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ORIGINAL ARTICLE

GPRC5A overexpression predicted advanced biological behaviors and poor prognosis in patients with gastric cancer

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Abstract G protein-coupled receptor, family C, group 5,
member A (GPRC5A) had received attentions for its role in
carcinogenesis and prognostic values in several types of can-
cer. However, the functional roles of GPRC5A in gastric can-
cer (GC) had never been elucidated. The expression levels of
GPRC5A were detected by real-time quantitative reverse tran-
scription PCR and Western blot in GC tissues and adjacent
non-tumor tissues. GPRC5A expression in tissue sections of
106 GC samples was evaluated using immunohistochemistry.
The staining results were compared with clinicopathological
factors and to the prognosis of GC patients. The mRNA and
protein expression levels of GPRC5A in gastric cancer tissues
were higher than those in adjacent non-tumor tissues. Positive
GPRC5A expression was significantly correlated with larger
size of primary tumor, diffuse type (Lauren's classification),
deeper serosal invasion, and more lymph node metastasis. In
addition, Kaplan–Meier curve analysis demonstrated that GC
patients with positive GPRC5A expression had poor progno-
sis than those with negative GPRC5A expression. GPRC5A
expression was identified as an independent factor of the over-
all survival in GC patients by multivariate Cox analysis. Fur-
ther, the overall survival difference existed between patients
with GPRC5A positive and negative groups in GC patients
with lymph node metastasis. Our results suggested that

elevated levels of GPRC5A played significant roles in GC
progression. GPRC5A could serve as a prognostic biomarker
of GC.

Keywords Gastric cancer · GPRC5A ·
Immunohistochemistry · Prognosis

Introduction

Gastric cancer (GC) was one of the most common malignant
tumors and the second leading cause of cancer death world-
wide [1]. Recent progress in early diagnosis, surgical tech-
niques, perioperative management, and the use of chemother-
apy had improved the outcomes of patients; however, GC
remained a major clinical challenge because of its high prev-
alence, poor prognosis, and limited treatment options [2–4].
Thus, there was an urgent need for identification of novel
markers for GC.

The G protein-coupled receptor, class C, group 5, member
A (GPRC5A), also known as retinoic acid-induced gene 3
(RAI3) or retinoic acid-induced gene 1 (RAIG1), was first
cloned in 1998 [5]. The GPRC5A gene was located on chro-
mosome 12p13-p12.3, and its promoter contained an RA re-
sponse element to which nuclear retinoid receptors (RAR and
RXR) can bind [6]. In recent years, GPRC5A had been receiv-
ing increasing attentions as it had been shown to play impor-
tant roles in human cancers, dysregulation of GPRC5A gene
had been associated with several cancers including lung can-
cer [7], breast cancer [8], and colorectal cancer [9]. In non-
small cell lung carcinoma, Tao and colleagues [6] reported
that the mRNA expression levels of GPRC5A were lower in
human lung tumors than in adjacent normal tissues. The mean
GPRC5A mRNA levels in different kinds of pathological type
were different; finally, they identified GPRC5A gene as a new

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tumor suppressor gene. Liu and colleagues [10] found that in oral squamous cell carcinoma, high levels of GPRC5A expression were detected in normal oral tissue; further combining with clinical features, they found that the GPRC5A expression was associated with pathologic differentiation grade. In the breast cancer, GPRC5A mRNA was up-regulated in breast cancers and breast cancer cell lines, and siRNA against high levels of GPRC5A gene can suppress breast cancer cell growth [11].

However, the carcinogenic roles of GPRC5A in GC remained unknown. In this study, the expression of GPRC5A in GC was estimated using real-time quantitative reverse transcription PCR (qRT-PCR), Western blot analysis, and immunohistochemistry. In addition, we identified the relationships between GPRC5A expression and clinicopathological factors, as well as its relation with the overall survival (OS) of GC patients.

