indicated that c-met overexpression is not related to positive lymph node, liver metastases, or peritoneal dissemination. Accordingly, c-met might be related to locally aggressive gastric cancer but not to metastatic disease. In contrast to the findings of Wu et al. (26), we could not detect any correlation between the histological features and c-met expression. Therefore, further investigations about histological subtypes of gastric carcinoma and c-met expression are necessary to elucidate the viewpoint.

In conclusion, we identified that c-met expression is increased in gastric carcinoma at both the mRNA and the protein levels. We suggest that overexpression of the c-met protooncogene may play an important role in the pathogenesis and progression of human gastric carcinoma. Moreover, c-met immunopositivity is an independent prognostic factor for patients with gastric cancer.

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