

# Bioinformatics Analysis Reveals Biomarkers With Prognostic Benefits in Diffuse Type Gastric Cancer

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## Primary research

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# Abstract

## Background

Gastric cancer (GC) is one of the most common malignancies in digestive system, among which the differentiation of diffuse type GC is relatively poor, the probability of distant metastasis and lymph node metastasis is relatively high, and the clinical prognosis is relatively poor. The purpose of this study is to explore potential signaling pathways and key biomarkers that drive the development of diffuse type GC.

## Methods

Using the “limma” package in R to screen Differentially expressed genes. Screening hub genes by PPI analysis. Immunohistochemistry analysis and qRT-PCR analysis was carried out to detect genes expression. Using Kaplan-Meier Plotter database analyzed the prognostic roles of hub genes.

## Results

A total of 355 DEGs consisting of 293 diffuse type DEGs and 62 intestinal type DEGs were selected according to screening criteria, 3 hub genes were chosen from diffuse type DEGs according to the degree of connectivity by using protein-protein interaction (PPI) networks and Cytoscape software including AGT, CXCL12 and ADRB2. Immunohistochemistry analysis and qRT-PCR results showed that the expression of three genes was related to the different GC lauren types. The Kaplan Meier analysis showed that the expression values of these three genes were related to prognosis of diffuse type GC.

## Conclusions

AGT, CXCL12 and ADRB2 might contribute to the progression of diffuse type GC, which could have potential as biomarkers or therapeutic targets for diffuse type GC.

## Background

Gastric cancer (GC) is one of the most common malignant tumors in the digestive system. According to the 2012 statistics of the IARC (international agency for research on cancer), between 1995 and 2009, the incidence rate and mortality rate of GC in all malignant tumors occupied fifth and third place respectively, more than 70% of these cases occur in developing countries and 50% in East Asia(1). Early diagnosis and treatment of GC can often achieve good results, but most patients were diagnosed by endoscopy only when they had symptoms. At this time, the disease was in advanced stage and already lost the best opportunity for surgery. Even after a complete R0 resection, one-third of patients will experience recurrence (2). Most GCs are adenocarcinomas, undeniably it's also a high heterogeneous disease with differences in epidemiology and histopathology. There is no evident breakthrough for the treatment of patients with advanced GC, surgery is still the main therapy for it, and majority cancer patients died of tumor recurrence and metastasis, the median overall survival (OS) is still shorter than 1 year(3).

There are many ways to classify GC by different classification system, such as the Bormann classification, the Lauren classification, and the World Health Organization (WHO) classification(4-6). Recent years, with the development of medicine and the deepening of understanding, some scholars tried to classify GC from molecular and genetic features level, such as The Cancer Genome Atlas (TCGA) classification(7) and Asian Cancer Research Group (ACRG) classification(8). Since the Lauren classification was proposed in 1965, it has been widely recognized by clinicians and pathologists and has been used up to now. The Lauren classification mainly divides GC into intestinal-, diffuse- and mix-types based on the tissue structure, biological behavior and epidemiological characteristics(5). In histology, intestinal type GC cells are large in size, clear in boundary, variable in morphologic and closely arranged, exhibiting tubular and glandular differentiation. On the contrary, diffuse type GC cells are typically scattered and often appeared as solitary cells or in small clusters due to lack of adhesion, this is the reason why it's hard to observe gland formation in tumor tissue and diffuse type GC is easy to dissemination. Mix type has all of the above characteristics. In epidemiology, intestinal type is the most common type, with the highest five-year survival rate, which is more common in men and the elderly, while diffuse type is more likely to happen in women and younger patients, with a lower 5-year survival rate(9-11). Mix type has the highest malignant degree because of its changeable biological behavior.

Recent years, with the development of medicine and bioinformatics, high-throughput sequencing has been applied as a common tool for medical research(12). Researchers could upload the data of gene expression profile chip to the Gene Expression Omnibus (GEO) datasets of NCBI. Reanalyzing and reintegrating those datasets could provide some meaningful clues for new research. A series of microarray datasets of GC have been developed in recent years(13-15) and a large number of meaningful differentially expressed genes (DEGs) have been found.

In this study, we downloaded GSE62254 dataset from the GEO and screened out DEGs by using "limma" and "survival" package in R. Subsequently, we performed the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs and found key biological features and signaling pathways. Moreover, we constructed a protein-protein interaction network of diffuse type DEGs and screened 3 hub genes out through Cytoscape tool. Finally, using the Kaplan Meier analysis to evaluate the overall survival of patients with aberrant expression levels of the hub genes.

## **Materials And Methods**

### **Data collection**

The gene expression profile GSE62254(8, 16), were downloaded from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>), which was based on GPL570 platform ([HG-U133\_Plus\_2] Affymetrix Human Genome U133 Plus 2.0 Array). GSE62254 contains 300 different Lauren subtypes GC samples.

### **Differentially Expressed Gene Analysis**

Using RMA algorithm in the R environment (v3.6.1)(17) to normalize and transform all the raw data to expression values. Differentially expressed genes(DEGs) between diffuse and intestinal subtypes samples were screened by using the “limma” package in R(18), the cut-off criterion were  $P < 0.05$  and  $|\log_2FC| > 0.585$ , which means  $\log_2FC$  of DEGs over 0.585 were identified as the diffuse subtype-specific genes, whereas that less than -0.585 were intestinal subtype-specific genes. To identify the gene associate with prognostic value, the “survival” package in R were used to make Cox regression analysis(19) and get the HRs and P-value of all genes in the GSE62254. The genes with  $P < 0.05$  were identified as OS (over survival) related genes. Then, the common two subtype genes and OS related genes were defined as diffuse/intestinal type DEGs, using Venny's online software (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) to draw Venn diagrams.

### **GO and KEGG enrichment analysis**

Using The Database for Annotation, Visualization and Integrated Discovery(DAVID, <http://david.ncifcrf.gov/>), which provides a comprehensive set of functional annotation tools for investigators to understand biological meaning behind large list of genes, to analysis the diffuse/intestinal type DEGs ontology functional annotation and KEGG pathway analysis. In order to get significant terms, set  $P$  value  $< 0.05$  as cut-off criterion.

### **PPI network construction and screening of hub gene of diffuse type GC**

Input the diffuse type DEGs into the Search Tool for the Retrieval of Interacting Genes (String) database for interaction network at the protein level, also known as protein–protein interaction (PPI) information, minimum required interaction score  $> 0.700$  (high confidence) was considered significantly. Then import the results into Cytoscape software to visualize the PPI network, using Cytohubba plugin to select the top 15 genes by four different algorithms, pick out and defined duplicate genes as hub genes.

### **Patients' information and tissues samples**

A total of 40 GC patients who received a gastrectomy in The Third Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China between December 2016 to July 2018 were recruited in this study. None of them received radiotherapy or preoperative chemotherapy before surgery. All specimens were handled and made anonymous according to the ethical and legal standards. Tissue samples were collected during the surgery for GC and were confirmed by tissue pathology examination. There were 20 cases for

diffuse and intestinal type gastric cancers, respectively. All fresh tumor tissues specimens were collected from formalin-fixed paraffin-embedded tissues of resection surgical procedures.

