

## Expression of phosphatase regenerating liver 3 is an independent prognostic indicator for gastric cancer

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### Abstract

**AIM:** To investigate the prognostic significance of phosphatase regenerating liver 3 (PRL-3) protein expression in gastric cancer.

**METHODS:** PRL-3 expression in paraffin-embedded tumor specimens from 293 patients with gastric cancer was studied retrospectively by immunohistochemistry. Monoclonal antibody specifically against PRL-3, 3B6, was obtained with hybridoma technique.

**RESULTS:** Positive PRL-3 expression was detected in 43.3% (127 of 293) of gastric cancer cases. High expression of PRL-3 was positively correlated with tumor size, depth of invasion, vascular/lymphatic invasion, lymph node metastasis, high TNM stage and tumor recurrence. Patients with positive PRL-3 expression had a significantly lower 5-year survival rate than those with negative expression (28.3% vs 51.9%,  $P < 0.0001$ ). Patients who received curative surgery, and with positive PRL-3 expression had a significant shorter overall survival and disease-free disadvantage over patients with negative expression (hazard ratio

of 16.7 and 16.6, respectively;  $P < 0.0001$  for both). Multivariate analysis revealed that PRL-3 expression was an independent prognostic indicator for overall and disease-free survival of gastric cancer patients, particularly for survival in TNM stage III patients.

**CONCLUSION:** PRL-3 expression is a new independent prognostic indicator to predict the potential of recurrence and survival in patients with gastric cancer at the time of tumor resection.

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**Key words:** Phosphatase regenerating liver 3; Gastric cancer; Prognosis; Recurrence; Antibody

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### INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies in the world with a high incidence and death rate. In China, it remains the most frequent cancer and the second cancer-related cause of death with a high case fatality<sup>[1]</sup>. TNM staging system is used worldwide to predict prognosis and direct therapeutic decisions of patients with GC. The 5-year survival rate in patients with stage I GC is close to 90% and around 10% for patients with stage IV GC<sup>[2]</sup>. However, the prognoses of patients with stage II and III GC are more heterogeneous and less predictable by staging criteria. Therefore, finding molecular markers that are able to predict the potential of tumor recurrence and prognosis of patients is extremely important for appropriate individualized therapy. Phosphatase regenerating liver 3 (PRL-3) (also known as PTP4A3) belongs to a newly

discovered group of phosphatase regenerating liver family which is implicated in oncogenic and metastatic processes<sup>[3,4]</sup>. The PRL family represents a protein tyrosine phosphatase superfamily possessing a unique COOH-terminal prenylation motif and a protein tyrosine phosphatase-active site signature sequence CX5R<sup>[5,6]</sup>. Three PRLs (PRL-1, -2, and -3) are highly homologous with similar amino acid sequence of 76%-87%<sup>[7-9]</sup>. Recent reports found that PRL-3 was consistently expressed at higher level of metastasis in liver compared to normal colorectal epithelia and primary cancer tissues<sup>[10,11]</sup>. A growing body of evidence showed that an excess of PRL-3 phosphatase is a key alteration contributing to the acquisition of metastatic properties of the tumor cells. For example, nontumorigenic or low metastatic cell lines transfected with wild type PRL-3 displayed higher cell motility and invasiveness and could induce metastatic tumor formation in mice, while cells expressing catalytically inactive mutant PRL-3 significantly reduced the migratory capability<sup>[12,13]</sup>. Knockdown of endogenous PRL-3 in cancerous cells using small interfering RNA or phosphatase inhibitors can abrogate cell motility and the ability to form metastasis-like tumors in mice<sup>[12-15]</sup>. PRL-3 was further demonstrated to be a useful indicator for tumor recurrence and patient outcome in several human cancers including colorectal cancer and breast cancer<sup>[16-19]</sup>. In gastric cancer, PRL-3 was found to be highly expressed in tumor metastatic lymph nodes and closely associated with the peritoneal metastasis<sup>[20-23]</sup>, but the prognostic impact of PRL-3 expression in gastric cancer still remains to be further investigated.

In this study, we detected the expression of PRL-3 in GC tissue samples by immunohistochemistry using a PRL-3 specific monoclonal antibody 3B6 to investigate PRL-3 protein expression in GC tissues and whether PRL-3 could be applied as a prognostic indicator for GC to predict the potential of tumor recurrence and patient outcome.

