Increased Expression Regulator of G-protein Signaling 1 Predicts Unfavorable Prognosis of Gastric Cancer

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Abstract

Background: Regulator of G-protein signaling 1 (RGS1) expression has been reported to be a prognostic marker for specific cancers, but its function in gastric cancer (GC) remains to be elucidated.

Methods: RNA-sequencing data and clinicopathologic characteristics of 434 GC patients were collected from The Cancer Genome Atlas (TCGA) database. the expression level of RGS1 in GC tissues are significantly higher than pan-cancer tissues. The high expression of RGS1 is associated with worse progression of GC. The diagnostic value of RGS1 was evaluated by calculating receiver operating characteristic curve (ROC). Cox proportional hazards regression modeling and Kaplan-Meier curves were used to explore clinicopathologic characteristics related to overall survival (OS) in GC patients. Immunohistochemistry (IHC) was used to evaluate the impact of RGS1 on prognosis in the study population in our center.

Results: The expression level of RGS1 in GC were significantly higher comparing with normal stomach tissues (p=0.016). The expression of RGS1 was significantly associated with poor differentiation (p=1.917e-06), T stage (p=1042e-04), N stage (p=0.049) and TNM stage (p=0.004). The area under the ROC curve was 0.610. High RGS1 expression was significantly associated with poor OS (p=0.002). Multivariate analysis shows high RGS1 expression(p=0.031) was independent poor prognostic factors of OS.

Conclusion: High RGS1 protein expression was correlated with poor clinical outcomes, which was also independent predictors of worse OS. RGS1 may be a promising target for a new GC therapy.

Introduction

GC is the fifth most frequently diagnosed cancer and the fourth leading cause of cancer-related deaths in the world[1]. Incidence are markedly elevated in Eastern Asia. Most of patients with gastric cancer are advanced or metastatic on initial presentation. Although the overall response rate to chemotherapy is great, the median survival is only 9 to 11 months[2, 3]. GC is biologically and genetically heterogeneous with a poorly understood carcinogenesis at a molecular level. Therefore, biomarkers for early and accurate diagnosis of GC would contribute to improve prognoses for these patients.

RGS proteins are GTPase-activating proteins (GAPs) for Gα subunits. The function of GAPs is to desensitize continual G-protein-coupled receptors (GPCRs) stimulation by accelerating intrinsic GTPase activities[4]. Recently, multiple studies reported that GPCRs was involved in progression and metastasis of multiple cancers[5]. As a result, RGS proteins probably play an important role in the pathophysiology of cancers[6]. RGS1 is a member of R4 family of RGS proteins. It is mainly expressed on immunocyte such as T and B lymphocytes[7, 8], natural killer cells[9] and dendritic cells[10]. Initially, RGS1 was focused on its inhibitory role in B lymphocytes trafficking[11]. Other studies found out that RGS1 plays a stimulatory role in proliferation, and metastasis of malignancies[12] then more studies focused on the relationship between RGS1 and malignancies. These malignancies have been confirmed the existence of RGS1 gene

amplification including melanoma, non-Hodgkin lymphoma, retinoblastoma, pancreatic and nasopharyngeal carcinoma[12-15] and correlated with a more advanced disease stage or a more aggressive phenotype in these studies. However, the clinical significance and prognostic value of RGS1 in GC remains unclear.

In this study, we aim to explore the diagnostic and prognostic value of RGS1 expression in GC patients from TCGA database. And the impact of RGS1 expression on survival of GC patients in the Department of General Surgery of Tianjin Medical University General Hospital was examined by IHC. Furthermore, the biological pathway involved in GC pathogenesis-associated the RGS1 regulatory network was evaluated using Gene Set Enrichment Analysis (GSEA).

Methods

RNA-sequencing data and bioinformatics analysis

The RNA-sequencing data and clinicopathologic characteristics of GC patients were collected from TCGA database (https://portal.gdc.cancer.gov/repository) on May 11th, 2021. The clinicopathologic characteristics and their survival time and status of 434 GC patients were downloaded from TCGA Gene Expression Quantification (https://portal. gdc. Cancer. gov/). The RNA-sequencing data (HTSeq-FPKM) of 407 cases were also collected at the same time, including 32 normal samples and 375 tumor samples. Box plots were used to present differences in RGS1 expression and variables. Besides, finally 365 cases were used in survival analysis, and 317 cases were used in cox proportional hazards regression modeling analysis.

Gene set enrichment analysis and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis

Gene set enrichment analysis is widely used to provide insights into high throughput gene expression data. The statistically significantly differential expression between low and high expression groups provides directions for future research[16]. In the present study, GSEA was used to identify significantly enriched pathways in the high RGS1 expression phenotype. Moreover, the pathway analysis was performed in KEGG pathway. P-value < 0.05 and false discovery rate (FDR)< 0.25 were considered as enriched.