Methods

Ethics statement

This research was approved by the Ethics Committee of the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, and a written informed consent was obtained from each patient involved in the study.

Patients and specimens

To analyze the expressions of GPRC5A, 30 fresh GC tissues and 30 adjacent non-tumor tissues from patients with GC between April 2014 and December 2014 at the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine were collected. Table 1 showed the clinicopathological characteristics of 30 GC patients. In addition, paraffin-embedded gastric cancer samples were collected between January 2005 and December 2009. The inclusion criteria

Table 1 The clinicopathological factors in 30 gastric cancer patients

Variables	Cases
Gender (male/female)	17/13
Age (≤60/>60)	19/11
Size of primary tumor(≤5/>5)	16/14
Location of primary tumor (lower 1/3/middle 1/3/upper 1/3/more than 1/3)	16/5/7/2
Degree of differentiation(well or moderate/poor)	4/26
Lauren's classification(intestinal type/diffuse type)	23/7
Serosal invasion(T1/T2/T3/T4)	2/3/1/24
Lymph node metastasis(N0/N1/N2/N3)	8/9/6/7

included the following: (1) histologically proven adenocarcinoma, (2) no history of gastrectomy or other malignancies, and (3) availability of complete follow-up data. The exclusion criteria are as follows: (1) patients who underwent palliative surgery and (2) patients who had distant metastasis or peritoneal dissemination that was confirmed during the operation. Based on these criteria, 106 GC patients were enrolled in the present study. Clinicopathological data, including gender, age at surgery, size of the primary tumor, location of the primary tumor, Lauren's classification, degree of differentiation, serosal invasion, lymph node metastasis, and GPRC5A expression, were obtained from medical records.

Real-time quantitative PCR

Expression of GPRC5A mRNA was determined by real-time quantitative PCR (qRT-PCR) using ABI SYBR Green Master Mix (Life Technologies, CA, USA). Total sample RNA was normalized to endogenous GAPDH mRNA. Primers designed and utilized for GPRC5A and GAPDH were as follows: (forward/reverse sequences) GPRC5A (5′-GTGGAGAACAGAGCCTACTCT/TGAGCTCAGATGACCAGACCT-3′) and GAPDH (5′-TGGGTGTGAACCATGAGAAGT/TGAGTCCTTCCACGATACCAA-3′). Thermal cycling conditions included an initial hold period at 95 °C for 2 min; this was followed by a two-step PCR program of 95 °C for 10 s and 65 °C for 40 s repeated for 40 cycles on an StepOnePlus system (ABI, CA, USA). The relative quantification of GPRC5A expression was normalized to GAPDH value ($2^{-\Delta\Delta CT}$ method).
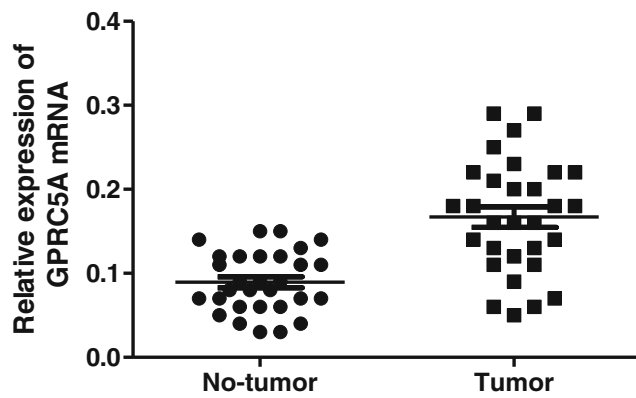
Western blot

