Identification of circRNA–miRNA–Immune-Related mRNA Regulatory Network in Gastric Cancer

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The pathogenesis of gastric cancer (GC) is still not fully understood. We aimed to find the potential regulatory network for ceRNA (circRNA–miRNA–immune-related mRNA) to uncover the pathological molecular mechanisms of GC. The expression profiles of circRNA, miRNA, and mRNA in gastric tissue from GC patients were downloaded from the Gene Expression Omnibus (GEO) datasets. Differentially expressed circRNAs, miRNAs, and immune-related mRNAs were filtered, followed by the construction of the ceRNA (circRNA–miRNA–immune-related mRNA) network. Functional annotation and protein–protein interaction (PPI) analysis of immune-related mRNAs in the network were performed. Expression validation of circRNAs and immune-related mRNAs was performed in the new GEO and TCGA datasets and in-vitro experiment. A total of 144 differentially expressed circRNAs, 216 differentially expressed miRNAs, and 2,392 differentially expressed mRNAs were identified in GC. Some regulatory pairs of circRNA–miRNA–immune-related mRNA were obtained, including hsa_circ_0050102–hsa-miR-4537–NRAS–Tgd cells, hsa_circ_0001013–hsa-miR-485-3p–MAP2K1–Tgd cells, hsa_circ_0003763–hsa-miR-145-5p–FGF10–StromaScore, hsa_circ_0001789–hsa-miR-1269b–MET–adipocytes, hsa_circ_0040573–hsa-miR-3686–RAC1–Tgd cells, and hsa_circ_0006089–hsa-miR-5584-3p–LYN–neurons. Interestingly, FGF10, MET, NRAS, RAC1, MAP2K1, and LYN had potential diagnostic value for GC patients. In the KEGG analysis, some signaling pathways were identified, such as Rap1 and Ras signaling pathways (involved NRAS and FGF10), Fc gamma R-mediated phagocytosis and cAMP signaling pathway (involved RAC1), proteoglycans in cancer (involved MET), T-cell receptor signaling pathway (involved MAP2K1), and chemokine signaling pathway (involved LYN). The expression validation of hsa_circ_0003763, hsa_circ_0001013, hsa_circ_0040573, FGF10, MET, NRAS, RAC1, MAP2K1, and LYN was consistent with the integrated analysis. In conclusion, the identified ceRNA (circRNA–miRNA–immune-related mRNA) regulatory network may be associated with the development of GC.

Keywords: gastric cancer, circRNA, miRNAs, mRNAs, diagnosis, immune, signaling pathway, protein–protein interaction
INTRODUCTION

Gastric cancer (GC) is one of the most serious malignant tumors (1). According to the GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries, the incidence of GC is 7.7% (2). In addition, with 2.26 million new cases estimated in 2020, GC (0.77 million) has become the most commonly diagnosed cancer worldwide, ranking only second to female breast cancer (3). Recurrence is the main cause of GC-related death. Although mortality is steadily decreasing, GC still leads to a poor diagnosis and prognosis for patients (4). In addition, the 5-year survival rate of patients is still very low in serious GC patients. It has been shown that obesity, active tobacco smoking, high meat and salt intake, low vegetable/fruit intake, Helicobacter pylori infection, and gut microbiota have been shown to be associated with an increased risk of GC (5–8). In addition, epigenetic alterations are associated with the processes of gastric carcinogenesis and metastasis (9). Clinically, surgery is the only curative treatment; however, some patients have inoperable disease at diagnosis (10). Hence, there is a need to elucidate the potential molecular mechanisms in the development of GC and to look for new molecular markers and therapeutic targets.

circRNAs usually result from splicing/back-splicing events via exon or intron circularization (11). circRNA can act as a molecular sponge of miRNA to regulate mRNA expression. It was reported that sponge circRNAs are part of a complex RNA-binding protein–circRNA–miRNA–mRNA interaction network and are involved in the establishment, chemoresistance, and progression of GC (12). hsa_circ_LARP4 can inhibit cell invasion of GC by sponging hsa-miR-424-5p and regulating large tumor suppressor protein that sponge circRNAs are part of a complex RNA-binding exon or intron circularization (11). circRNA can act as a molecular sponge of miRNA to regulate mRNA expression. It was reported that sponge circRNAs are part of a complex RNA-binding protein–circRNA–miRNA–mRNA interaction network and are involved in the establishment, chemoresistance, and progression of GC (12).