Patient population of collected cohort

Tumor tissue analysis was performed on patients from the Department of General Surgery of Tianjin Medical University General Hospital. All of the 58 patients who underwent radical gastrectomy from 2018 to 2019 were identified in this study. All patients were diagnosed as GC or carcinoma of the gastroesophageal junction (GEJ) Siewert type II or III. Patients who received neoadjuvant chemotherapy were excluded. All patients' specimens were collected and archived under protocols approved by the institutional Review Boards of Tianjin Medical University General Hospital. A portion of each patient's

tumor was fixed in 10% buffered formalin for at least 24 hours and processed into paraffin blocks. Clinicopathologic data were collected from institution's database. Pathologic stage was determined according to the 8th edition American Joint Committee on Cancer (AJCC) staging system.

Immunohistochemistry and Evaluation of Immunohistochemistry Staining of collected cohort

Since reverse transcription quantitative PCR (RT-qPCR) had been employed to detect RGS1 expression in tumor tissue in previous research, so RGS1 expression was analyzed by immunohistochemistry. First of all, all the samples of tumors were stained by H&E to ensure that an appropriate amount of tumors were present. After that, one representative tumor-tissue-containing block was selected to underwent IHC. Briefly, samples were fixed in formalin, dehydrated through a graded alcohol series, and embedded into paraffin sections which were placed on charged slides. The thickness of each slide is 5μm. All the slides need be stored in oven which maintained 56℃. These slides were rehydrated by graded alcohol and deparaffinized with xylene. Next, these slides were blocked by hydrogen peroxide for 25 minutes. Subsequently, antigen retrieval was performed in a 95℃ EDTA buffer for 15 minutes. Wait until it returns to room temperature. These slides were incubated with 5% BSA for 30 minutes. Afterwards the slides were incubated with primary anti-RGS1 antibody (ab117077, abcam) for one night at 4℃ in a wet box. In the second day, after the slides naturally warmed up to room temperature, added reaction enhancing solution incubating for 20 minutes. After the secondary antibody reaction, the slides were stained by DAB and hematoxylin. Finally the slides were sealed in neutral balsam and observed under microscope. The images of RGS1 IHC staining was shown in Figure 1.

Immunostained tissue slides were examined and scored independently and evaluated by two experienced pathologists who were blinded to patient outcomes. Antigen expression was defined as the presence of specific staining in the cytoplasm (for cancers) or gland cells (for adjacent non-tumor tissues). The evaluation was based on the staining intensity and staining extent. Staining intensity was scored as 0 (negative), 1 (weak positive), 2 (positive) and 3 (strong positive). Depending on the percentage of positively stained cells, staining extent was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The staining intensity score multiplied by the staining extent score makes the final staining score which ranges from 0 to 12. According to the cut-off value of ROC curves, RGS1 staining was scored low or high. Low RGS1 was defined as a total score ≤ 2.

Follow-up

All patients were followed up post-gastrectomy in every three months for the first two years, and in every six months for the next year. Each follow-up visit included a physician examination and laboratory tests, such as serum CEA and CA19-9. Abdominal enhanced CT and chest X-ray or chest CT were also performed. The median follow-up time was 35 months (range 2 to 50 months).

Statistical Analysis

All statistical analyses were performed using R software (V.3.5.2) and IBM SPSS version 26.0.0 (IBM Corporation Armonk, NY). The relationship between clinicopathologic characteristics and the RGS1 expression were performed by Kruskal-Walis H test. Correlations between RGS1 expression and clinical information were evaluated by $\chi 2$ test and Fisher test (categorical variables) and student's t-test (continues variables). Kaplan-Meier estimates were used to generate overall survival plots. On the other hand, the long-rank test was used to analyze statistical differences between these curves. Cox proportional hazards regression modeling was used in univariate and multivariate survival analyses. Significant difference was assumed as p value < 0.05.

Results

RGS1 expression in GA tissues

RGS1 expressions in GC tissues were explored using the RNA-seq data from TCGA database. It is shown in Fig 2 that the RGS1 expression of GC tissues was obviously higher than normal gastric tissues (p=0.016).

Clinicopathologic Characteristics

The clinicopathologic characteristics of 375 GC patients form TCGA database were retrospectively collected and analyzed. As shown in Fig 3(a-g), the expression of RGS1 was analyzed with variables like age, gender, differentiation, AJCC TNM stage. The results showed the expression of RGS1 was significantly associated with differentiation (p=1.917e-06), T stage (p=1042e-04), N stage (p=0.049) and TNM stage (p=0.004). These findings revealed that the high expression of RGS1 was correlated with advanced stage of GC than the low expression of RGS1.

Diagnostic value of RGS1 expression in GA patients

To evaluate the diagnostic efficacy of RGS1, we drafted a ROC curve according to the RNA-seq data of 317 GC patients from TCGA database. As shown in Fig 4, the area under the ROC curve was 0.610, which means RGS1 expression can be used to distinguish GC tissue from normal gastric tissue.