### **Immunohistochemical analysis**

Immunohistochemistry was performed to determine the expressions of AGT, CXCL12 and ADRB2 in human diffuse type GC tissues and intestinal type GC tissues. Paraffin-embedded tissue were passed through dimethylbenzene and gradient ethanol solution to deparaffinize and rehydrate the sections. Antigen retrieval was performed by heating the sections in a microwave oven in 10 mM sodium citrate-hydrochloric acid buffer (pH 6.0) for about 15 minutes, while 0.3% peroxidase quenching solution was used to block endogenous peroxidase activity. After blocking for 30 min with 10% skim milk, each sections were incubated with mouse anti-human AGT antibody (1:50, NO.79299, LifeSpanBioSciences, USA) rabbit anti-human CXCL12 antibody( 1:200, ab9797, Abcam, UK) or abbit anti-human ADRB2 antibody (1:100, ab182136, Abcam, UK) overnight at 4°C. Next, incubate slides with secondary antibody at room temperature for 30 minutes. The sections then were recruited with 3,3'-diaminobenzidine (DAB) solution, and every slide were counterstained with hematoxylin, dehydrated, and sealed with cover slips. The stained sections were examined under an optical microscope. The intensity of staining were assigned as follows: 0 (negative, 0–25%, no positive malignant cells), 1 (weak, 0–25% positive malignant cells), 2 (moderate, 25–75% positive malignant cells), and 3 (strong, 75–100% positive malignant cells).

### **RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)**

qRT-PCR was used to verify and compare the expression levels of three mutated genes (AGT, CXCL12 and ADRB2) between diffuse type GC tissues and intestinal type GC tissues. Total RNA was extracted with the trizol reagent (Invitrogen, USA) according to the manufacturer's instructions, and RNA purity was detected using a microplate reader (Infinite M1000 PRO, TECAN). A PrimeScript RT reagent kit (Takara, Japan) was used for the complementary DNA synthesis reactions. Using SYBR® Premix ExTaq™ (Takara, Japan) to perform the qRT-PCR in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as standardized references. Primers were as follows:

AGT

Forward Primer 5'-CCCTGGCTTTCAACACCTAC-3';

Reverse Primer 5'-CTGTGGGCTCTCTCATCC-3';

CXCL12

Forward Primer 5'-GATTGTAGCCCGGCTGAAGA-3'

Reverse Primer 5'-TTCGGGTCAATGCACACTTGT-3'

ADRB2

Forward Primer 5'-AACTGGTTGGGCTATGTCAA-3'

Reverse Primer 5'-GTTAGTGCCTGTCAGGGAG-3'

GAPDH

Forward Primer 5'-TGTGGGCATCAATGGATTTGG-3'

Reverse Primer 5'-ACACCATGTATTCCGGGTCAAT-3'

### **Kaplan Meier analysis of Hub genes**

Using Kaplan Meier Plotter (<https://kmplot.com/analysis/>), which could assess the effect of 54k genes on survival in 21 cancer types, to verify the overall survival (OS) analysis of hub genes in diffuse type GC. In order to improve the reliability of result, we select GSE62254 and GSE15459 datasets as research target respectively, set P value<0.05 as cut-off criterion.

## **Results**

The flowchart of the bioinformatics analytical methods is presented in Figure 1. The GSE62254 database totally included 300 different Lauren subtypes GC samples. 265 samples were single out with definite Lauren subtypes and certainly survival data, including 128 diffuse type GC samples and 137 intestinal type GC samples. The details information of these samples are shown in supplementary materials (Table S1). According to the screening criteria of  $|\log_{2}FC| \geq 0.585$  and adjusted P value < 0.05, 584 differentially expressed genes (DEGs), including 458 up regulated genes in diffuse type and 122 down regulated genes in intestinal type, were screened out by using the “limma” package in R, presented these DEGs in volcano plot (Figure 2A). To identify the gene associate with prognostic value, using the “survival” package in R to make Cox regression analysis and get the HRs and P-value of all genes in the GSE62254. 7389 genes with P < 0.05 were identified as OS related genes. Using online webpage tool, Venn, to construct the Venn diagram of the DEGs and OS related genes. A total of 293 diffuse type DEGs and 62 intestinal type DEGs were picked out for further research (Figure 2B).

Uploaded the diffuse type and intestinal type DEGs list respectively to the online website DAVID to analyze the GO function and KEGG pathway analysis, the results were considered as a significant one if P value < 0.05. GO analysis showed that the diffuse type DEGs were mainly enriched in cell adhesion (ontology: BP), extracellular exosome (ontology: CC), and calcium ion binding (ontology: MF), while the

intestinal type DEGs were mainly enriched in cell division (ontology: BP), nucleus (ontology: CC) and protein binding (ontology: MF). Details of the results are shown in Figure 3 and Figure 4. The top 15 results from the GO enrichment analysis of the subtype-specific DEGs are shown in Table 1.

As for KEGG pathway analysis, the results of the analysis are shown in Figure 5 and Table 2. The diffuse type DEGs were mainly enriched in cGMP-PKG signaling pathway, while the intestinal type DEGs were mainly enriched in Cell cycle.

To explore and identify subtype-specific genes in diffuse type GC further, the 293 diffuse type DEGs were uploaded to STRING online database to analyze and construct a protein-protein interaction (PPI) network, it was identified that 112 nodes and 182 interactions were involved in the PPI network (Figure 6). Downloaded the results and analyzed in Cytoscape software, the top 15 hub genes were ranked by using the four different algorithms of the CytoHubba plugin according to the predicted scores. The gene overlapped was considered as significant and a total of 3 overlapping hub genes were determined for further analysis, included AGT, CXCL12, ADRB2 (Table 3).

Immunohistochemistry analysis showed that the distribution density of AGT, CXCL12 and ADRB2 is related to different GC Lauren types. Compared with intestinal type GC tissues, the expression of AGT, CXCL12 and ADRB2 showed strongly stained in diffuse type GC tissues (Figure 7).

As for the results of qRT-PCR, it is consistent with the results of immunohistochemistry analysis. The expression levels of AGT, CXCL12 and ADRB2 were significantly higher in the diffuse type GC tissues than in the intestinal type GC tissues ( $p < 0.01$  for AGT,  $p < 0.001$  for CXCL12,  $p < 0.01$  for ADRB2) (Figure 8).

To evaluate the prognostic value of the 3 hub genes in diffuse type GC, we performed a Kaplan-Meier prognosis analysis for overall survival (OS) at Kaplan Meier Plotter (<https://kmplot.com/analysis/>). In order to improve the reliability of result, we select GSE62254 and GSE15459 datasets as research target respectively. The results showed that the high expression of AGT (logrank  $p=0.0048$ ), CXCL12 (logrank  $p=0.0027$ ) and ADRB2 (logrank  $p=0.014$ ) indicated a poor prognosis for diffuse type GC patients according to GSE62254. In GSE15459, the high expression of AGT (logrank  $p=0.00056$ ) and ARDB2 (logrank  $p=0.0012$ ) presented similar results, indicated a poor prognosis for diffuse GC type patients, while the expression of CXCL12 (logrank  $p=0.093$ ) was not correlated with prognosis (Figure 9).

## Discussion

The GC is a highly heterogeneous disease. Since the Lauren classification was proposed in 1965, it has been widely recognized by clinicians and pathologists and has been used up to now. For many years, the value of histopathologic classification in evaluating the prognosis of GC is very limited, and Lauren classification is considered to be the most valuable clinicopathological classification. There are

significant differences between different Lauren subtype(20-22), which suggested that some specific biomarkers might play an important role during genesis and development of GC. Although there are many studies on the biological mechanism of GC, there are few studies on specific GC subtypes.

