Identification and Characterization of the Roles of CircCASP9 in Gastric Cancer Based on a CircRNA–miRNA–mRNA Regulatory Network

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**Identification and Characterization of the Roles of CircCASP9 in
Gastric Cancer Based on a CircRNA–miRNA–mRNA Regulatory
Network**

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23 **Abstract**

24 **Background:** Circular RNAs (circRNAs) play crucial roles in gastric cancer (GC). This study aimed
25 to construct a new circRNA-miRNA-mRNA competing endogenous RNA (ceRNA) network for GC
26 and validate a specific circRNA in GC tissues and cell lines.

27 **Methods:** We obtained circRNA profiles from an in-house circRNA microarray and the Gene
28 Expression Omnibus (GEO) database, and microRNA (miRNA) profiles from StarBase and
29 CircBank. Messenger RNA profiles were downloaded from GEO. We then constructed a ceRNA
30 network using the extracted circRNA (circCASP9), miRNA (miR-589-5p) and 52 mRNAs. The
31 expression of circCASP9 was confirmed in GC tissues and cell lines, using quantitative reverse
32 transcription polymerase chain reaction. The function of circCASP9 was validated in GC cell lines,
33 using the cell counting kit-8, ethynyl deoxyuridine, transwell, and colon assays. The sponge function
34 of circCASP9 for miR-589-5p was verified using dual luciferase assays.

35 **Results:** A novel ceRNA network was established with one downregulated circRNA, one
36 upregulated miRNA, and 52 downregulated mRNAs. CircularCASP9 was downregulated in GC
37 tissues and cell lines and sponged miR-589-5p. Furthermore, circCASP9 inhibited GC proliferation,
38 migration, and invasion.

39 **Conclusions:** We identified the expression and anticancer effects of the core circRNA circCASP9 in
40 a novel circRNA-miRNA-mRNA ceRNA regulatory network of GC. This present study may provide
41 a novel therapeutic target and insight understanding of the mechanism of gastric cancer.

42 **Keywords:** gastric cancer, circular RNA, circCASP9, regulatory network, tumorigenesis

43 **Background**

44 Gastric cancer (GC) ranks fifth among the most prevalent cancers and accounts for ~33% of cancer-
45 related mortality globally [1]. Most patients with GC have an advanced stage of the disease at the
46 time of diagnosis [2]. Despite progress in GC treatment, the 5-year survival rate remains at 20–25%
47 [3]. Thus, exploring the mechanism of tumorigenesis and tumor development is important for the
48 early diagnosis and therapy of GC. Circular RNAs (circRNAs) are a recently discovered category of
49 endogenous noncoding RNAs, formed by the covalent bonding of 5' and 3' ends and generated by
50 reverse splicing introns or exons [4, 5]. Circular RNAs are primarily located in the cytoplasm and are
51 competing endogenous RNAs (ceRNAs). Circular RNAs can competitively sponge microRNAs
52 (miRNAs) to regulate mRNA expression, thereby affecting the genesis and progression of tumors
53 [6-8]. For instance, circREPS2 inhibits GC progression by combining with miR-558 [9]. The
54 circRNA MCTP2 suppresses cisplatin resistance in GC by absorbing miR-99a-5p [10]. Circular
55 LMO7 promotes GC progression by acting on miRNA-30a-3p [11]. Other circRNAs associated with
56 GC should be identified to further increase the understanding of the potential molecular mechanisms
57 of GC.

58 Here, we generated data from a new microarray and the online databases, to identify a candidate
59 circRNA associated with GC. Based on predicted interactions among circRNAs, miRNAs, and
60 mRNAs, we established a novel ceRNA network. Moreover, we explored the expression and function
61 of circCASP9 and verified the sponge function of circCASP9 to miR-589-5p in the network.

62 **Methods**

63 **Data sources**

64 Our circRNA microarray was manufactured by CapitalBio Technology (Beijing, China). Two
65 additional circRNA expression datasets, GSE89143 and GSE83521, were collected from the Gene
66 Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) [12]. Circular RNAs that

67 could not be transformed to standard names in circBase were excluded [13]. MicroRNA profiles were
68 obtained from the StarBase (<http://starbase.sysu.edu.cn/>), circBank (<http://www.circbank.cn/>), and
69 CircInteractome (<https://circinteractome.nia.nih.gov/>) databases [14-16]. The mRNAs were acquired
70 from the StarBase, MiRDB (<http://www.mirdb.org/>), and TargetScan databases
71 (<http://www.targetscan.org/>) [17, 18], and mRNA expression profiles were downloaded from the
72 GEO dataset GSE33651. The network diagrams for circRNAs were downloaded from the Cancer-
73 Specific circRNA Database (CSCD) (<http://gb.whu.edu.cn/>) [19].

74 **CircRNA microarray analysis**

75 Our circRNA microarray of five pairs of fresh GC and paracancerous tissues was manufactured by
76 CapitalBio Technology (Beijing, China). Total RNA was extracted with TRIzol reagent (Invitrogen,
77 Carlsbad, CA, USA). CircRNAs were enriched after the total RNA was digested with RNase R
78 (Epicentre; Illumina, Inc., San Diego, CA, USA). After amplification, the enriched circRNAs were
79 transcribed into fluorescent cDNA. Then, the cDNA was hybridized using Hybridization Oven
80 (Agilent Technologies, G2545A). Next, the washed microarray was scanned by Agilent Microarray
81 Scanner (Agilent Technologies, G2565A). Finally, the microarray data was collected by Agilent
82 Feature Extraction (v10.7) and were normalized by Agilent GeneSpring (Agilent Technologies, CA,
83 USA).