Tissues were added to 1 mL of 100 mmol/L Tris/HCl (pH 7.5), 100 mmol/L NaCl, 0.5 % sodium deoxycholate, 1 mmol/L ethylenediaminetetraacetic acid, 1 % Nonidet P-40, and 0.1 % sodium dodecyl sulfate and protease inhibitor. After blocking was performed, 50 μg of the sample was incubated for 60 min with rabbit anti-GPRC5A (Abcam, ab155557, 1:1000 dilution) at room temperature. A gel imager system (Asia Xingtai Mechanical and Electrical Equipment Company, Beijing, China) was used to analyze images and determine gray values.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were freshly cut (4 μm). The sections were deparaffinized in xylene and rehydration in a graded series of alcohols. The endogenous peroxidase activity was blocked by exposure to 3 % H_2O_2 for 5~10 min at room temperature. Then, the sections were
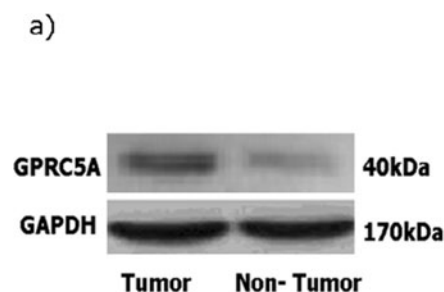
Fig. 1 Relative expression of GPRC5A mRNA in gastric cancer tissues and adjacent non-tumor tissues. The paired sample *t* test showed a significant difference between the two groups (*P*<0.05)

immersed in sodium citrate buffer (pH 6.0) and steamed at high power for 30 min. The sections were blocked with 1 % BSA, and immunohistochemical reactions were performed using the rabbit anti-human antibody detecting GPRC5A (Abcam, ab155557, 1:100 dilution) and HRP-conjugated secondary antibody. Finally, the visualization signal was developed with 3,3′-diaminobenzidine solution, and all slides were counterstained with 20 % hematoxylin. The slides were dehydrated and mounted on cover slips. For negative controls, PBS was used in place of the primary antibody.

Two independent pathologists randomly reviewed and scored each stained tissue section. In brief, 5×100 fields were evaluated per slide, and at least 100 cells were evaluated per field. We used a scoring standard for GPRC5A protein expression, and both distribution and intensity were considered. The positive staining percentage was scored as: 0 (0–9 %), 1 (10–25 %), 2 (26–50 %), 3 (51–75 %), or 4 (76–100 %). Intensity was scored as: 0 (negative staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The combined total score was multiplied by these two sets of numbers, ranging from 0 to 12. When the multiplication product of the two scores was ≥6, the samples were considered positively stained.

Follow-up

All patients were followed up every 6 months for 2 years, then every year or until death. Ultrasound, computed tomographic scans, chest X-ray, and endoscopy were performed at every visit. The median follow-up for the entire cohort was 42 (range, 3–78) months.
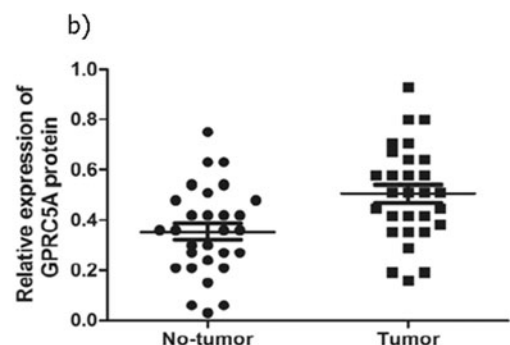
Statistical analysis

The mRNA and protein expression levels of GPRC5A in GC tissues compared with adjacent non-tumor tissues were analyzed using the paired sample *t* test. Associations between GPRC5A expression and clinicopathological factors were analyzed using the chi-square test. Univariate and multivariate Cox regression models were adopted to evaluate prognostic significance of clinicopathological factors. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analyses. *P*<0.05 was regarded as statistically significant.

Results

The GPRC5A mRNA and protein expression levels in gastric cancer

We performed qRT-PCR to analyze the GPRC5A mRNA in 30 fresh GC tissues and 30 adjacent non-tumor tissues. We found that the relative expression levels of GPRC5A mRNA in GC tissues were higher than those in adjacent non-tumor tissues (0.17 ± 0.06 vs 0.09 ± 0.03, *P*<0.05) (Fig. 1).