To explore the Lauren subtype-specific genes of GC, our study selected GSE62254 dataset and screened 266 samples of diffuse or intestinal GC with certainly survival data out. A total of 598 DEGs were screened out by using R including 293 diffuse type DEGs and 62 intestinal type DEGs. To deeply explore the biological pathways and functions involved by these DEGs, we performed GO and KEGG analysis. To find the key genes for diffuse type GC progression from the numerous DEGs, we identified the top 15 hub genes through the PPI network and Cytoscape by using 4 different algorithms and took the overlapped genes as the research object, including AGT, CXCL12, ADRB2. In order to validate the present results, we used Kaplan-Meier curves to analyze the association of the 3 hub genes expression with OS, and the results showed that all the 3 hub genes were related to the OS of diffuse type GC in other datasets. Therefore, the results indicate that these 3 hub genes may be new diagnostic and prognostic biomarkers for diffuse type GC.

The gene AGT encodes pre-angiotensinogen or angiotensinogen precursor protein, which mainly expressed in the liver and cleaved by the enzyme renin in response to lowered blood pressure. The resulting product, angiotensin 1 (Ang $\text{I}$ ), is cleaved by angiotensin converting enzyme (ACE) to produce angiotensin 2(Ang $\text{II}$ ) followed. In another word, the product of AGT constitutes a key component of Renin-Angiotensin-System (RAS). RAS could be involved in arterial hypertension, kidney disease, and other cardiovascular conditions in previous studies(23-25), with the deepening of research, more and more clinical studies support RAS signaling promoted cancer growth and dissemination(26). RAS components expressed in many cell types of the tumor microenvironment and directly affected cell proliferation, invasion, migration, metastasis, apoptosis, angiogenesis, cancer-associated inflammation and immunomodulation(26, 27), it could direct or indirect promote tumor growth in many ways, for instance, regulating cancer-associated fibroblasts (CAFs)(28) and promoting VEGF-mediated angiogenesis(29, 30) in solid tumors. Integrating characteristics of diffuse type GC and our analysis result, we speculated that AGT might be a potential indicator for the diagnosis and prognosis of diffuse type GC.

The CXCL12 gene, also known as stromal cell-derived factor 1 (SDF1), encodes a stromal cell-derived alpha chemokine member of the intercrine (chemokine CXC) family. The encoded protein, chemokine CXCL12, binds mainly to the receptors CXC receptor 4 (CXCR4)(31-33), play an essential role in many diverse cellular functions. CXCR4 is widely expressed on hematopoietic cells, embryonic pluripotent stem cells and several types of tissue-committed stem cells(34), which have direct or indirect proangiogenic properties. Proven evidence shows the CXCL12/CXCR4 axis is associated with tumor progression,

angiogenesis, metastasis, and survival. CXCL12 overexpression will enhance the proliferation and invasion of colon cancer cells through the MAPK/PI3K/AP-1 signaling pathway(35). Serve as a hub gene, high CXCR4 expression could be a biomarker indicating poor prognosis for hepatocellular carcinoma patients(36). CXCL12/CXCR4 antagonists, such as plerixafor or BKT140, have already produced and display encouraging results in anti-cancer activity(37). However, according to our result, relationship between the expression of CXCL12 gene and its prognosis in human diffuse type GC between GSE62254 and GSE15459 presented quiet different results. In my opinion, the main reason is that the situation may be different according to different characteristics such as race, gender, helicobacter pylori infection. The differences between two datasets need more comprehensive and precise studies in the future to explain.

The gene ADRB2 encodes beta-2-adrenergic receptor, which belongs to the G protein-coupled receptor superfamily. ADRB2 protein can increase cAMP, and downstream L-type calcium channel interaction via adenylate cyclase stimulation through trimeric Gs proteins, and then mediate physiological response such as bronchodilation and smooth muscle relaxation. In recent years, some studies have pointed out that ADRB2 also plays an important role in many cancers. ADRB2 signaling could negatively regulated autophagy, leading to hypoxia-inducible factor-1 $\alpha$  stabilization and induced sorafenib resistance of HCC (hepatocellular carcinoma)(38). Moreover, ADRB2 expression was associated with HCC outcomes(39). In prostate cancer, intact sympathetic nerves were essential for tumor formation, and the ADRB2 high expression level can activate an angio-metabolic switch and affects the phenotype of the prostate cells and thereby their ability to migrate and invade(40). In GC, chronic stress caused by stress hormone-induced activation of the ADRB2 signaling pathway plays a crucial role in GC progression and metastasis(41), ADRB2 signaling can regulates GC progression(42). Our study found that ADRB2 is highly expressed in diffuse type GC and has diagnostic and prognostic value.

## Conclusions

In summary, we screened out 293 diffuse type DEGs from the GSE62254 dataset, which may contain hub genes contributing to the pathogenesis of diffuse type GC. The GO and KEGG enrichment analyses revealed that the DEGs were mainly enriched in extracellular exosome and cGMP-PKG signaling pathway. Through survival analysis, three of the top 15 hub genes including AGT, CXCL12, ADRB2, their high expressions are associated with a reduced survival time of patients with diffuse type GC. Through Immunohistochemistry and qRT-PCR, we found that the expression of previous hub genes in diffuse type GC tissues was high. According to literature, these genes have a specific association with tumor invasion, metastasis and angiogenesis. Although we could not perform experimental research to probing potential oncogenic mechanisms, take previous reports into consideration, we suggest a hypothesis carefully that AGT, CXCL12, ADRB2 overexpression contributed to unfavorable prognosis in diffuse type GC.

## Declarations

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## Funding

Not applicable

## Availability of data and materials

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

## Authors' contributions

GW conceived and designed the present study. SL and CY collected, extracted and analyzed the data. SL and FD performed the experiments. SL wrote the manuscript. YC, FD and YZ reviewed the final manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Written informed consent was obtained from each patient and the study was approved by the Ethics Committee of The First People's Hospital of Hefei, Anhui Medical University.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Consent for publication

Not applicable.

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## Tables

Table 1

Top 15 GO enrichment terms associated with the subtype-specific DEGs

<b>A. Diffuse type DEGs top 15 enriched GO terms.</b>			
<b>Category</b>	<b>Term</b>	<b>Count</b>	<b>P-value</b>
<b>BP</b>	cell adhesion	17	6.64E-04
<b>BP</b>	nervous system development	14	1.86E-04
<b>BP</b>	negative regulation of cell proliferation	14	0.003535
<b>BP</b>	multicellular organism development	14	0.02974
<b>BP</b>	inflammatory response	13	0.006531
<b>CC</b>	extracellular exosome	54	0.013967
<b>CC</b>	extracellular space	52	5.74E-11
<b>CC</b>	extracellular region	52	2.60E-08
<b>CC</b>	extracellular matrix	22	1.34E-09
<b>CC</b>	proteinaceous extracellular matrix	21	1.39E-09
<b>MF</b>	calcium ion binding	20	0.002718
<b>MF</b>	heparin binding	16	2.78E-09
<b>MF</b>	receptor binding	12	0.006744
<b>MF</b>	growth factor activity	8	0.005364
<b>MF</b>	collagen binding	6	0.001096

<b>B. Intestinal type DEGs top 15 enriched GO terms.</b>			
<b>Category</b>	<b>Term</b>	<b>Count</b>	<b>P-value</b>
<b>BP</b>	cell division	14	7.40E-11
<b>BP</b>	mitotic nuclear division	12	3.94E-10
<b>BP</b>	positive regulation of cell proliferation	8	7.78E-04
<b>BP</b>	chromosome segregation	7	8.73E-08
<b>BP</b>	sister chromatid cohesion	6	2.15E-05
<b>CC</b>	nucleus	35	2.54E-06
<b>CC</b>	cytoplasm	25	0.020284
<b>CC</b>	cytosol	20	0.004431
<b>CC</b>	nucleoplasm	16	0.021557
<b>CC</b>	condensed chromosome kinetochore	6	7.60E-06
<b>MF</b>	protein binding	39	0.002772
<b>MF</b>	ATP binding	11	0.01662
<b>MF</b>	protein kinase binding	5	0.030146
<b>MF</b>	chemokine activity	4	4.86E-04
<b>MF</b>	microtubule binding	4	0.027519

Notes:GO, gene ontology. DEGs, differentially expressed genes. BP, biological process. CC, cellular component. MF, molecular function.