MATERIALS AND METHODS

Data Retrieval and Analysis

In this study, the expression profiles of circRNA, miRNA, and mRNA were downloaded from the Gene Expression Omnibus (GEO) datasets by searching keywords [“gastric cancer” (All Fields) AND “Homo sapiens”(organ) AND “gse”(Filter)]. The following datasets were selected: 1) dataset must be genome-wide transcriptome data of mRNA/miRNA/circRNA; 2) data were obtained from tumor tissues of the GC group and paracancer control group; and 3) both standardized and raw datasets were considered. Finally, two circRNA expression datasets (GSE83521 and GSE89143), two miRNA expression datasets (GSE93415 and GSE158315), and two mRNA expression datasets (GSE66229 and GSE65801) were selected (Table 1).

Screening of Differentially Expressed circRNAs, miRNAs, and Immune-Related mRNAs

The probe and ID of circRNA/miRNA/mRNA were mapped one by one. After scale standardization, the dataset was merged and batch effects were removed via the ComBat function of sva package (R-4.0.5). The metaMA and limma packages were used to identify circRNAs/miRNAs/mRNAs. The default parameter of the Pvalcombination command of the metaMA package was used to make the difference. p-values and effect sizes (ES, the effectSize obtained from the metaMA package) from the combined.ES = 1 and FDR <0.05 were the screening criteria for circRNAs/miRNAs/mRNAs. In

TABLE 1 | Detailed information of selected datasets of circRNA, miRNA, and mRNA expression datasets.

| GEO accession | Author | Platform | Samples (N:GC) | Year | Tissue |
|---------------|--------|----------|----------------|------|--------|
| GSE83521 | Yan Zhang | GPL19978 Agilent-069978 Arraystar Human CircRNA microarray V1 | 6:6 | 2017 | Gastric tissue |
| GSE89143 | Jianmin Guo | GPL19978 Agilent-069978 Arraystar Human CircRNA microarray V1 | 3:3 | 2017 | Gastric tissue |
| GSE93415 | Marek Sierżega | GPL19071 Exiqon miRCURY LNA microRNA array; 7th generation REV - hsa, mmu, and mco; batch 208520-22; lot 35101-35101 (miRBase 19.0) | 20:20 | 2017 | Gastric tissue |
| GSE158315 | Yuping Wang | GPL18058 Exiqon miRCURY LNA microRNA array, 7th generation (miRBase v18, condensed Probe_ID version) | 5:5 | 2021 | Gastric tissue |
| GSE66229 | Michael Nebozhyn | GPL570 (H3-1333_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array | 100:300 | 2015 | Gastric tissue |
| GSE65801 | Hao Li | GPL14550 Agilent-028004 SurePrint G3 Human GE 8 x 60K Microarray (Probe Name Version) | 32:32 | 2015 | Gastric tissue |

N, paracancer control group; GC, gastric cancer group.
addition, immune-related mRNAs were downloaded from the ImmPort database (https://www.immport.org/shared/home). Those immune-related mRNAs were obtained by intersection of differentially expressed mRNAs and immune-related mRNAs in the ImmPort database. xCell (21) was used to calculate the distribution of immune cells in each sample based on the ssGSEA method. xCell score was organized into immune cell infiltration matrix to calculate the types of immune cells that differed between the GC group and the normal control group. The Pearson correlation coefficient method was used to calculate the correlation between mRNAs and differential immune cells.

Construction of the ceRNA (circRNA–miRNA–Immune-Related mRNA) Regulatory Network

The TargetScan (http://www.targetscan.org/vert_71/) software was utilized to predict targeted relationship between differentially expressed circRNAs and differentially expressed miRNAs. In addition, the miRWalk software (http://mirwalk.umm.uni-heidelberg.de/interactions/) was applied to predict targeted relationship between miRNAs and mRNAs. The relationship pairs of miRNA–mRNA verified in at least one database (TargetScan, miRDB, and MiRTarBase) were selected. The overlapping mRNAs were obtained between predicted mRNAs (in the miRNA–mRNA relationship pairs) and immune-related differential expression mRNAs. The ceRNA (circRNA–miRNA–immune-related mRNA) regulatory network was constructed by fusing with the circRNA–mRNA relationship pairs and miRNA–immune-related mRNA relationship pairs.

Functional Annotation of Immune-Related mRNAs in the ceRNA Regulatory Network

To investigate the function of immune-related mRNAs in the regulatory ceRNA network, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed by using the DAVID database (https://david.ncifcrf.gov/tools.jsp). FDR <0.05 was considered as statistical significance.

Protein–Protein Interaction Network of Immune-Related mRNAs in the ceRNA Regulatory Network

To further explore the interaction between immune-related mRNAs in the ceRNA regulatory network, protein–protein interaction (PPI) was performed by using the STRING database. The results were imported using the Cytoscape software (http://www.cytoscape.org/). The CytoHubba plug-in was used to filter core immune-related mRNAs by intersecting the first 10 mRNAs of each algorithm (degree, MNC, MCC, and EPC). In addition, the ROC analysis was carried out to assess the diagnostic value of core immune-related mRNAs in the PPI network.