In addition, GPRC5A protein levels were measured by Western blotting. The results showed GPRC5A bands at the expected size of 40 kDa (Fig. 2a). The relative protein expression values of GPRC5A in GC tissues were higher than those in adjacent non-tumor tissues (0.51 ± 0.18 vs 0.35 ± 0.17, *P*<0.05) (Fig. 2b).

Fig. 2 **a** Expression of GPRC5A protein in gastric cancer tissues and adjacent non-tumor tissues. **b** The paired sample *t* test showed a significant difference between the two groups (*P*<0.05)

Fig. 3 **a**, **b** GPRC5A protein was positively expressed in gastric cancer tissues. **c**, **d** GPRC5A protein was negatively expressed in gastric cancer tissues. Magnification=×100 (**a**, **c**) or ×400 (**b**, **d**)
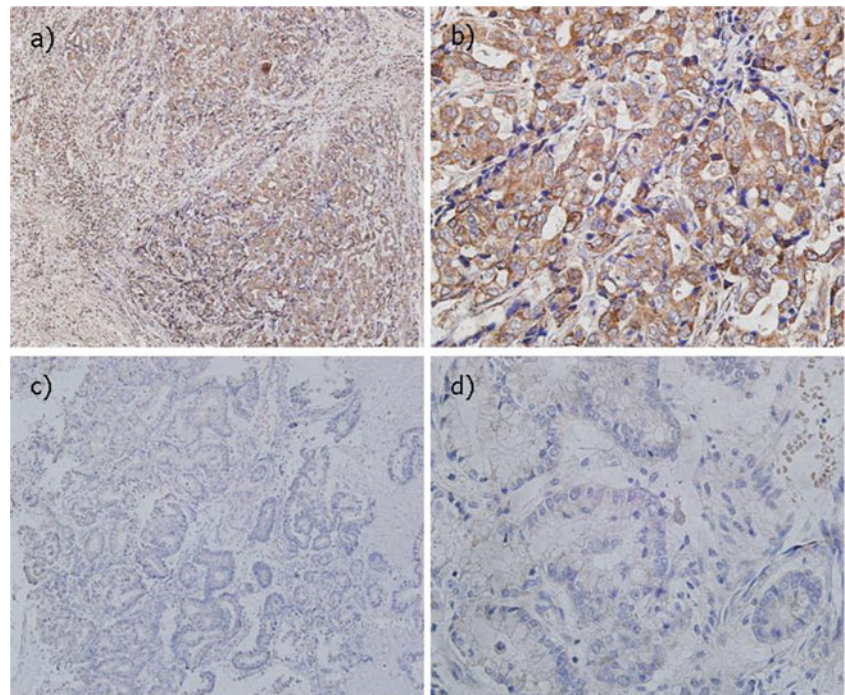
Table 2 Correlation between GPRC5A protein expression and clinicopathological factors in gastric cancer patients

Variables	Number of each group	GPRC5A expression		Chi-square value	P value
		Negative	Positive		
Gender				0.044	0.834
Male	61	27	34		
Female	45	19	26		
Age				0.008	0.928
≤60	64	28	36		
>60	42	18	24		
Size of primary tumor				12.518	<0.001
≤5	65	37	28		
>5	41	9	32		
Location of primary tumor				4.947	0.176
Lower 1/3	40	15	25		
Middle 1/3	20	7	13		
Upper 1/3	41	23	18		
More than 1/3	5	1	4		
Degree of differentiation				0.052	0.820
Well/moderate	10	4	6		
Poor	96	42	54		
Lauren's classification				8.190	0.004
Intestinal type	80	41	39		
Diffuse type	26	5	21		
Serosal invasion				10.640	0.014
T1	11	5	6		
T2	10	6	4		
T3	9	8	1		
T4	76	27	49		
Lymph node metastasis				8.799	0.032
N0	45	27	18		
N1	22	7	15		
N2	7	2	5		
N3	32	10	22		