Table 2  
KEGG pathway analysis of subtype-specific DEGs.

Category	Term	Count	P-value
Diffuse type DEGs	hsa04022:cGMP-PKG signaling pathway	11	2.19E-05
	hsa04924:Renin secretion	7	1.26E-04
	hsa00350:Tyrosine metabolism	4	0.00896
	hsa04610:Complement and coagulation cascades	5	0.01021
	hsa04270:Vascular smooth muscle contraction	6	0.01456
	hsa05414:Dilated cardiomyopathy	5	0.01983
	hsa04360:Axon guidance	6	0.02008
	hsa04970:Salivary secretion	5	0.02142
	hsa04261:Adrenergic signaling in cardiomyocytes	6	0.02758
	Intestinal type DEGs	hsa04110:Cell cycle	4
hsa05134:Legionellosis		3	0.0095
hsa04062:Chemokine signaling pathway		4	0.0137
hsa05132:Salmonella infection		3	0.02153
hsa04060:Cytokine-cytokine receptor interaction		4	0.02776
	hsa04668:TNF signaling pathway	3	0.03451

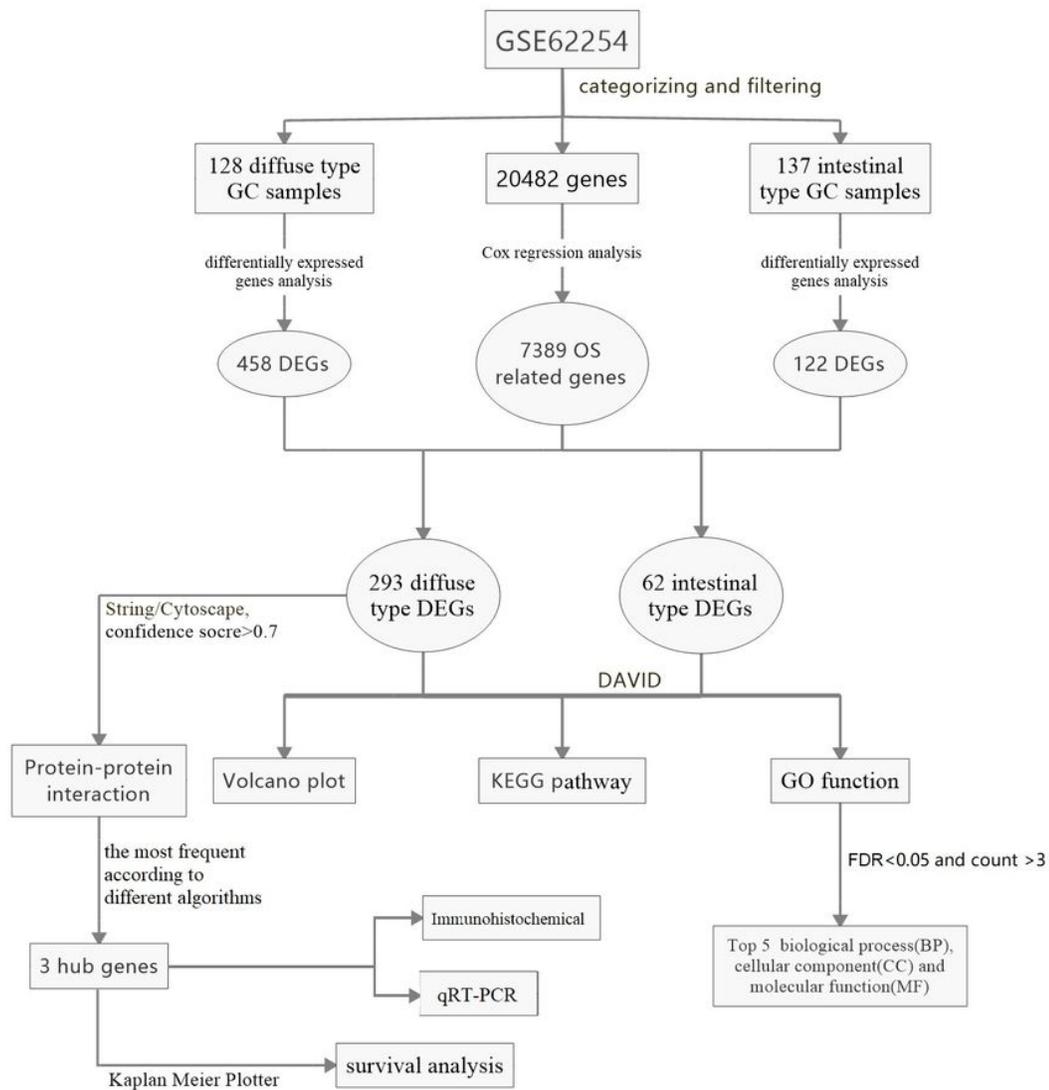
Notes:KEGG,Kyoto Encyclopedia of Genes and Genomes. DEGs, differentially expressed genes.

Table 3  
Hub genes for diffuse type DEGs ranked in Cytohubba plugin of Cytoscape

Category	Rank methods in cytoHubba			
	Degree	EPC	Closeness	EcCentricity
Gene symbol top 15	<b>AGT</b>	<b>AGT</b>	<b>AGT</b>	PRKAR2B
	<b>CXCL12</b>	<b>CXCL12</b>	<b>CXCL12</b>	ITGA1
	IGF1	S1PR1	PRKAR2B	GRP
	EDNRB	EDNRB	EDNRB	ACTG2
	FBN1	CXCL13	IGF1	MYH11
	IGFBP5	ARHGEF25	<b>ADRB2</b>	<b>AGT</b>
	CCL19	GRP	ITGA1	<b>CXCL12</b>
	MYH11	CX3CR1	FGF2	LMOD1
	TAC1	TAC1	CCL19	<b>ADRB2</b>
	GRP	EDNRA	CXCL13	ITGA8
	<b>ADRB2</b>	CCL19	CX3CR1	CAV1
	ITGA1	P2RY12	S1PR1	THBS4
	SCG2	HTR2B	P2RY12	EDNRA
	EDNRA	<b>ADRB2</b>	FGF13	SLIT2
	CXCL13	PRKAR2B	TAC1	ROBO1