Survival outcome and multivariate analysis

As demonstrated in Fig 5, The overall survival of patients who expressed high RGS1 were poor compared with those with the low RGS1 expression (p=0.011). The following factors including age, advanced N stage, advanced AJCC stage and RGS1 expression were significantly poor prognostic factors in univariate analysis. Covariates for the multivariate analysis were selected based on the results of the univariate analysis and clinical reference. According to the multivariate analysis results, high RGS1 expression was significantly associated with poor overall survival (p=0.031). Age and advanced AJCC stage were also associated with poor overall survival (Table 2).

GSEA identifies functions and signaling pathways

To analyze the biological characteristics shared by biopsies displaying diferent RGS1 expression levels and predict the functions and pathways in which RGS1 may be involved, we performed the GSEA assay. KEGG analysis indicated that genes belong to the following processes: B cell receptor signaling pathway, chemokine signaling pathway, glyoxylate and dicarboxylate metabolism, hematopoietic cell lineage, jak stat signaling pathwy and leukocyte transendothelial migration were significantly enriched for in the RGS1 high-expressing GC samples. As shown in Fig 6.

RGS1 expression of collected cohort

In previous study, higher expression of RGS1 was significantly related to worse OS (p=0.017) at the genetic level[17]. According to clinicopathologic characteristics of 58 patients in the Department of General Surgery of Tianjin Medical University General Hospital (Table 2), ages of these patients were ranged from 51 to 84 (mean, 67.9±7.76 years). There were more male than female patients (41 to 17). More than half of the patients (70.7%) were under radical distal gastrectomy. Meanwhile 43 patients (74.1%) were poorly differentiated. Most of the patients were advanced disease. The median follow-up duration of this cohort group was 35 months (range, 2-50 months), and 22 patients (37.9%) died of their disease. These patients were divided into two groups according to the cut-off value of the IHC results of RGS1 protein. There was significant difference in CEA between the low- and high-level groups (4.65±6.5 vs 2.38±1.5). However, there were few significant correlations between RGS1 expression and other clinicopathologic characteristics such as age, gender, BMI, tumor size, tumor location, T stage, N stage, AJCC stage or type of surgery. As shown in Fig 7, compared with the low RGS1 expression group, median OS time of the high RGS1 expression group was shorter (19.5 to 36.5 months, respectively). In addition, the high RGS1 expression group showed a worse 3-year-survival rate than the low RGS1 expression group (45.8% vs 73.5%, respectively). The overall survival of patients which expressed high RGS1 was poor compared with those with the low RGS1 expression (p=0.017). Next, multivariate analysis of factors were performed. Table 3 shows the following factors and RGS1 expression are significantly negative prognostic factors on univariate analysis, which involved advanced T stage, advanced AJCC stage. These variables were involved in the multivariate analysis. According to the multivariate analysis result, high RGS1 expression was still significantly associated with poor overall survival (p=0.022). At the same time, advanced T stage and AJCC stage were also associated with poor overall survival.

Discussion

Gastric cancer is one of the major threats to human health because of high incidence and mortality[1]. With significant advances in our understanding of the molecular and immune pathogenesis of GC, chemotherapy and several molecular targeted drugs have been established as standard treatments for advanced gastric cancer[2], it benefits certain population of GC patients. Unfortunately, the median survival of most GC patients is not well extended[2, 3]. Thus, we still need a better therapy marker in gastric cancer.

In the present study, the clinical data and RNA-sequencing data were downloaded from TCGA database. It is indicated that the RGS1 expression was evaluated in GC tissues from the result of comparison of the expression level of RGS1 in normal tissues and in GC tissues. According to the analysis of RGS1 expression with variables, the high expression of RGS1 was also related with worse differentiated type of tumors, advanced AJCC stage, advanced T stage and N stage. Notably, Kaplan-Meier curves illustrated that the high RGS1 expression patients had significantly shorter OS. Univariate and multivariate analysis of TCGA database indicated that the high RGS1 expression was an independent negative prognostic factor in GC. ROC curve confirmed the diagnostic value of RGS1 expression in gastric cancer. Besides, It was indicated in cox proportional hazards regression modeling that age and AJCC TNM stage were also independent prognostic factors of overall survival in GC patients. The results of single center analysis were basically consistent with all the results above. However, some results in collected cohort did not match the bioinformatics results, we hypothesize this may due to the lack of samples that were used in this cohort.