## MATERIALS AND METHODS

### Patients

This retrospective study enrolled patients who underwent clinical surgery for primary gastric cancer at the Department of Surgery, Beijing Cancer Hospital, Peking University School of Oncology between July 1994 and December 2000. Patients with inadequate histologic specimens or missing clinical information were excluded. A total of 293 patients were finally included. There were 194 males and 99 females, with ages ranging from 25 to 82 (mean  $\pm$  SD, 58  $\pm$  17.1 years). Two hundred (68.3%) patients received curative resection (R0) with radical lymph node dissection; the remaining 93 (31.7%) patients with microscopic or macroscopic tumor residues were given palliative resection (R1/R2). Site distribution of the primary tumor was 153 at antrum, 52 at cardia or fundus, and 88 at corpus. Tumor size ranged from 5 to 120 mm (mean, 43.8 mm).

### Histology

Data were collected from clinical case report record and follow-up database. Tumor staging was based on the clinical evaluation and postoperative pathological reports. TNM staging was on the basis of the 1997 fifth edition of AJCC/UICC TNM staging criteria for gastric cancer<sup>[24]</sup>. The tumors were histologically classified according to the WHO classification criteria.

### Follow-up

None of these patients had received radiotherapy or chemotherapy preoperatively. All the patients were followed up at regular intervals of 6 mo after surgery until June 2006 with a minimum of five years. Tumor recurrence was clinically defined as the reappearance of tumor after curative surgery. The overall survival time was calculated from the date of surgery to the date of last visit or death and the disease-free survival time from the date of resection to relapse.

### Immunostaining of PRL-3

Tumor tissue specimens from the 293 patients were routinely fixed in 10% formalin and embedded with paraffin. Paraffin embedded tissue samples were cut into 4  $\mu$ m sections. The sections were put in an oven at 60°C for 5 h and cooled down overnight before they were deparaffinized in xylene. The sections were then dehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwaving the sections in 1 mmol/L EDTA (PH 8.0) for 15 min. PRL-3 monoclonal antibody 3B6 (a generous gift from Prof. Shou, Beijing Institute for Cancer Research, China)<sup>[25]</sup> was used as the primary antibody at a dilution of 1:100 overnight at 4°C. The Powervision two-step histostaining reagent PV-6002 (Dako, Glostrup, Denmark) was applied as the secondary antibody. The sections were visualized with diaminobenzidine and counterstained with hematoxylin. Each incubation step was followed by washing with phosphate-buffered saline. For negative control, the primary antibody was omitted from the reaction sequence. Sections of liver metastasis from colon cancer with known strong PRL-3 immunoreactivity were used as positive controls. The number of tumor cells with cytoplasm staining of PRL-3 was counted without knowledge of the clinicopathological data, and > 5% positive tumor cells were defined as positive PRL-3 expression<sup>[19]</sup>.

### Statistical analysis

Statistical analyses were performed with SAS 8.1 software. The  $\chi^2$  test was used to analyze the association between PRL-3 expression and clinicopathological features of GC. Cumulative survival rates and differences in survival curves were estimated by Kaplan-Meier method with the log-rank test. The effect of PRL-3 on survival was analyzed using the Cox proportional hazard regression model adjusted for clinical and histopathologic features. Two-sided *P* values

**Table 1 Association between PRL-3 expression and clinicopathological features**

Factors	Patients	PRL-3 expression		P value
		Positive (n = 127)	Negative (n = 266)	
Gender				
Male	194	85	109	NS
Female	99	42	57	
Age (yr)				
< 60	149	66	83	NS
≥ 60	144	61	83	
Tumor size (cm)				
≥ 5	118	63	55	0.004
< 5	175	64	111	
Depth of invasion				
T1	22	5	17	0.008 <sup>a</sup>
T2	46	15	31	0.009 <sup>b</sup>
T3	175	77	98	0.042 <sup>c</sup>
T4	50	30	20	
Lymph node metastasis				
N0	82	30	52	< 0.0001 <sup>d</sup>
N1	120	42	78	
N2	51	33	18	
N3	40	22	18	
Distant metastasis				
Present	42	21	21	NS
Absent	251	106	145	
Vascular/lymphatic invasion				
Present	162	89	73	< 0.0001
Absent	131	38	93	
Differentiation				
Poor	194	91	103	NS
Well/Moderate	99	36	63	
Surgical curability				
Curative	200	79	121	NS
Not curative	93	48	45	
Recurrence <sup>e</sup>				
Absent	177	63	114	0.002
Present	23	16	7	
TNM stages				
I	42	13	29	0.007 <sup>f</sup>
II	52	17	35	0.008 <sup>g</sup>
III	99	43	56	
IV	100	54	46	