Expression Validation of Differentially Expressed circRNAs and Immune-Related mRNAs

In order to further validate the expression of identified circRNAs and immune-related mRNAs, electronic validation was performed. The GSE93541 dataset (involving tumor tissues from three cases and three normal controls) and the GSE141977 dataset (involving plasma from three cases and three normal controls) were used for expression validation of identified circRNAs. Additionally, the Cancer Genome Atlas (TCGA) dataset (involved tumor tissues from 375 cases and 32 normal controls) was used to validate the expression of identified mRNAs. The expression result of these circRNAs and mRNAs was shown by the box plots. Statistical significance was ascribed to p-value <0.05.

In addition, qRT-PCR was used to further validate the expression of identified circRNAs and immune-related mRNAs. Five GC patients and five normal controls were enrolled. The blood sample of these individuals was collected. All participating individuals provided informed consent with the approval of the Ethics Committee of Zhejiang Hospital (2021-164K). Total RNA of the blood sample was extracted and DNA was synthesized using FastQuant cDNA first-strand synthesis kit. qRT-PCR was performed in the SuperReal PreMix Plus (SYBR Green). Relative circRNA/mRNA expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. $2^{-\Delta\Delta Ct}$ >1 and $2^{-\Delta\Delta Ct}$ <1 represented upregulation and downregulation, respectively.

RESULTS

Differentially Expressed circRNAs, miRNAs, and Immune-Related mRNAs

A total of 144 differentially expressed (55 upregulated and 89 downregulated) circRNAs, 216 differentially expressed (105 upregulated and 111 downregulated) miRNAs, and 2,392 differentially expressed (1,329 upregulated and 1,063 downregulated) mRNAs were obtained in GC. The top 10 differentially expressed circRNAs were identified, such as hsa_circ_00145062 (upregulation), hsa_circ_0006089 (upregulation), hsa_circ_0001789 (upregulation), hsa_circ_0018004 (upregulation) and hsa_circ_003763 (downregulation) (Table 2). hsa-miR-4537 was one of top 10 downregulated miRNAs (Table 3). The top 10 differentially expressed miRNAs are listed in Table 4. The heat map of the top 100 differentially expressed circRNAs, miRNAs, and mRNAs is shown in Figures 1A–C, respectively. In addition, 1,793 immune-related mRNAs were obtained from the ImmPort database. It was noted that 159 immune-related mRNAs were also differentially expressed mRNAs in GC (Figure 2).

cRNA (circRNA–miRNA–Immune-Related mRNA) Regulatory Network

A total of 2,037 negatively regulated targeting relationship pairs of circRNA–miRNA (involving 143 circRNAs and 198 miRNAs) and 142 negatively regulated targeting relationship pairs of miRNA–immune-related mRNA (involving 97 miRNAs and 58 immune-related mRNAs) were identified via the TargetScan software and miRWalk, respectively. The ceRNA (circRNA–miRNA–immune-related mRNA) regulatory network (involving 137 circRNAs, 96 miRNAs,
and 58 immune-related mRNAs) was obtained by fusing with the circRNA–miRNA relationship pairs and miRNA–immune-related mRNA relationship pairs (Figure 3). Some ceRNA relationship pairs were identified, such as hsa_circ_0045602-hsa-miR-4538–WNT5A and hsa_circ_0018004–hsa-miR-199a-5p–KL.

**Enrichment Analysis of Immune-Related mRNAs in the ceRNA Regulatory Network**

Fifty-eight immune-related mRNAs in the regulatory ceRNA network were used for functional enrichment analysis. GO analysis showed that signal transduction, extracellular space, and growth factor activity were the most significantly enriched biological process, cytological component, and molecular function, respectively (Figure 4). According to the KEGG analysis, Rap1 and Ras signaling pathways [involved NRAS proto-oncogene, GTPase (NRAS), and fibroblast growth factor 10 (FGF10)], Fc gamma R-mediated phagocytosis and cAMP signaling pathway [involved Rac family small GTPase 1 (RAC1)], proteoglycans in cancer [involved MET proto-oncogene, receptor tyrosine kinase (MET)], T-cell receptor signaling pathway [involved mitogen-activated protein kinase