The exact role of RGS1 in pathogenesis of GC are still not clear. However, early study demonstrated that all RGS proteins have RGS domain, which is main structure to interact with Gα subunits to accelerate GTP hydrolysis[18, 19] up to 1000-fold[20]. After that G protein signaling through downstream effectors were interrupted[21-23]. To the best of our knowledge, signaling pathways mediated by GPCRs are critical mediating oncogenic processes, some members of GPCRs have been found RGS-mediated signaling pathways. For instance, activated PAR1 signaling was regulated by AP-2 through recruitment of RGS proteins[24]. As a member of R4 family of RGS proteins, RGS1 proteins share the same RGS domain, it could have similar function as GAP. Furthermore, another group demonstrates that RGS1 can regulate the non-GAP function of Gαs to promote the activation of AKT and ERK, which finally makes contribution to melanoma proliferation and invasion. Therefore, RGS1 can promote cancer proliferation and invasion through both GAP and non-GAP function. Besides, multiple pieces of evidence indicated that RGS1 may act as an important biomarker in GC and other malignancies. First, it has been confirmed that RGS1 expression was up regulated in many malignancies, and high expression of RGS1 correlated with advanced disease stage or worse prognosis[12-15]. Second, the RGS1 gene is located at 1q31, as we all know, which has been proven to be amplified in several malignancies[12-15, 25]. Third, the powerful role of RGS1 as a prognostic factor for GC rigorously demonstrated by Cox regression analyses of OS. High expression of RGS1 was shown as an independent negative prognostic predictor. Respectively, in the analysis of TCGA cohort, RGS1 has the similar capability to predict OS compared with AJCC stage.

On the other hand, to further investigate the roles of RGS1 in the causation of GC, GSEA was performed using TCGA data. GSEA results showed that genes involved in B cell receptor signaling, T cell receptor signaling, Toll like receptor signaling, natural killer cell mediated cytotoxicity, chemokine signaling, cytokine cytokine-receptor signaling pathway and so on, were associated with high RGS1 expression. Since RGS protein was found in 1995. RGS1 has been studied intensely by many groups. It had been proven that RGS1 can participate in mediating chemokine-induced B-cell migration. However, in these studies about RGS1-/- mice and RGS1-/- B cells models, it seems that RGS1 can promotes the process of B cells homing to lymph nodes, which suppress the migratory activity of B cells[11, 26, 27]. Another group

pays attention to T lymphocytes, it is confirmed that regulatory CD4+ T cells express higher RGS1 than other subpopulations of T lymphocytes, in the opposite, the migratory activity of regulatory CD4+ T cells was weakened[8, 28]. Thus, high expression of RGS1 eventually increases the immune-evasion in GC. However, the exact mechanisms of RGS1 contributing to proliferative, invasive, and metastatic capacity of GC cells still need additional studies.

In conclusion, the present study analyzed clinical information and gene expression data using bioinformatics algorithms. The study also identified that high expression of RGS1 was a potential independent prognostic factor of OS. Finally, we estimated that our study might be conducive to further research on the complicated mechanisms of RGS1 participating in initiation and progression of GC, which might identify RGS1 proteins as potential therapy targets.

Declarations

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Not applicable

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Hai Lin and Feng Zhu performed the data processing and data analysis, and they contributed equally to the manuscript. Jun Wang and Yubiao Liu participated in the discussion of research ideas. Weihua Fu led the design of the study and was corresponding author. All authors have read and approved the manuscript.

Ethics approval and consent to participate

This study was approved by the ethical committee of Tianjin Medical University General Hospital and informed consent was obtained from all patients. The ethical number is NO. IRB2020-WZ-134.

Patient consent for publication

Consent for publication was obtained from all patients.

Competing interests

The authors have declared that no competing interest exists.

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Tables

Table 1. Univariate and multivariate analysis of the relationship between RGS1 expression and OS in TCGA cohort.

Variable	Univariate analysis	Multivariate analysis		
	p-value	HR	95%CI	p-value
Age(years))	0.005	1.041	1.020-1.063	<0.001
Gender	0.062			
Differentiation	0.230			
AJCC stage*	0.001	1.719	0.767-3.852	0.034
T stage	0.088			
N stage	0.036			
M stage	0.022			
RGS1(H/L)	0.012	1.848	1.059-3.224	0.031
*AJCC, American Joint Committee on Cancer.				

Table 2, Clinicopathological characteristics of high and low RGS1 expression groups in validation cohort.

Characteristics	RGS1		
	Low(n=34), n (%)	High(n=24), n (%))	P-value
Age(years)	67.74±7.7	68.08±8.00	0.924
Gender			0.545
Male	23(67.6)	18(75.0)	
Female	11(32.4)	6(25.0)	
Body mass index(kg/□)	24.0±3.8	23.4±3.2	0.643
Type of surgery			0.653
DG	23(67.6)	18(75)	
TG	9(26.4)	4(16.7)	
PG	2(6.0)	2(8.3)	
Tumor size(cm)	3.8±2.1	4.4±2.1	0.735
Tumor location			0.433
U	4(11.8)	5(20.8)	
M	6(17.6)	2(8.3)	
L	24(70.6)	17(70.8)	
Differentiation, n			0.166
Poorly differentiated	28(82.4)	15(62.5)	
Moderately differentiated	2(5.9)	5(20.8)	
Highly differentiated	4(11.7)	4(16.7)	
T stage, n			0.226
1	4(11.7)	4(16.7)	
2	9(26.5)	2(8.3)	
3	16(47.1)	11(45.8)	
4	5(14.7)	7(29.2)	
N stage, n			0.645
0	12(35.3)	6(25.0)	
1	5(14.7)	5(20.8)	
2	8(23.5)	4(16.7)	