Correlation between GPRC5A expression and clinicopathological features

GPRC5A protein was mainly localized in the cytoplasm of GC cells, and the positive staining was not seen in the smooth muscles, vessels, and stromal fibroblasts (Fig. 3). Of 106 GC specimens, we found 60 cases (56.6 %) were with positive GPRC5A expression. Table 2 summarized associations between the status of GPRC5A expression in GC tissue and various clinicopathological features. Positive GPRC5A expression was significantly correlated with larger size of primary tumor ($P<0.001$), diffuse type (Lauren's classification) ($P=0.004$), deeper serosal invasion ($P=0.014$), and more lymph node metastasis ($P=0.032$). By contrast, GPRC5A expression was not correlated with gender, age, location of primary tumor, and degree of differentiation ($P>0.05$).

Survival analysis for gastric cancer patients

Univariate analysis showed significant relationships between the OS and size of primary tumor ($P=0,023$), serosal invasion ($P=0,013$), lymph node metastasis ($P<0.001$), and GPRC5A expression ($P<0.001$), but not with gender, age, location of primary tumor, degree of differentiation, and Lauren's

Table 3 Univariate and multivariate analyses of overall survival in gastric cancer patients

Variables	5-YSR (%)	Univariate analysis		Multivariate analysis	
		Chi-square value	P value	HR value	P value
Gender		0.367	0.545		
Male	41.9				
Female	55.4				
Age		0.097	0.755		
≤60	49.9				
>60	46				
Size of primary tumor		5.155	0.023		
≤5	53.5				
>5	38.2				
Location of primary tumor		5.471	0.14		
Lower 1/3	59.9				
Middle 1/3	43.3				
Upper 1/3	41.1				
More than 1/3	20				
Degree of differentiation		2.671	0.102		
Well/moderate	68.6				
Poor	45.3				
Lauren's classification		0.17	0.681		
Intestinal type	50				
Diffuse type	46.8				
Serosal invasion		10.755	0.013		
T1	100				
T2	58.3				
T3	44.4				
T4	38.3				
Lymph node metastasis		46.163	<0.001	1.294	<0.001
N0	72.6				
N1	54.5				
N2	14.3				
N3	15.6				
GPRC5A expression		12.892	<0.001	0.457	0.01
Negative	64.5				
Positive	34.5				

5-YSR 5-year survival rates
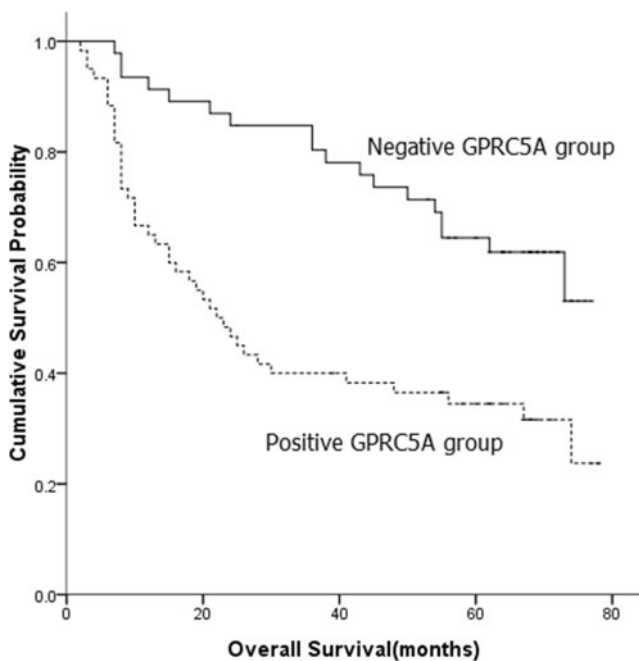
Fig. 4 The overall survival of the gastric patients with positive GPRC5A expression was lower than the patients with negative GPRC5A expression (*P*<0.001)

classification. Significant variables in univariate analysis were detected by multivariate analysis using the Cox proportional hazards model. We confirmed lymph node metastasis (HR= 1.294, *P*<0.001) and GPRC5A expression (HR=0.457, *P*= 0.010) as independent prognostic factors of overall survival (Table 3). Patients with positive and negative GPRC5A

expression showed 5-year overall survival rates of 34.5 and 64.5 %, respectively, (Fig. 4).