NS: Not significant; <sup>a</sup>T1-T2 vs T3-T4; <sup>b</sup>T1-T3 vs T4; <sup>c</sup>T1 vs T2-4; <sup>d</sup>N0-N1 vs N2-N3; <sup>e</sup>200 cases received curative surgery; <sup>f</sup>I - II vs III-IV; <sup>g</sup>I - III vs IV.

of less than 0.05 were considered to be statistically significant.

## RESULTS

### Patient outcome

Forty-two patients were classified as stage I, 52 as stage II, 99 as stage III and 100 as stage IV. A total of 194 cases were poorly differentiated, 69 cases were moderately differentiated and the remaining 30 cases were well differentiated.

The follow-up period for survivors ranged from 2 to 120 mo (median, 31 mo). The 5-year overall survival rate was 41.7% in the entire cohort of patients, 92.9% in stage I, 72.5% in stage II, 32.5% in stage III and 12.3% in stage IV patients. One hundred and five patients remained alive and disease-free, 15 patients were alive with disease. One hundred and seventy patients died

of GC, and 3 patients died of other causes. Among the 200 patients who received curative surgery, 23 patients had tumor recurrence with 3 in peritoneum, 3 in lymph node, 7 in liver, 3 in other organs (2 ovarian, 1 lung), 4 in multiple organs and 3 in remnant stomach.

### PRL-3 expression in GC and its relation with clinicopathological features

PRL-3 immunostaining was predominantly localized in the cytoplasm of normal or tumor epithelial cells. PRL-3 stained cells in normal epithelia were mainly observed in the neck of gastric glands (Figure 1). Among the 293 GC specimens analyzed, 127 (43.3%) tumors had positive PRL-3 expression. The rate of positive PRL-3 expression was significantly higher in stage III and IV than in stage I and II (48.7% vs 31.9%,  $P = 0.007$ ). High expression of PRL-3 was correlated closely with large tumor size, depth of invasion in gastric wall, lymph node metastasis, vascular/lymphatic invasion and recurrent frequency. No significant correlation was observed between PRL-3 expression and sex, age, distant metastasis, grade of differentiation and surgical curability (Table 1).

### Univariate survival analysis of prognostic impact of PRL-3 expression

Kaplan-Meier method with log-rank test revealed that patients with positive PRL-3 expression had a significantly lower cumulative 5-year overall survival rate than those with negative expression (28.3% vs 51.9%,  $P < 0.0001$ ). Among the 99 patients with stage III GC, those with positive PRL-3 expression had a lower survival rate than those with negative expression (18.6% vs 43.2%,  $P = 0.0004$ , Figure 2). Among the 200 patients who received curative surgery, patients whose tumor had positive PRL-3 expression had worse disease-free status and poorer overall survival (hazard ratio, 16.6 and 16.7 respectively;  $P < 0.0001$  for both) than those with negative expression (Figure 3). Among patients who received palliative resection or patients in stages other than stage III, PRL-3 showed no significant correlation with prognosis.