3	9(26.5)	9(37.5)	
AJCC stage, n			0.670
I	9(26.5)	4(16.7)	
II	9(26.5)	7(29.2)	
III	16(47.0)	13(54.1)	
CEA	4.65±6.5	2.38±1.5	0.038
CA-199	57.46±170.6	84.13±217.4	0.368
DG, distal gastrectomy. TG, total gastrectomy. PG, proximal gastrectomy. U, upper third. M, middle third. L,lower third. AJCC,American Joint Committee on Cancer. CEA, carcinoembryonic antigen. CA-199, carbohydrate antigen 199.			

Table 3. Univariate and multivariate analysis of the relationship between RGS1 expression and OS in validation cohort.

Variable	Univariate analysis	Multivariate analysis		
	p-value	HR	95%CI	p-value
Age(≤65years/>65 years)	0.182			
Gender(female/male)	0.491			
BMI	0.758			
Type of surgery(PG/DG/TG)	0.826			
Tumor size(≤4.4cm/>4.4cm)	0.114			
Tumor location(M/L/U)	0.580			
Differentiation type(PD/HD/MD)	0.799			
RGS1(H/L)	0.022	2.674	1.154-6.196	0.022
T stage	0.008	7.134	1.669-30.502	0.008
N stage	0.071			
AJCC stage	0.003	3.001	1.457-6.180	0.003
CEA	0.418			
CA-199	0.715			

Figures

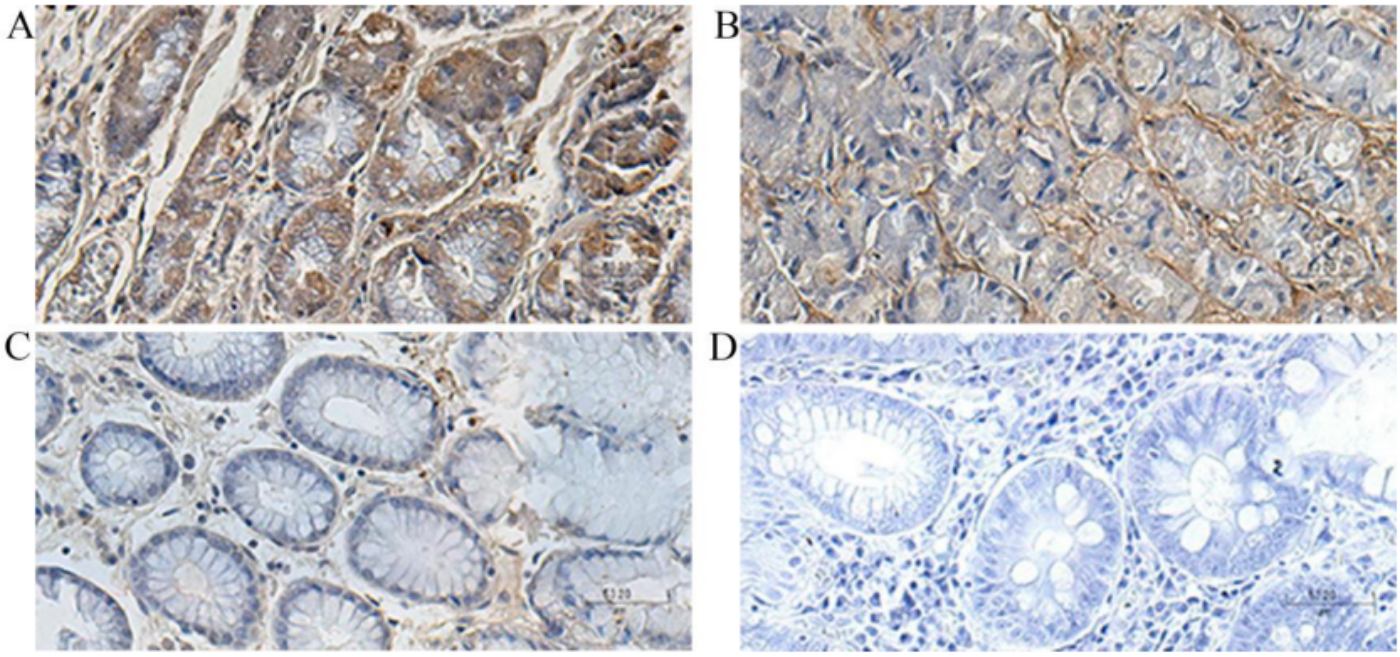

Figure 1

Immunohistochemical staining for RGS1 in representative tissue samples. A. Gastric cancer with positive staining intensity. B. Gastric cancer with weak positive staining intensity. C. Gastric cancer with negative staining intensity. D. normal gastric tissue with negative staining intensity.