### Table 2 | The top 10 upregulated and downregulated circRNAs in GC.

| ID                | Symbol                          | Combined.ES | p-value    | FDR          | Up/down |
|-------------------|---------------------------------|-------------|------------|--------------|---------|
| hsa_circRNA_102191 | hsa_circ_0045602                | 3.118562462 | 1.07E-06   | 0.000830662  | Up      |
| hsa_circRNA_104947 | hsa_circ_0007613                | 3.076836769 | 1.43E-06   | 0.000830662  | Up      |
| hsa_circRNA_101882 | hsa_circ_00340573               | 3.859880212 | 1.82E-06   | 0.000830662  | Up      |
| hsa_circRNA_102592 | hsa_circ_00523001               | 3.180132727 | 2.28E-06   | 0.000830662  | Up      |
| hsa_circRNA_101471 | hsa_circ_00343998               | 2.865182594 | 3.78E-06   | 0.000830662  | Up      |
| hsa_circRNA_102614 | hsa_circ_00060899               | 2.831849087 | 3.94E-06   | 0.000830662  | Up      |
| hsa_circRNA_104589 | hsa_circ_0001789                | 3.306599675 | 4.20E-06   | 0.000830662  | Up      |
| hsa_circRNA_100641 | hsa_circ_0019054                | 2.89673611  | 4.77E-06   | 0.00087084   | Down    |
| hsa_circRNA_101875 | hsa_circ_0040481                | 2.642927097 | 9.51E-06   | 0.001488245  | Down    |
| hsa_circRNA_102777 | hsa_circ_0055521                | 2.42548772  | 2.78E-05   | 0.003099928  | Down    |
| hsa_circRNA_100571 | hsa_circ_0018004                | -3.196809378| 9.51E-07   | 0.000830662  | Down    |
| hsa_circRNA_104599 | hsa_circ_0001793                | -3.624031186| 2.17E-06   | 0.000830662  | Down    |
| hsa_circRNA_400666 | hsa_circ_0092330                | -2.878804101| 3.17E-06   | 0.000830662  | Down    |
| hsa_circRNA_101651 | hsa_circ_00368941               | -2.829679697| 3.88E-06   | 0.000830662  | Down    |
| hsa_circRNA_001914 | hsa_circ_0000902                | -2.987032494| 7.15E-06   | 0.001245059  | Down    |
| hsa_circRNA_101965 | hsa_circ_0000740                | -2.632340492| 1.06E-05   | 0.001531677  | Down    |
| hsa_circRNA_103442 | hsa_circ_0003763                | -2.780691514| 1.88E-05   | 0.00256875  | Down    |
| hsa_circRNA_400056 | hsa_circ_0092297                | -2.597537412| 2.21E-05   | 0.00275069  | Down    |
| hsa_circRNA_100382 | hsa_circ_0002377                | -2.69612581 | 2.26E-05   | 0.00275069  | Down    |
| hsa_circRNA_104661 | hsa_circ_0004386                | -2.495771422| 2.39E-05   | 0.00275019  | Down    |

**ES, effect size; FDR, false discovery rate.**

### Table 3 | The top 10 upregulated and downregulated miRNAs in GC.

| Symbol          | Combined.ES | p-value | FDR          | Up/down |
|-----------------|-------------|---------|--------------|---------|
| hsa-miR-181a-5p | 2.621880675 | 8.01E-13| 6.84E-10     | Up      |
| hsa-miR-181b-5p | 2.541945488 | 4.05E-12| 1.15E-09     | Up      |
| hsa-miR-23a-3p  | 2.283816919 | 1.17E-10| 1.11E-08     | Up      |
| hsa-miR-331-3p  | 2.121044968 | 1.04E-09| 6.82E-08     | Up      |
| hsa-miR-320c    | 2.029389583 | 1.58E-09| 8.73E-08     | Up      |
| hsa-miR-25-3p   | 1.94259155  | 4.78E-09| 2.55E-07     | Up      |
| hsa-miR-320d    | 1.961475455 | 6.29E-09| 2.98E-07     | Up      |
| hsa-miR-92a-3p  | 1.928060578 | 7.08E-09| 3.02E-07     | Up      |
| hsa-miR-21-3p   | 1.858730342 | 1.58E-08| 5.18E-07     | Up      |
| hsa-miR-320e    | 1.90482778  | 1.72E-08| 5.44E-07     | Up      |
| hsa-miR-4279    | -2.564714404| 1.76E-12| 7.51E-10     | Down    |
| hsa-miR-3124-3p | -2.474608778| 5.60E-12| 1.19E-09     | Down    |
| hsa-miR-4728-3p | -2.455455298| 1.21E-11| 2.07E-09     | Down    |
| hsa-miR-4835    | -2.39092709 | 1.54E-11| 2.19E-09     | Down    |
| hsa-miR-4537    | -2.418295745| 5.96E-11| 7.27E-09     | Down    |
| hsa-miR-8425-5p | -2.298345262| 8.63E-11| 9.21E-09     | Down    |
| hsa-miR-5196-3p | -2.172908344| 3.75E-10| 3.20E-08     | Down    |
| hsa-miR-877-3p  | -2.202864847| 4.62E-10| 3.51E-08     | Down    |
| hsa-miR-4290    | -2.27123672 | 4.90E-10| 3.51E-08     | Down    |
| hsa-miR-4288    | -2.219462883| 1.40E-09| 8.54E-08     | Down    |

**ES, effect size; FDR, false discovery rate.**
kinase 1 (MAP2K1), and chemokine signaling pathway [involved LYN proto-oncogene, Src family tyrosine kinase (LYN)] were significantly enriched signaling pathways (Figure 4 and Table 5).