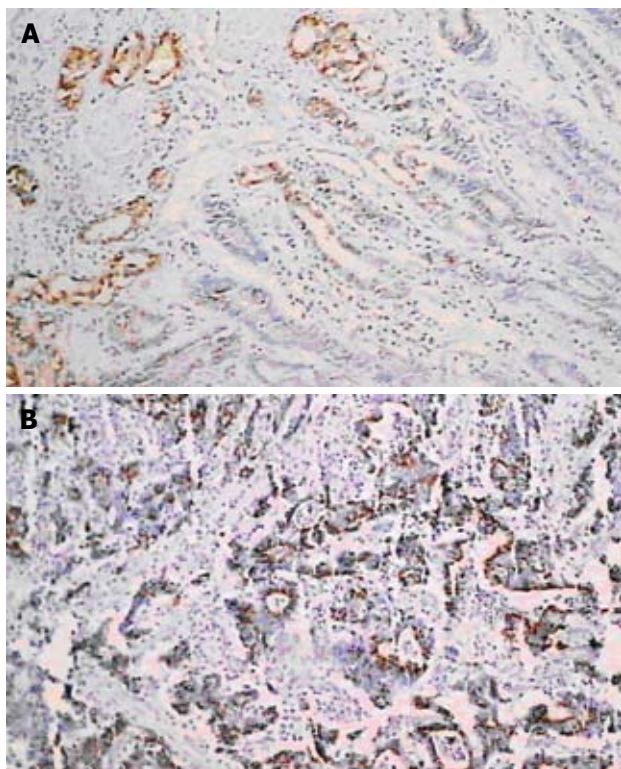
### Multivariate survival analysis of prognostic impact of PRL-3 expression

Multivariate analysis by extended Cox regression model revealed that PRL-3 expression remained an independent prognostic factor after adjusting for sex, age, tumor location, tumor size, depth of invasion, lymph node metastasis, distant metastasis, TNM staging, vascular/lymphatic invasion, and surgical curability. PRL-3 expression was a significantly independent prognostic factor for the overall survival of all 293 GC patients. For the 200 patients who received curative resection, PRL-3 expression was found to be an independent prognostic factor for both disease-free and overall survival. The results are shown in Table 2.

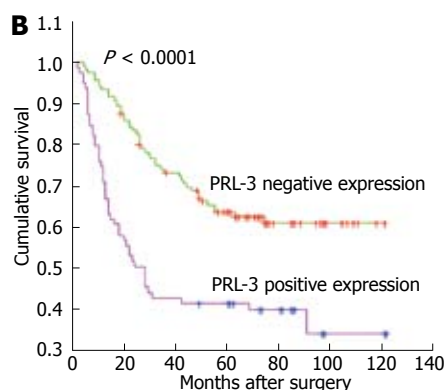
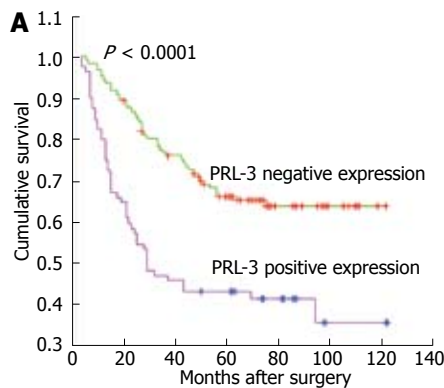
## DISCUSSION

In this study, we detected the protein expression

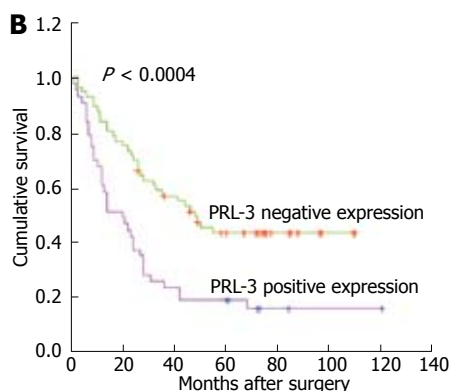
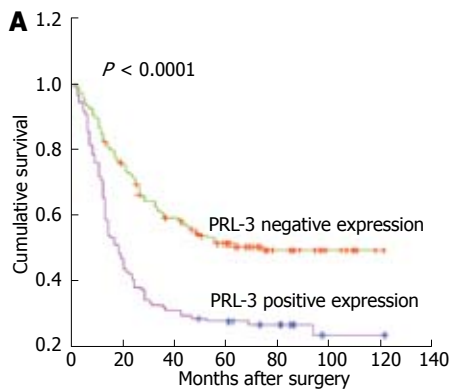




**Figure 1 Immunohistochemical staining.** A: PRL-3 is negative or weak in adjacent (3 cm away from the tumor) normal gastric epithelial mucosa ( $\times 40$ ); B: In positive cases, PRL-3 expression in cancer cell cytoplasm is strong ( $\times 200$ ).