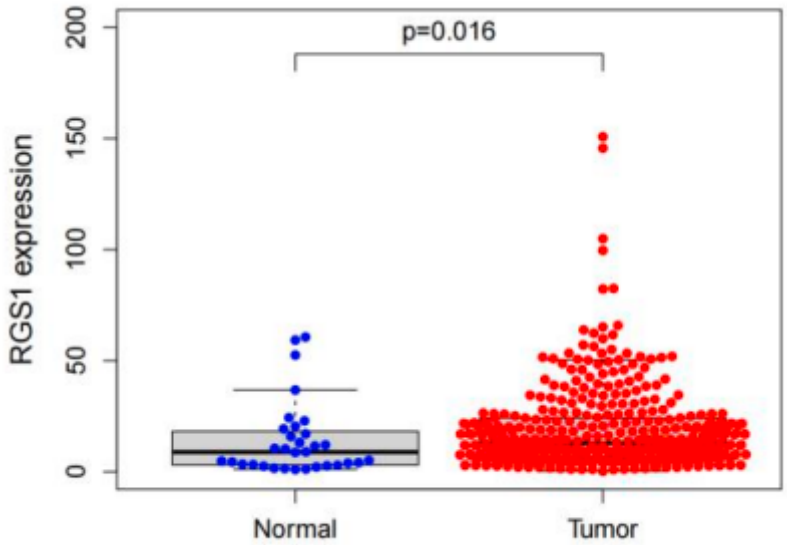

Figure 2

The expression of RGS1 in GC tissue and normal gastric tissue

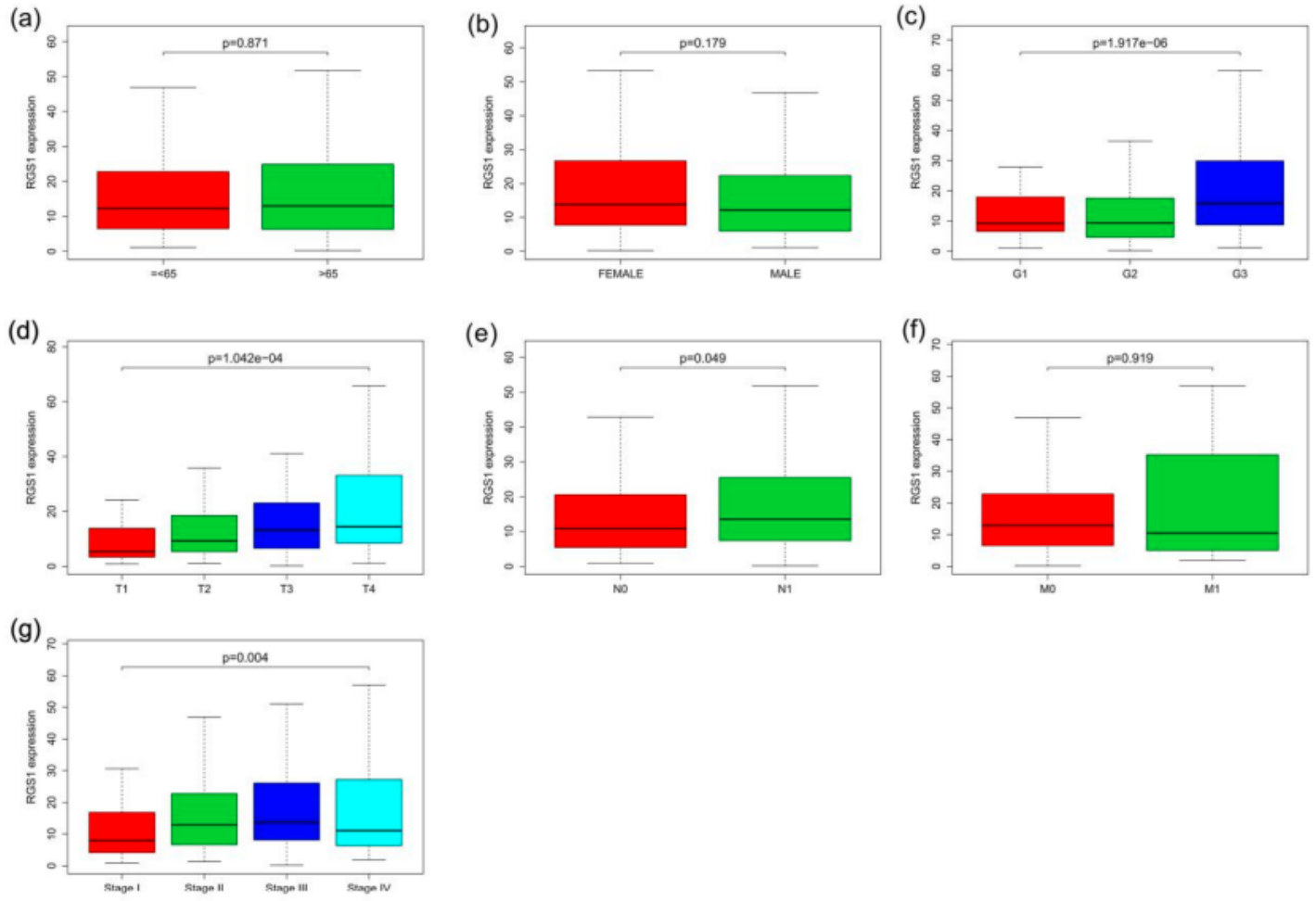

Figure 3

Association between RGS1 expression and clinicopathologic characteristics. a: age, b: gender, c: differentiation, d: T stage, e: N stage, f: M stage, g: AJCC TNM stage.