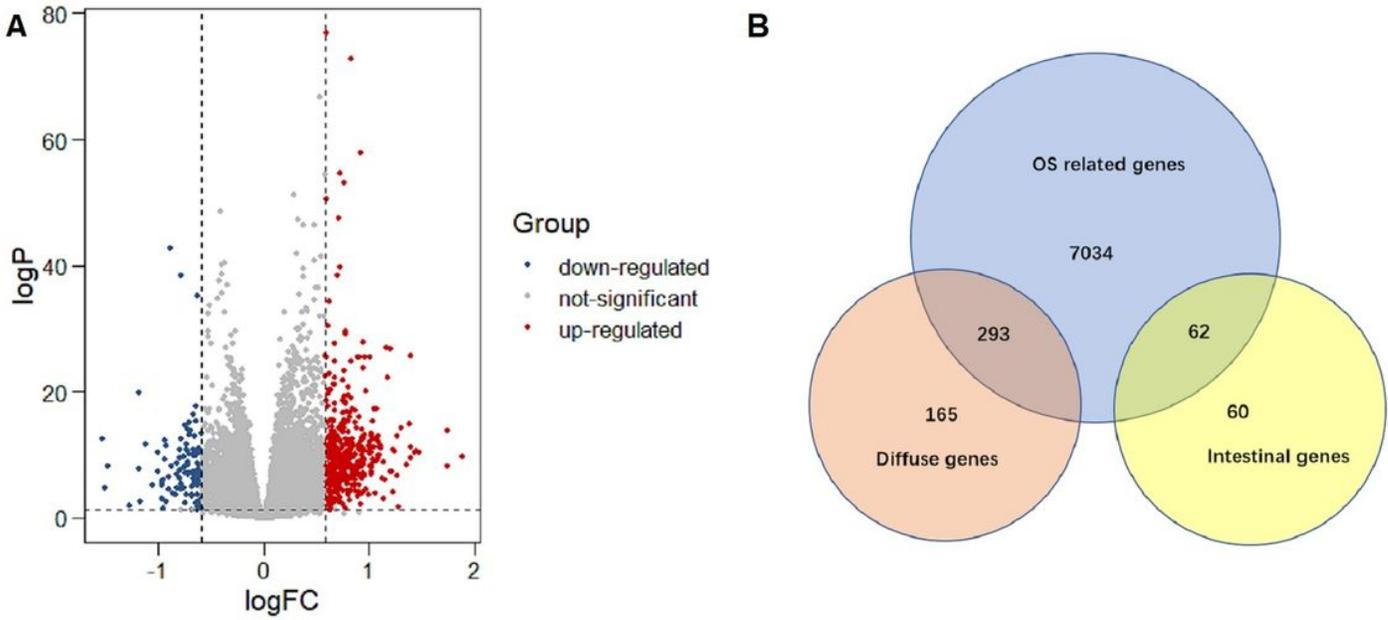
Bold gene symbols were the overlap hub genes in top 15 by four ranked methods respectively in cytoHubba. EPC: Edge percolated component.

## Figures



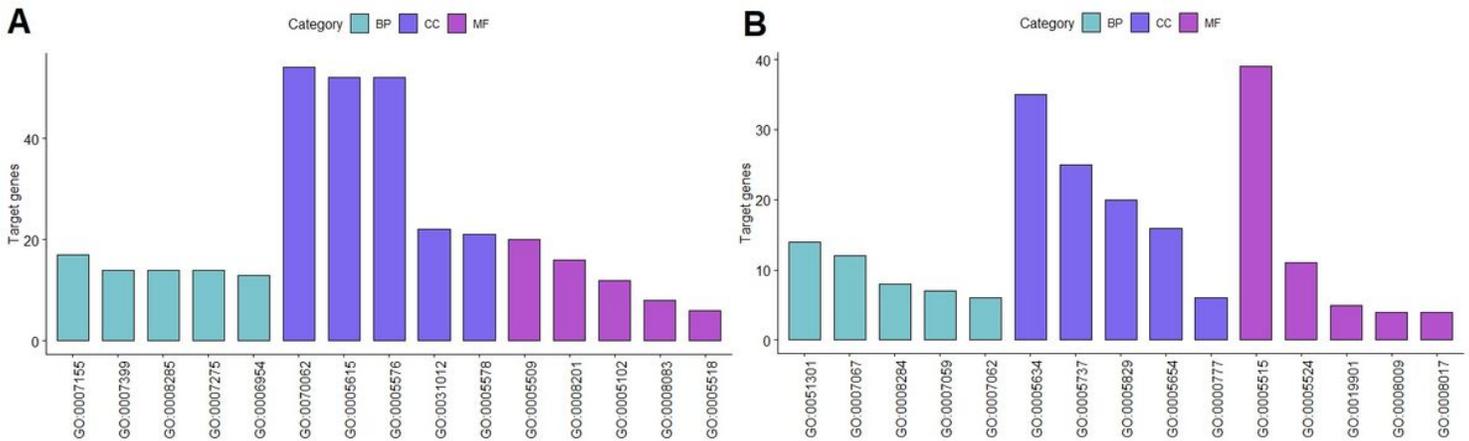
**Figure 1**

The flowchart of the bioinformatics analytical methods. GC, Gastric cancer; OS, over survival; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization and Integrated Discovery; FDR, false discovery rate