When stratified by lymph node metastasis, GC patients without lymph node metastasis had no overall survival difference between patients with GPRC5A positive and negative (*P*=0.385), while in patients with lymph node metastasis, significant differences were observed in the overall survival between GPRC5A positive expression and negative expression groups (*P*=0.013) (Fig. 5).

Discussion

GPRC5A mRNA was expressed at high levels in the normal lung and at low levels in other normal tissues such as the kidney, placenta, colon, testis, and ovary in normal human beings [12]. The aberrant expressions of GPRC5A gene were known to be involved in tumorigenesis and tumor progression in many types of human cancer. Zougman and colleagues [9] reported that the GPRC5A gene was expressed at lower levels in normal colon tissue than in colon cancer tissues. GPRC5A mRNA levels were lower in non-small cell lung cancer tissues than in adjacent normal tissues [6]. It was not clear whether GPRC5A acted as an oncogene or tumor suppressor in hepatocellular carcinoma (HCC), Zheng and colleagues [13] found that mRNA and protein expression levels of GPRC5A were both significantly higher in HCC than in normal liver tissues and overexpression of GPRC5A was present in 73.6 % of HCC tissues. However, Xin and colleagues [14] found that
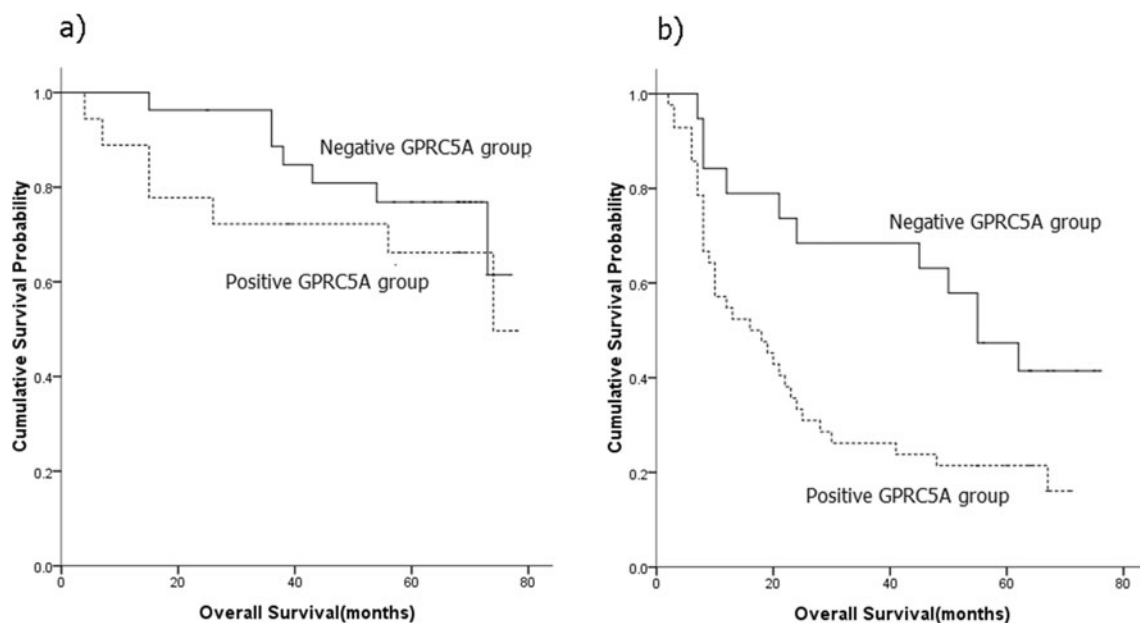
Fig. 5 a In gastric cancer patients without positive lymph node metastatic, no overall survival difference existed between GPRC5A positive and negative groups (*P*=0.385). **b** Positive GPRC5A