PPI Network of Immune-Related mRNAs in the ceRNA Regulatory Network

In order to further explore the interaction between immune-related mRNAs in the regulatory ceRNA network, the PPI network was established (Figure 5). Six core immune-related mRNAs were identified by four algorithms, namely, FGF10, MET, NRAS, RAC1, MAP2K1, and LYN (Figure 6). It is worth mentioning that FGF10 (AUC = 0.764), MET (AUC = 0.829), NRAS (AUC = 0.840), RAC1 (AUC = 0.797), MAP2K1 (AUC = 0.813), and LYN (AUC = 0.817) had a potential diagnostic value for GC (Figure 7). It was indicated that these mRNAs had a diagnostic potential for GC. In addition, the ceRNA subnetwork (involving 59 circRNAs and 10 miRNAs) based on the above six mRNAs was constructed (Figure 8). The ceRNA subnetwork contains 75 nodes and 124 edges. Some ceRNA relationship pairs were identified, such as hsa_circ_0050102–hsa-miR-4537–NRAS, hsa_circ_0001013–hsa-miR-485-3p–MAP2K1, hsa_circ_0003763–hsa-miR-145-5p–FGF10, hsa_circ_001789–hsa-miR-1269b–LYN, hsa_circ_0040573–hsa-miR-3686–RAC1, and hsa_circ_0006089–hsa-miR-5584-3p–LYN. In addition, xCell was used to calculate the distribution of immune cells in each sample based on the ssGSEA method. The types of immune cells were different in the GC group from those in the normal control group. For example, Tgd cells and adipocytes were the most upregulated and downregulated types of immune cells in GC (Figure 9). The Pearson correlation coefficient method was used to calculate the correlation between above six mRNAs of differential immune cells (Figure 10). The result showed that NRAS, RAC1, and MAP2K1 were positively correlated with Tgd cells. FGF10 was positively correlated with StromaScore. LYN and MET were negatively correlated with neurons and adipocytes, respectively.
To further validate the expression of identified circRNAs and immune-related mRNAs, electronic validation was performed. The GSE93541 dataset (Figure 11A) and the GSE141977 dataset (Figure 11B) were used for expression validation of hsa_circ_0003763, hsa_circ_0004928, and hsa_circ_0040573. The result showed that hsa_circ_0003763 was downregulated and hsa_circ_0004928 and hsa_circ_0040573 were upregulated.
FIGURE 4 | GO (A) and KEGG (B) enrichment analyses of immune-related mRNAs in the regulatory ceRNA network in GC. BP, biological process; CC, cytological component; MF, molecular function.

TABLE 5 | KEGG enrichment analysis of immune-related mRNAs in the ceRNA regulatory network in GC.