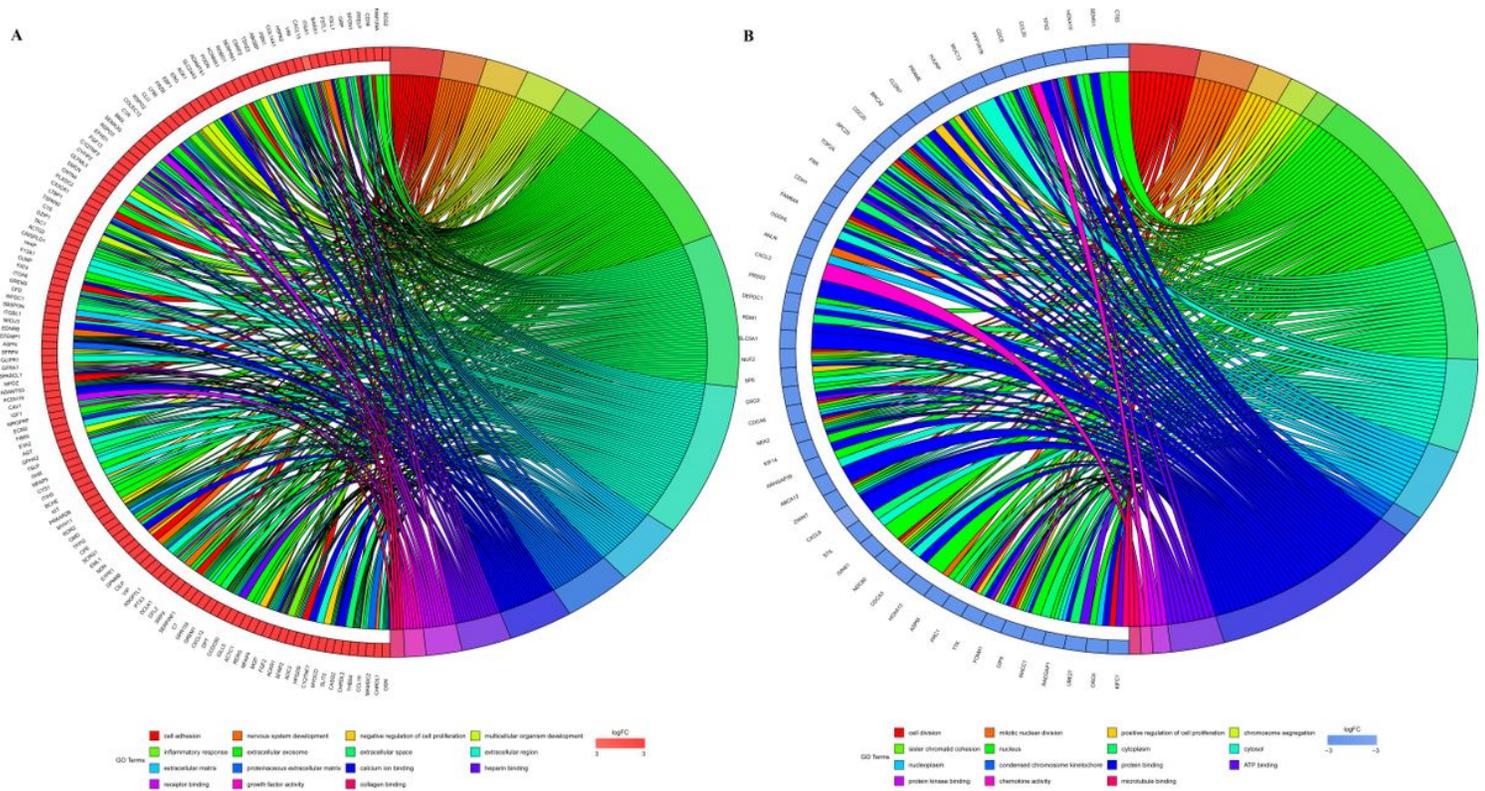
**Figure 2**

(A) The volcano map of DEGs from GSE62254 dataset. The red plots represent the genes highly related with diffuse subtype GC whereas the blue ones represent the genes upregulated in intestinal subtype GC. The screening criteria for logFC was 0.585. (B) Venn diagrams of subtype-specific DEGs screened through the intersection of OS related genes with intestinal and diffuse type GC genes respectively.



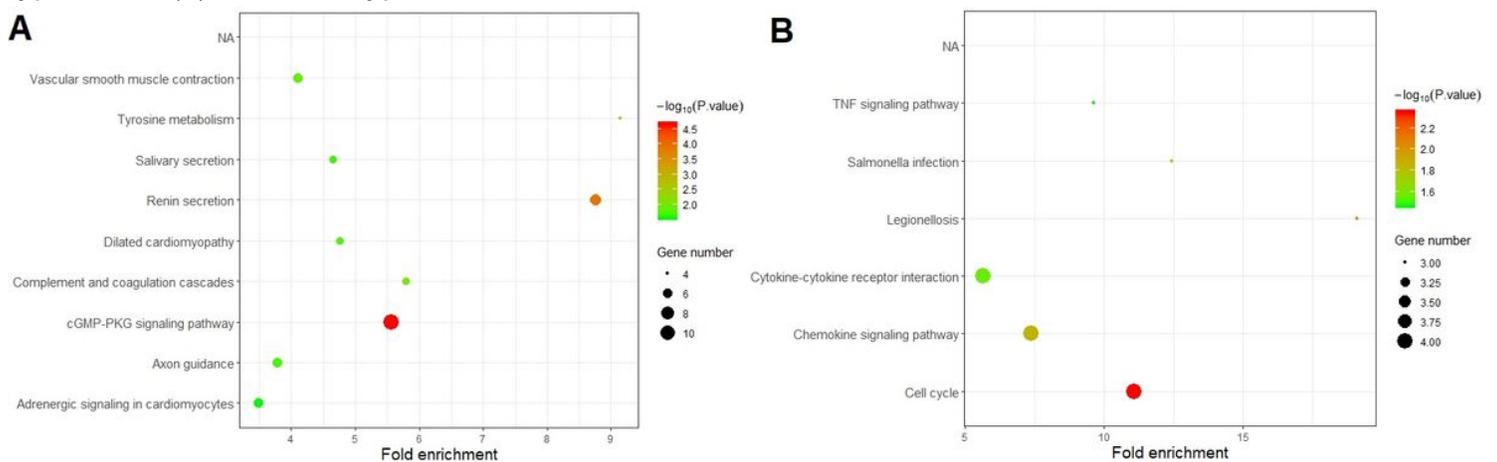
**Figure 3**

Top 15 enriched GO terms. (A) diffuse type DEGs with the top 15 enriched GO terms. (B) intestinal type DEGs with the top 15 enriched GO terms.