expression was found to have poorer prognosis in gastric cancer patients with positive lymph node metastatic (*P*=0.013)

mRNA level of GPRC5A gene was decreased in HCC cell lines. In gastrointestinal tumors, Wu and colleagues reported that the GPRC5A gene, as a new transcriptional target of p53, was elevatedly expressed in pancreatic ductal adenocarcinoma [15]. Cheng and colleagues [16] found that GPRC5A, which was associated with cancer cell proliferation, was also elevatedly expressed in gastric cancer. In our study, we found that the GPRC5A mRNA and protein levels were higher in gastric cancer tissues compared to adjacent non-tumor tissues, which was in line with the previous finding by Cheng et al. These results supported the hypothesis that GPRC5A functioned as an oncogene in gastric cancer.

Besides the results mentioned above, the GPRC5A expression data obtained from immunohistochemistry detection were analyzed for correlation with clinicopathological factors. Some studies had reported that GPRC5A expression levels were not associated with tumor stage, lymph node status, or histological tumor type [17], while other studies found that GPRC5A was a good predictor of tumor size in breast cancer [11]. In this study, we found that positive GPRC5A expression was significantly correlated with larger size of primary tumor, diffuse type, deeper serosal invasion, and more lymph node metastasis, which indicates that GPRC5A overexpression may affect the invasion, metastasis, and progression of GC, and overexpression of GPRC5A gene could indicate the aggressive behavior of cancer.

In the aspect of prognostic values of GPRC5A expression, Zheng and colleagues [13] reported that positive GPRC5A expression was associated with lower overall and lower disease-free survival rate in HCC patients after curative hepatectomy. While in colorectal cancer, Zougman and colleagues [9] found that when compared to negative GPRC5A expression group, positive GPRC5A expression group was significantly associated with disease recurrence. However, Jörissen and colleagues [17] reported that GPRC5A expression was not associated with overall survival and recurrence-free survival in breast cancer. In present study, Kaplan–Meier curves proved that patients with positive GPRC5A expression presented poorer overall survival than patients with negative GPRC5A expression. Multivariate Cox regression analysis confirmed that GPRC5A expression, along with lymph node metastasis, was an independent prognostic factor of GC prognosis. Additionally, in GC patients with lymph node metastasis, we found that the overall survival difference existed between patients with GPRC5A positive and negative groups. These findings suggested that GC patients with GPRC5A overexpression may be a high-risk group with poor survival and will need more aggressive additional systemic therapy.

GPRC5A played important roles in cancer cell differentiation, proliferation, and distal metastasis. However, the mechanism of the roles of GPRC5A was complicated. Deng and colleagues [18] had shown that down-expression of GPRC5A led to the activation of NF-κB in lung cancer cell lines, further promoting tumorigenesis. Other papers reported that the GPRC5A gene was related with STAT3 signaling pathway in the development of cancers [19]. GPRC5A also interacted with multiple proteins including EIF4A1 and HSPA9. Some studies even found GPRC5A was co-fractionated with GTF2F2, MPV17, NOLC1, SLC25A3, and EGFR in vivo [20, 21]. GPRC5A was also involved in regulating the cell cycle. The markers FEN1, MCM2, CCND1, and UBE2C were upregulated following loss of GPRC5A expression in cancers [22]. Future studies in this field were necessary because understanding of GPRC5A function in malignant transformation had the potential to improve prognostication in gastric cancer patients.

In conclusion, the results of this study showed that positive GPRC5A expression was related to more aggressive clinicopathological features and inferior outcome in gastric cancer. The expression of GPRC5A may serve as a potential prognostic biomarker in gastric cancer.

Conflicts of interest None

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