| Term                          | Count | p-value | FDR | mRNAs                                                                 |
|-------------------------------|-------|---------|-----|----------------------------------------------------------------------|
| hsa05200:Pathways in cancer   | 16    | 8.63E−09| 9.34E−07 | MAP2K1, PRKCB, F2R, WNT5A, TGFBR1, PGF, NRAS, FGF14, TRAF3, CDK4, AGR1, RAC1, FGF13, BID, MET, FGF10 |
| hsa04060:Cytokine-cytokine receptor interaction | 11    | 2.17E−08| 1.47E−04 | GHR, CXCL9, TNFSF12A, TNFSF15, TNFSF9, LIF, TNFSF11, TNFSF10B, LIFR, IL1RAP, TGFBR1 |
| hsa05218:Melanoma             | 7     | 5.03E−09| 4.17E−04 | MAP2K1, NRAS, FGF14, CDK4, FGF13, MET, FGF10 |
| hsa04015:Rap1 signaling pathway | 10   | 5.43E−08| 3.84E−04 | MAP2K1, NRAS, FGF14, PRKCB, F2R, RAC1, FGF13, MET, Pgf, FGF10 |
| hsa04014:Ras signaling pathway | 10   | 9.89E−08| 7.43E−04 | MAP2K1, NRAS, FGF14, PRKCB, F2R, RAC1, FGF13, MET, Pgf, FGF10 |
| hsa04810:Regulation of actin cytoskeleton | 9     | 4.54E−07| 3.34E−04 | MAP2K1, NRAS, FGF14, CDK4, FGF13, MET, Pgf, FGF10 |
| hsa04660:Fc epsilon RI signaling pathway | 11   | 4.75E−07| 3.53E−04 | GHR, MAP2K1, NRAS, FGF14, CDK4, FGF13, MET, Pgf, FGF10 |
| hsa04664:Fc epsilon RI signaling pathway | 6    | 6.55E−07| 4.17E−04 | LYN, MAP2K1, NRAS, PRKCB, RAC1, VAV2 |
| hsa04650:Naive killer cell mediated cytotoxicity | 7     | 1.12E−06| 8.09E−04 | MAP2K1, NRAS, PRKCB, TNFSF10B, RAC1, BID, VAV2 |
| hsa05211:Renal cell carcinoma | 5     | 7.88E−07| 5.07E−04 | MAP2K1, NRAS, RAC1, PK3, MET |
| hsa04662:B-cell receptor signaling pathway | 5     | 9.50E−07| 6.23E−04 | LYN, MAP2K1, NRAS, RAC1, VAV2 |
| hsa04010:MAPK signaling pathway | 8     | 8.00E−07| 5.43E−04 | MAP2K1, NRAS, FGF14, PRKCB, RAC1, FGF13, TGFBR1, FGF10 |
| hsa04612:Antigen processing and presentation | 5     | 0.001338| 0.009003 | MAP2K1, NRAS, PRKCB, RAC1, VAV2 |
| hsa05205:Proteoglycans in cancer | 7     | 0.001573| 0.009003 | MAP2K1, NRAS, PRKCB, WNT5A, PLAU, RAC1, MET |
| hsa04510:Focal adhesion | 7     | 0.00183 | 0.009003 | MAP2K1, PRKCB, RAC1, PK3, MET, Pgf, VAV2 |
| hsa04666:Fc receptor signaling pathway | 5     | 0.001937| 0.009003 | MAP2K1, NRAS, RAC1, VAV2 |
| hsa04620:T-cell receptor signaling pathway | 5     | 0.003655| 0.012318 | MAP2K1, NRAS, PRKCB, WNT5A, PLAU, RAC1, MET |
| hsa05223:Non-small cell lung cancer | 4     | 0.005345| 0.012318 | MAP2K1, NRAS, PRKCB, CDK4 |
| hsa04062:Chemokine signaling pathway | 6     | 0.006326| 0.012318 | MAP2K1, CXCL9, NRAS, RAC1, VAV2 |
| hsa04730:Long-term depression | 4     | 0.006842| 0.012318 | MAP2K1, NRAS, PRKCB, CDK4 |
| hsa04370:VEGF signaling pathway | 4     | 0.006797| 0.012318 | MAP2K1, NRAS, PRKCB, RAC1 |
| hsa04071:Phospholipid signaling pathway | 5     | 0.006981| 0.012318 | MAP2K1, NRAS, PRKCB, RAC1, BID |
| hsa05214:Glio | 4     | 0.008903| 0.012318 | MAP2K1, NRAS, PRKCB, CDK4 |
| hsa05212:Pancreatic cancer | 4     | 0.008903| 0.012318 | MAP2K1, CDK4, RAC1, TGFBR1 |
| hsa04024:AMPK signaling pathway | 6     | 0.008199| 0.012318 | MAP2K1, FSHB, F2R, RAC1, ADRB2, VAV2 |
| hsa05220:Chronic myeloid leukemia | 4     | 0.010713| 0.012318 | MAP2K1, NRAS, CDK4, TGFBR1 |
| hsa04550:Signal pathways regulating pluripotency of stem cells | 5     | 0.01188| 0.012318 | MAP2K1, NRAS, WNT5A, LIF, TGFBR1 |

FDR, false discovery rate.
in GC. Although there was no significant difference (may be caused by the small sample size), the expression trend was consistent with the integrated analysis results. In addition, the TCGA dataset was used to validate the expression of FGF10, MET, NRAS, RAC1, MAP2K1, and LYN (Figure 12). The result showed that FGF10 was significantly downregulated. MET, NRAS, RAC1, MAP2K1, and LYN were remarkably upregulated in GC, which is in line with the integrated analysis results.

To validate the expression of two circRNAs (hsa_circ_0003763 and hsa_circ_0004928) and four immune-related mRNAs (FGF10, MET, RAC1, and LYN), blood samples from five GC patients and five normal controls were collected for qRT-PCR (Figure 13). The clinical information of these individuals is listed in Table 6. The qRT-PCR result showed that RAC1 and LYN were significantly upregulated in GC, which is consistent with the integrated analysis results, while the expression trends of hsa_circ_0004928, MET, hsa_circ_0003763, and FGF10 were the same with the integrated analysis results without statistical significance.