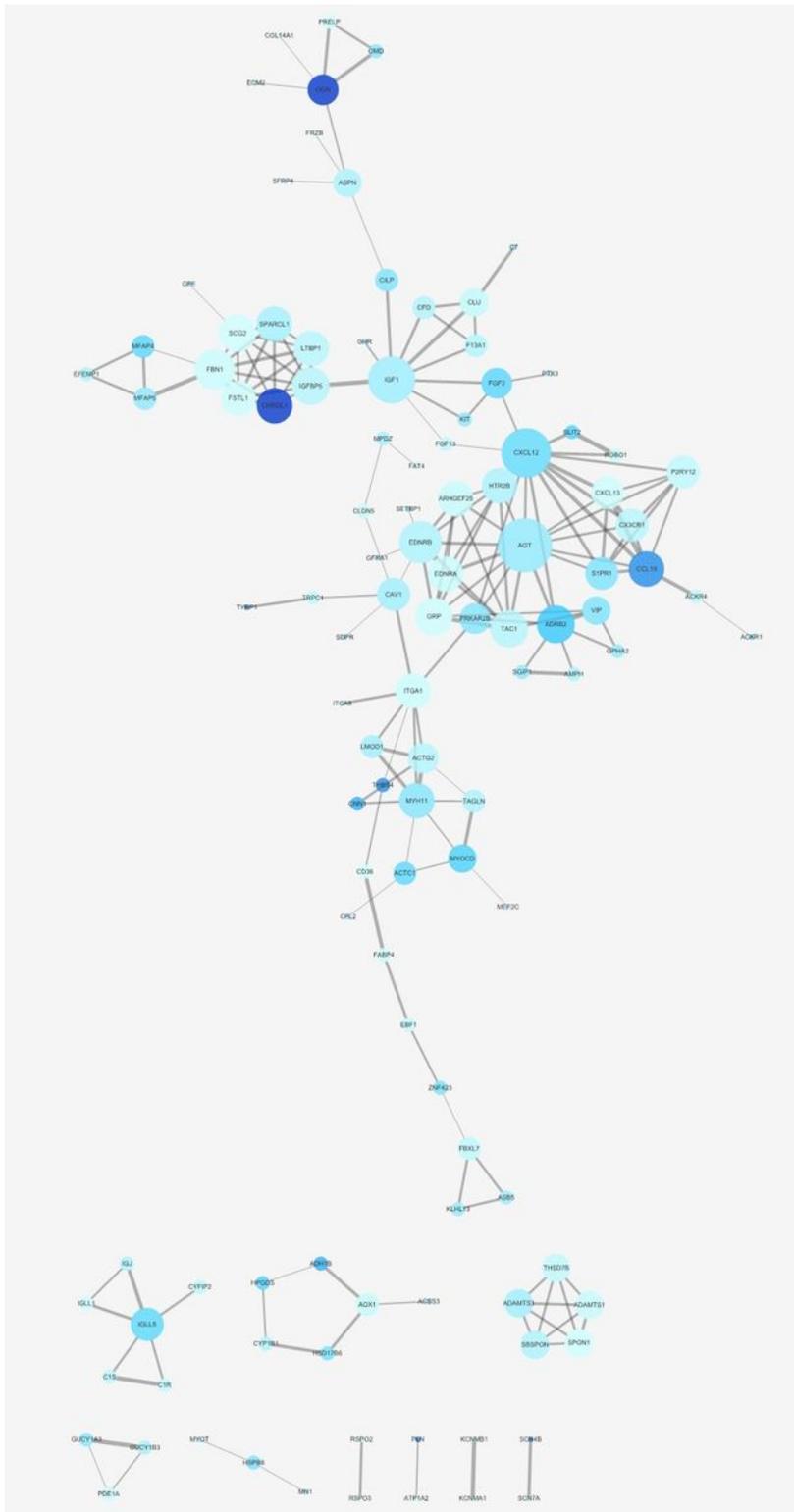
**Figure 4**

Distribution of subtype-specific DEGs in corresponding GC for different GO-enriched functions. (A) diffuse type DEGs. (B) intestinal type DEGs.



**Figure 5**

KEGG pathway enrichment analysis of the subtype-specific DEGs. (A) diffuse type DEGs. (B) intestinal type DEGs.



**Figure 6**

Protein–protein interaction (PPI) network of diffuse type DEGs. Node color: the deeper the blue represents the higher the logFC of gene, Node size:the larger means the more genes connected.

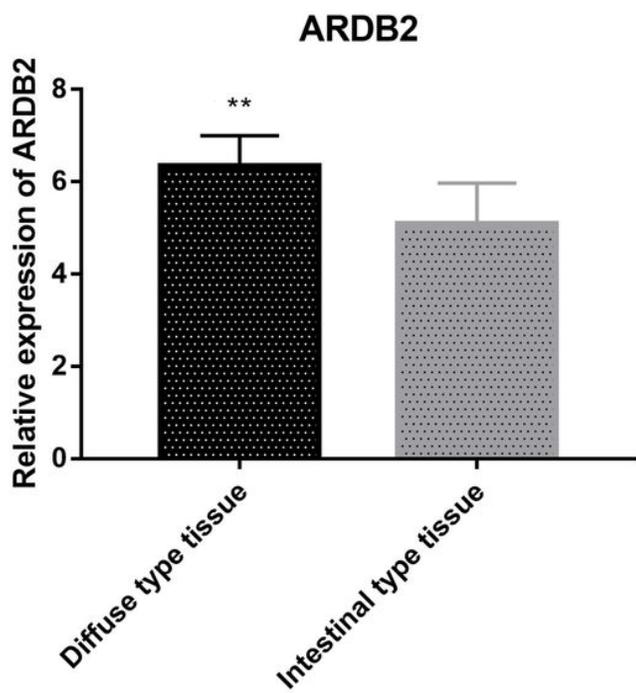
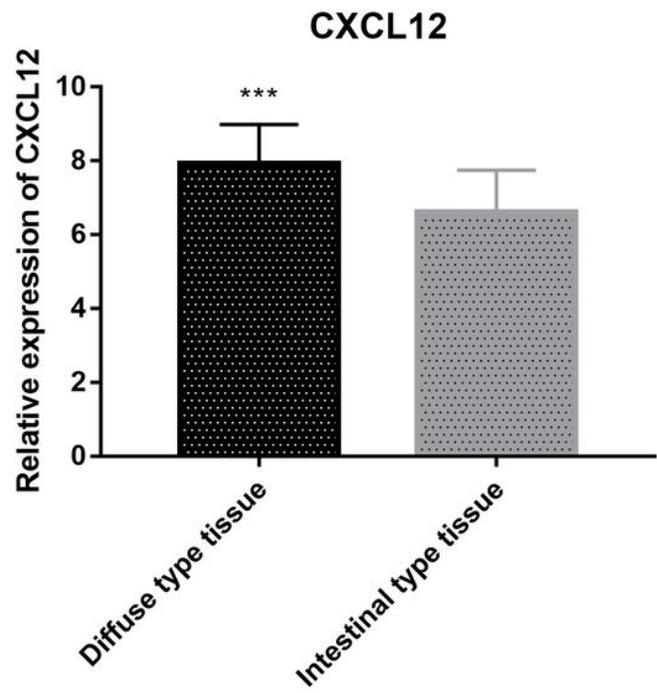
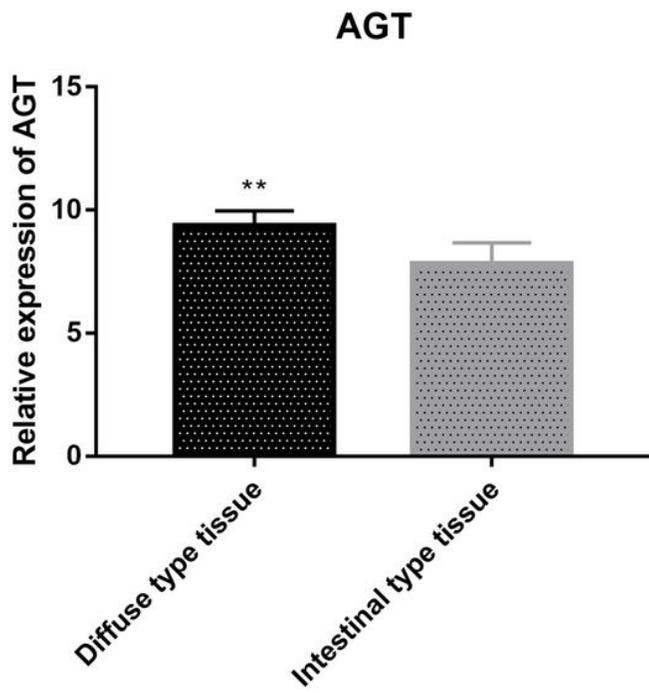
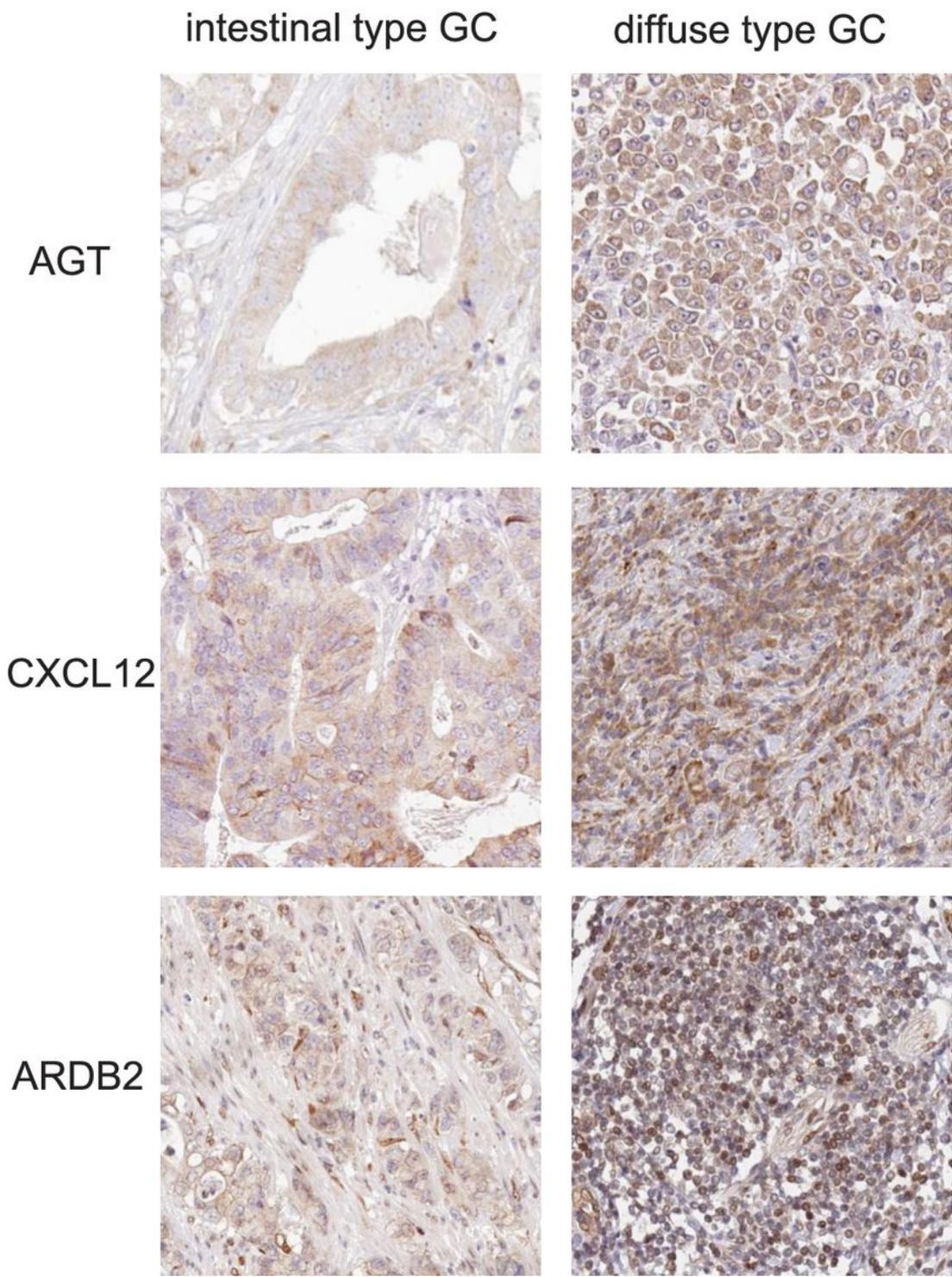