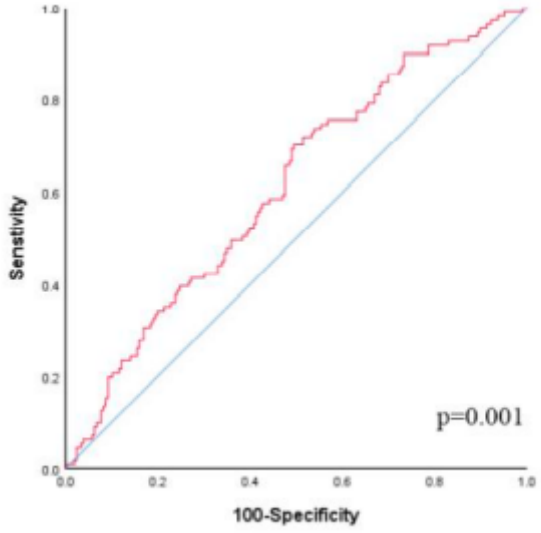

Figure 4

Receiver operating characteristic (ROC) curve for RGS1 expression

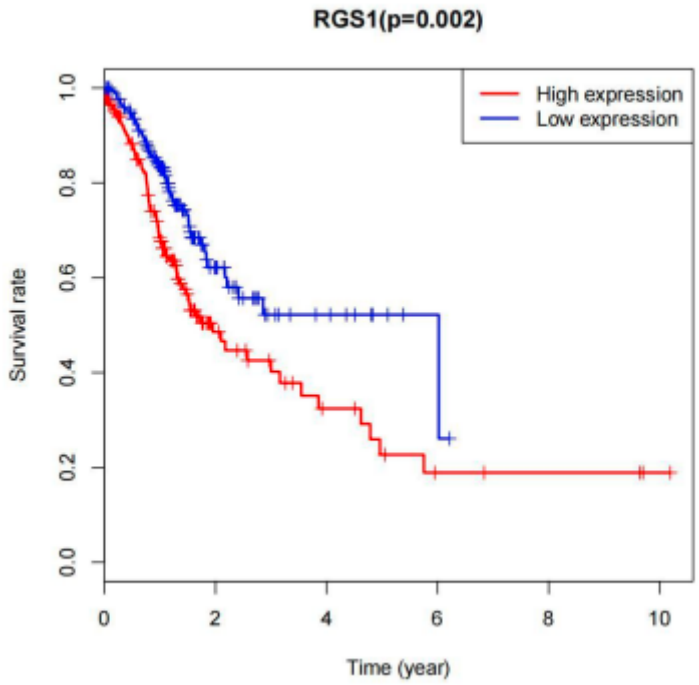

Figure 5

RGS1 expression and overall survival in TCGA cohort