**DISCUSSION**

Up to now, no articles about hsa_circ_0050102 have been reported in any diseases. hsa_circ_0003763 was associated with pancreatic ductal adenocarcinoma (22). hsa_circ_0003763 was upregulated in hepatocellular carcinoma tissues and facilitated the invasion of hepatocellular carcinoma cells (23). hsa-miR-4537 was associated with vitreoretinal lymphoma (24). hsa-miR-145-5p played key roles in GC epithelial cells and affected cell invasion and migration of GC cells (25). In GC cells, hsa-miR-145-5p has been verified as the direct target of has_circ_0000376 (26). It has been demonstrated that hsa_circ_DLST can act as the sponge of has-miR-502-5p to regulate the NRAS/MAP kinase/ERK kinase 1/2 signaling pathways in GC cells (27). FGF10, a secreted factor, stimulated the proliferation of lung cancer cells (28). In GC, FGF10 was involved in several signaling pathways (29). Tgd cells were related to the prognosis of patients with stomach adenocarcinoma (30). In this study, we found regulatory pairs of hsa_circ_0050102–hsa-miR-4537–NRAS–Tgd cells and hsa_circ_0003763–hsa-miR-145-5p–FGF10–StromaScore in GC, among which, NRAS and FGF10 had potential diagnostic value for patients. In addition, NRAS and FGF10 were involved in both Rap1 and Ras signaling pathways in GC. Rap1 played important roles in the metastasis and invasion of various tumor cells by regulating cytoskeleton remodeling. In GC, some differentially expressed angiogenesis-related genes were associated with the Rap1 signaling pathway (31). Genes involved in the Ras signaling pathway were found in approximately 40% of GC patients (32). It has been suggested that regulatory networks of hsa_circ_0050102–hsa-miR-4537–NRAS–Tgd cells and hsa_circ_0003763–hsa-miR-145-5p–FGF10–StromaScore may play important roles in the proliferation, migration, and invasion of GC by involving signaling pathways of Rap1 and Ras.

hsa_circ_0040573 was found in human umbilical vein endothelial cell line (33). It was shown that RAC1 was an essential effector of GC malignant transformation and metastasis (34, 35). In gastric cancer tissues, the expression of RAC1 was increased, which was significantly related to TNM
stage (36). Herein, we found the regulatory pairs of hsa_circ_0040573–hsa-miR-3686–RAC1–Tgd cells in GC. It was noted that RAC1 had potential diagnostic value for patients. Moreover, RAC1 was associated with Fc gamma R-mediated phagocytosis and cAMP signaling pathway. Fc gamma R-mediated phagocytosis was found in plasma exosomes from GC patients (37). It has been demonstrated that there was a significant positive correlation between nuclear factor kappa B (NF-κB) and cAMP-regulated phosphoprotein expression level in GC tissues (38). Thus, it can be seen that hsa_circ_0040573, RAC1, and Tgd cells played a crucial role in malignant transformation, metastasis, and TNM stage of GC.

The expression of hsa_circ_0001789 was found in GC (39). hsa-miR-1269b was downregulated in basal cell carcinoma and GC (40, 41). MET can be internalized by macrophages to educate them toward a protumorigenic phenotype (42). In addition, the

FIGURE 7 | ROC analysis of six core immune-related mRNAs in the PPI network in GC.

FIGURE 8 | The ceRNA subnetwork based on six core immune-related mRNAs in the PPI network in GC. Circle, V shape, and rectangle represent differentially expressed circRNA, miRNA, and mRNA, respectively. Red and green represent upregulation and downregulation, respectively. The black border represents the top 10 up/downregulated circRNAs/miRNAs/mRNAs.
upregulated paracrine hepatocyte growth factor can bind the c-MET receptor on the migrated GC cells to facilitate the proliferation of metastatic GC cells (43). In fat tissues, adipocytes regulated cancer development by their effects on the microenvironment. A previous report preliminarily showed that omental adipocytes could promote GC cell invasiveness (44). Herein, we found a regulatory pair of hsa_circ_0001789–hsa-miR-1269b–MET–adipocytes in GC. MET had a potential diagnostic value for patients. Furthermore, MET was involved in proteoglycans in cancer in GC. In gastric epithelia, the basal surface of cells was surrounded by the basement membrane, which was mainly composed of glycoproteins (45–47). The proteoglycan signaling pathway has been found in GC (48). It was indicated that the regulatory pair of hsa_circ_0001789–hsa-miR-1269b–MET–adipocytes played roles in cell proliferation and invasiveness of GC.