**Figure 3 Patients who underwent curative surgery.** A: Overall survival; B: Disease-free survival. Significant differences were observed between the PRL-3 negative and positive groups.



**Figure 2 Overall survival curve.** A: Entire cohort of 293 patients; B: Patients with stage III. Significant differences were observed between the two groups with PRL-3 negative and positive expression.

of PRL-3 in GC tissues using the highly specific monoclonal antibody 3B6 prepared by Peng *et al.* PRL-3

had higher rates of positive expression in advanced stages and PRL-3 expression was positively correlated with tumor size, depth of invasion, and lymph node metastasis vascular/lymphatic invasion at the time of surgery and recurrence. These results suggest that PRL-3 may play a crucial role in invasion, progression and metastasis of GC. The present analyses revealed that PRL-3 was an independent prognostic indicator for overall and disease-free survival of GC. Among patients with advanced TNM stages especially stage III, patients with positive PRL-3 expression have more frequent recurrence and poorer survival, adjuvant therapies such as radiotherapy and chemotherapy may be necessary after curative surgery. Evaluation of PRL-3 expression status may identify a subset of patients with GC who require more intensive treatment.

Miskad *et al.*<sup>[20]</sup> reported that PRL-3 was highly expressed in metastatic lymph nodes of GC and high expression of PRL-3 was closely associated with tumor stage. Wang *et al.*<sup>[21]</sup> found that the high expression of PRL-3 in lymph node metastases had a negative impact on the prognosis of patients with GC. Li *et al.*<sup>[22-23]</sup> reported that PRL-3 expression was correlated with peritoneal metastasis and poor prognosis in GC patients. The superiority of our study may be the use of antibody specifically against PRL-3 and the relatively extensive clinical data which facilitated the analysis from multiple angles. One limitation of our study is that the relatively small sample hindered the analysis in stage I and stage II patients.

Table 2 Multivariate analysis of PRL-3 expression by Cox proportional hazard model

Factors	Overall survival						Disease-free survival		
	All patients (n = cases)			Curatively resected (n = 200)			Curatively resected (n = 200)		
	P	Relative risk	95% CI	P	Relative risk	95% CI	P	Relative risk	95% CI
PRL-3	< 0.001	1.76	1.30-2.40	< 0.001	2.35	1.54-3.59	< 0.001	2.46	1.62-3.73
T	< 0.001	3.16	1.79-5.58	0.003	3.04	1.47-6.29	0.004	2.76	1.38-5.52
N	< 0.001	3.29	1.93-5.60	< 0.001	3.99	2.21-7.20	< 0.001	4.67	2.59-8.43
D	0.04	1.38	1.01-1.88	0.32	1.23	0.82-1.87	0.015	1.69	1.11-2.57
S	< 0.001	2.24	1.54-3.26	-	-	-	-	-	-

PRL-3 expression, positive *vs* negative; T: Depth of invasion, T3-4 *vs* T1-2; N: Lymph node metastasis, present *vs* absent; D: Tumor size,  $\geq 5$  cm *vs*  $< 5$  cm; S: Surgical curability, palliative *vs* curative.

Attributed to the high sequence similarity of three PRLs and the wide expression of PRL-1 and PRL-2 in normal tissues and cancer cell lines, commercial polyclonal antibody against PRL-3 used in previous studies could potentially cross-react with PRL-1 and PRL-2. Monoclonal antibody specifically reacting with PRL-3 is extremely important to exclude the interference of PRL-1 and PRL-2 and therefore allows us to accurately evaluate the prognostic implication of PRL-3 expression<sup>[25,26]</sup>. To prepare specific PRL-3 monoclonal antibody, Peng *et al.*<sup>[25]</sup> obtained the monoclonal antibody 3B6 with hybridoma technique, and confirmed its specificity with ELISA and Western blotting assays. High specificity of the monoclonal antibody 3B6 against PRL-3 was demonstrated. The applicability of the monoclonal antibody has been further confirmed by two other studies investigating the prognostic impact of PRL-3 expression in colorectal cancer and breast cancer<sup>[16,19]</sup>.