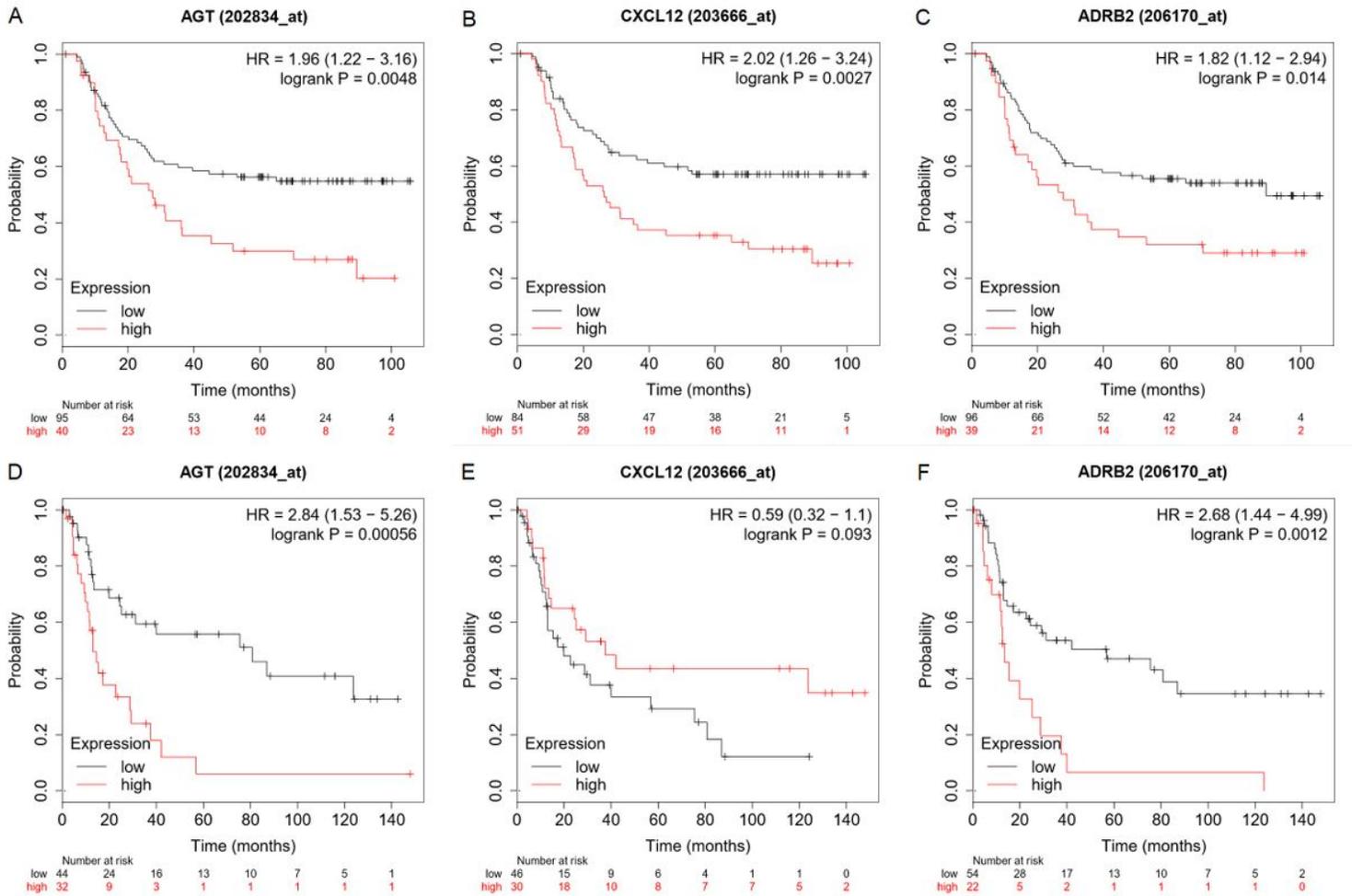
Figure 7

Immunohistochemical detection of AGT, CXCL12 and ADRB2 expression in different GC lauren type tissues. Original magnifications, ×400.



**Figure 8**

Verification results of quantitative real-time polymerase chain reaction (qRT-PCR) of AGT, CXCL12 and ADRB2. \*\* Indicates a significant difference between diffuse type GC tissues and intestinal type GC tissue ( $p < 0.01$ ). \*\*\* Indicates an extremely significant difference between diffuse type GC tissues and intestinal type GC tissues ( $p < 0.001$ )



**Figure 9**

Survival analysis curves of hub genes in diffuse type GC, where the x-axis represents the overall survival time and the y-axis represents the survival rate (A-C) Survival analysis comparing overall survival and the expression state of (A) AGT, (B) CXCL12 and (C) ADRB2 based on GSE62254 database (D-F) Survival analysis comparing overall survival and the expression state of (D) AGT, (E) CXCL12 and (F) ADRB2 based on GSE15459 database

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableSI.xlsx](#)