hsa_circ_0001013, upregulated in GC, regulated the expression of fibrillin 1 through competing with miRNA response elements of hsa-miRNA-182-5p, which led to metastasis in GC (49). hsa-miR-485-3p, a key regulator of gastrointestinal stromal tumors, served as a molecular biomarker and potential therapeutic target for this malignant disease (50). The role of hsa-miR-485-3p has been found in GC cells (51). MAP2K1 was involved in the PD-L1 pathway in GC (52). In our study, we found the regulatory network of hsa_circ_0001013–hsa-miR-485-3p–MAP2K1–Tgd cells in GC, among which, MAP2K1 had a potential diagnostic value for GC patients. In addition, MAP2K1 was enriched in T-cell receptor signaling pathway. Our result indicated that hsa_circ_0001013, hsa-miR-485-3p, MAP2K1, and Tgd cells may be associated with tumor metastasis in the development of GC.

hsa-miR-5584-3p played important roles in tongue squamous cell carcinoma by interacting with hsa_circ_087212 (53). LYN was significantly downregulated in gastric gastrointestinal stromal tumors with high-grade malignancy (54). In this study, we found the relationship between hsa_circ_0006089, hsa-miR-5584-3p, and LYN. Moreover, LYN had a potential diagnostic value and was negatively correlated with neurons. In addition, LYN was involved in chemokine signaling pathway. It was suggested that chemokines and their specific receptors can play an important role in GC progression via promotion of angiogenesis, invasion, survival, and metastasis (55). This indicated that the regulatory network of hsa_circ_0006089–hsa-miR-5584-3p–LYN–neurons played important roles in angiogenesis, invasion, survival, and metastasis of GC.

Besides the above circRNA–miRNA–immune-related mRNA regulatory network, some other circRNA–miRNA–mRNA regulatory networks were also found in GC, such as hsa_circ_0045602–hsa-miR-4538–WNT5A and hsa_circ_0018004–hsa-miR-199a-5p–KL. hsa_circ_0045602 was upregulated in GC tumor tissues (56). The expression of hsa-miR-4538 was decreased in acute myeloid leukemia (57). hsa-miR-199a-5p level was significantly increased in GC tissues.
Moreover, a higher miR-199a-5p expression level of hsa-miR-199a-5p promoted the invasion of GC cells and was related to increased likelihood of lymph node metastasis (58). Thus, it can be seen that these molecules may be involved in tumor cell invasion and metastasis of GC.

In conclusion, our study found several ceRNA (circRNA–miRNA–immune-related mRNA) regulatory networks including hsa_circ_0003763–hsa-miR-4537–NRAS–Tgd cells, hsa_circ_0001013–hsa-miR-485-3p–MAP2K1–Tgd cells, hsa_circ_0001789–hsa-miR-1269b–MET–adipocytes, hsa_circ_0003763–hsa-miR-145-5p–FGF10–StromaScore, hsa_circ_0001789–hsa-miR-1269b–MET–adipocytes, hsa_circ_0003763–hsa-miR-4537–NRAS–Tgd cells, and hsa_circ_0006089–hsa-miR-5584-3p–LYN–neurons in GC, among which FGF10, MET, NRAS, RAC1, MAP2K1, and LYN had a potential diagnostic value for GC patients. In addition, some signaling pathways were identified, such as Rap1 and Ras

FIGURE 11 | Expression validation of hsa_circ_0003763, hsa_circ_0004928, and hsa_circ_0040573 in the GSE93541 dataset (A) and GSE141977 dataset (B). Ns, no significant difference.

FIGURE 12 | Expression validation of FGF10, MET, NRAS, RAC1, MAP2K1, and LYN in the TCGA dataset. **p < 0.01; ***p < 0.001; ****p < 0.0001.
signaling pathways (involved NRAS and FGF10), Fc gamma R-mediated phagocytosis and cAMP signaling pathway (involved RAC1), proteoglycans in cancer (involved MET), T-cell receptor signaling pathway (involved MAP2K1), and chemokine signaling pathway (involved LYN). Our study may provide a novel field for understanding the molecular mechanisms of GC at the immunological levels. However, there are limitations to our study. Firstly, larger numbers of samples in qRT-PCR are needed. Secondly, an in-vitro gastric cell line experiment in cultures and tissues from healthy and gastric cancer patients is further needed to confirm the results of the in-silico analyses. Thirdly, the cell reporter assay for miRNA–circRNA–mRNA interaction is further needed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/ supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

All participating individuals provided informed consent with the approval of the Ethics Committee of Zhejiang Hospital (2021-164K). The patients/participants provided their written informed consent to participate in this study.
AUTHOR CONTRIBUTIONS
GZ was the major contributor in the subject design. GZ contributed administrative support. PL provided the materials and samples and collected and sorted the data. ZW and GZ contributed to the data analysis and interpretation. All authors read and approved the final manuscript.

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