PRL-3 has been confirmed to be an important metastatic instrumental molecule. Although the actual signal transduction pathways in which PRL-3 is implicated are largely unknown, Rho signaling pathway molecules which are regulators of motility and invasion have been identified as potential candidate targets of PRL-3. PRL-3 transfectants displayed altered extracellular matrix adhesive property and up-regulated integrin-mediated cell spreading efficiency<sup>[14,27]</sup>. Peng *et al.*<sup>[28]</sup> recently found that PRL-3 activates the mitogen-associated protein kinase pathway by binding a cell membrane protein in cell migration and invasion. PRL-3 was also found to be associated with membrane structures including ruffles, protrusions, and some vacuolar-like membrane extensions which have been demonstrated to play a role in cell movement and invasion<sup>[29-31]</sup>. Besides, PRL-3 may be involved in triggering angiogenesis and establishing microvasculature *in vitro*<sup>[32-34]</sup>. These findings suggest that PRL-3 may be implicated with the key steps of tumor metastasis including tumor cell invasion and survival in circulation and vasculature formation.

In addition to its role in predicting tumor recurrence and prognosis, PRL-3 has a potential value of being a candidate for metastasis tailored therapies. Since primary tumors can be surgically resected, the metastatic tumors are the main cause responsible for a high case fatality. PRL-3 was highly expressed in tumor metastasis and

found to play a key role in tumor metastatic process<sup>[10-21]</sup>. PRL-3 may serve as a potential therapeutic target for cancer metastases. Inhibition of PRL activity might be carried out using phosphatase inhibitors targeting the consensus phosphatase motif, farnesyltransferase inhibitors, interference RNA or monoclonal antibody as well<sup>[9,35-37]</sup>. Recent progress in active recombinant PRL-3 production and findings on PRL-3 structure will undoubtedly facilitate the development of PRL-3 inhibitors<sup>[38-40]</sup>. Detection of PRL-3 expression would be able to provide supportive information for anti-cancer therapy.

In conclusion, PRL-3 is closely associated with tumor invasion and lymphatic metastasis and is identified as a new prognostic indicator to predict tumor recurrence and patient survival in GC.

## COMMENTS

### Background

It is established that phosphatase regenerating liver 3 (PRL-3) is consistently expressed in liver metastasis of colon cancer. A recent study reported that PRL-3 expression was related to peritoneal metastasis of stomach cancer.

### Research frontiers

In a few studies, the association of PRL-3 expression with prognosis of cancers was investigated and demonstrated that this is related to poor prognosis of breast and stomach cancer. The antibody used in many studies was commercial polyclonal antibody that could cross-react with PRL-1 and PRL-2.

### Innovations and breakthroughs

A recent study showed that PRL-3 was expressed in 70.4% of 637 GCs. PRL-3 expression was correlated with peritoneal metastasis. Patients with PRL-3 negative expression had a better survival rate than those with positive PRL-3 at all stages. In this study, PRL-3 was found to express in 43.3% (127/293) of primary tumor tissues of GC. PRL-3 protein expression was demonstrated to be an independent predictor for poor prognosis in GC. In addition, a specific monoclonal antibody to PRL-3 was used, allowing us to accurately evaluate the prognostic significance of PRL-3 expression in GC.

### Applications

Defection of PRL-3 protein expression in primary tumor tissues of GC might be helpful in identification of GC patients with poor prognosis who should receive more intensive treatment. In addition, PRL-3 may serve as a potential therapeutic target for GC.

### Terminology

PRL-3 (also known as PTP4A3) is a member of phosphate of regeneration liver (PRL) family including PRL-1, PRL-2 and PRL-3 which are implicated with oncogenic and metastatic processes of tumors. The excess of PRL-3 phosphates is an alteration contributing to the acquisition of metastatic properties of tumor cells. The monoclonal antibody 3B6 used in this study is a specific monoclonal antibody to PRL-3 generated by Peng *et al.*, Beijing Institute for Cancer Research.

**Peer review**

The authors examined the expression of PRL-3 protein in primary tumor tissues of 293 cases of GC. It revealed that patients with negative PRL-3 expression had a higher 5-year survival rate than those with positive PRL-3 expression. PRL-3 is an independent prognostic indicator for GC. The findings are of clinical significance.

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