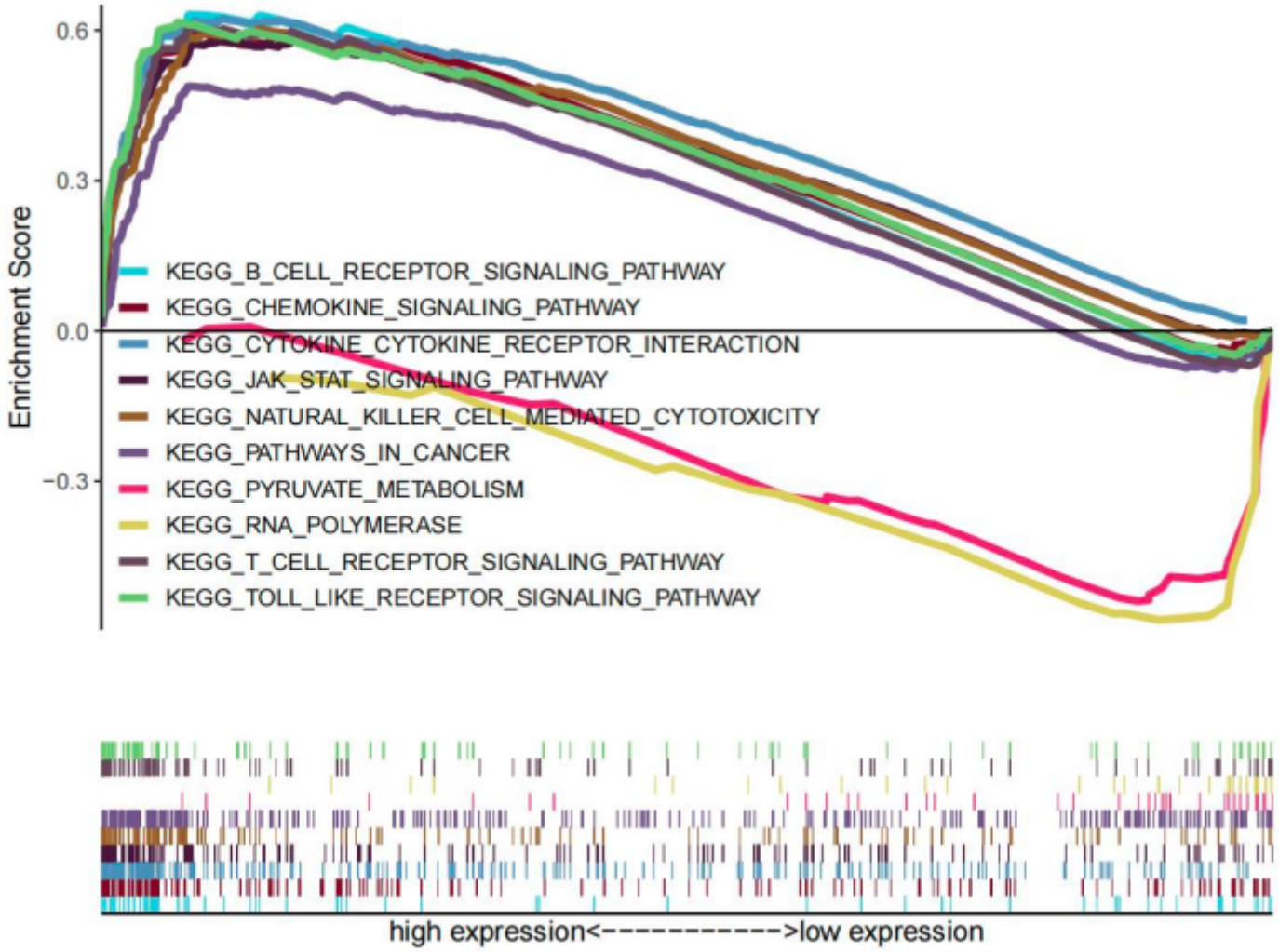

Figure 6

Enrichment plots from the gene set enrichment analysis (GSEA)

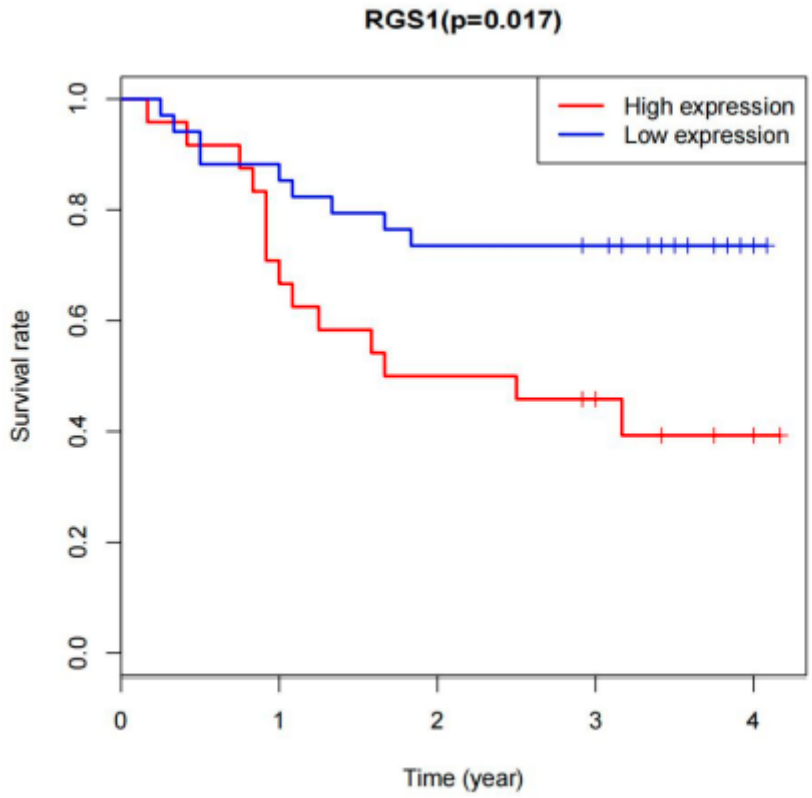

Figure 7

RGS1 expression and overall survival in collected cohort