84 **Clinical specimens**

85 We obtained 40 pairs of GC and paracancerous tissues from the First Affiliated Hospital of
86 Chongqing Medical University, between 2017 and 2020. None of the patients had received
87 preoperative chemoradiotherapy. The tissue samples were immediately stored at -80 °C after
88 surgical resection and validated by two pathologists.

89 **Cell culture**

90 The human GC cell lines AGS, MGC-803, and MNK-45, as well as a human gastric epithelial cell
91 line (GES-1) (Type Culture Collection of the Chinese Academy of Sciences, Beijing, China) were
92 incubated in RPMI 1640 (Biological Industry Ltd., Haemek, Israel), containing 10% fetal bovine
93 serum (FBS) (Biological Industry Inc.), at 37°C under a 5% CO₂ atmosphere.

94 **Construction of a circRNA–miRNA–mRNA network**

95 We identified target circRNAs (TCs) using our circRNA microarray and the GSE89143 and
96 GSE83521 datasets. The upregulated and downregulated circRNAs identified from overlap among
97 the three microarrays were confirmed as TCs, using the “limma” R package ($|\log_2[\text{fold-change}]|$
98 $[\log_2FC] > 2$ and $\text{adj.p} < 0.05$). The miRNAs sponged by circRNAs were predicted using StarBase
99 and circBank. Differentially expressed miRNAs were extracted from StarBase and were regarded as
100 target miRNAs (TMIs) ($FC > 2$, $p < 0.05$). Differentially expressed mRNAs (DEMs) were identified
101 in the GSE33651 dataset using the “limma” R package ($|\log_2FC| > 1$ and $\text{adj.p} < 0.05$). Interactions
102 between MiRNA–mRNA were collected using StarBase, MiRDB, and TargetScan. The intersection
103 of predicted mRNAs from the three databases was obtained to identify target mRNAs (TMs). The
104 circRNA–miRNA–mRNA network was generated using Cytoscape version 3.8.0
105 (<https://cytoscape.org/>) [20].

106 **Functional enrichment analysis of TMs**

107 Overrepresented gene annotation terms in biological process (BP), cellular component (CC), and
108 molecular function (MF) categories were determined with gene ontology (GO) analysis [21].
109 Pathway enrichment was evaluated using the Kyoto Encyclopedia of Genes and Genomes (KEGG),
110 with thresholds for significance of $p < 0.05$ and $q < 0.05$ [22]. The results were visualized using the
111 clusterProfiler package in R [23].

112 **RNA extraction and qRT-PCR**

113 Total RNA isolated using TRIzol (Takara, Japan) was reverse transcribed using the PrimeScript RT
114 Reagent Kit (RR037A; Takara Bio Inc., Kusatsu, Japan) and analyzed with qRT-PCR using TB Green
115 Premix Ex TaqII (RR820A; Takara Bio Inc.). Divergent primers for circCASP9 were manufactured
116 by GeneSeed (Guangzhou, China), and other primers were purchased from Sangon Biotech
117 (Shanghai, China). Relative expression was determined using the $2^{-\Delta\Delta ct}$ method.

118 **Oligonucleotide transfection**

119 CircCASP9-specific small interfering RNA (si-circCASP9) and normal control small interfering
120 RNA (si-NC) were designed by GenePharma (Shanghai, China). RiboFECT™ CP (RiboBio,
121 Guangzhou, China) was used to transfect siRNA, as described by the manufacturer. The silencing
122 efficiency of si-circCASP9 was verified with qRT-PCR. Table 1 shows the primer sequences.

123 **Fluorescence in situ hybridization (FISH) assay**

124 We detected Cy3-labeled circCASP9 probes (GenePharma) in GC cells using FISH kits (RiboBio).
125 Probes specific to circCASP9 were mixed and incubated overnight, then nuclei were stained with
126 4',6-diamidino-2-phenyl indole (DAPI). Images were acquired using an LSM800 laser scanning
127 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Table 1 shows the sequences
128 of Cy3-labeled circCASP9 probes.

129 **Cell proliferation and colony-formation assays**

130 Transfected GC cells (2×10^3 /well) were cultured in 96-well plates, then incubated with 10 μ L of the
131 CCK-8 reagent (MedChemExpress, Monmouth Junction, NJ, USA) for 2 h. Absorbance at 450 nm
132 was detected at 0, 24, 48, and 72 h thereafter.

133 Transfected GC cells (6×10^2 /well) were cultured in 6-well plates for 8 d, fixed in
134 paraformaldehyde (Servicebio, Wuhan, China), and stained with crystal violet (Solarbio Co., Ltd.,

135 Beijing, China). Thereafter, colony formation was assayed using a CanoScan 9000F Mark II scanner
136 (Canon Inc., Tokyo, Japan).

137 **Ethynyl deoxyuridine assays**

138 Transfected GC cells (2×10^3 /well) were cultured in 96-well plates until they reached the logarithmic
139 growth phase. Cell viability was evaluated using Ethynyl Deoxyuridine (EdU) Labeling/Detection
140 Kits (RiboBio), as described by the manufacturer. Images were captured using an LSM800 laser
141 scanning confocal microscope.

142 **Migration and invasion assays**

143 Corning® Transwell® cell culture chambers (Corning Inc., Corning, NY, USA) were embedded in
144 100 μ L of 1 mg/mL Matrigel matrix (BD Biosciences, San Jose, CA, USA). Thereafter, transfected
145 GC cells (3×10^4 /well) in 300 μ L of serum-free medium, were seeded in the upper chambers and
146 incubated for 24 h, with 700 μ L of medium containing 10% FBS in the lower chambers. Thereafter,
147 the cells were fixed in paraformaldehyde, stained with crystal violet, then visualized using an
148 inverted microscope (Leica).

149 **Dual-Luciferase reporter assay**

150 The circCASP9-wild-type (WT) plasmid with the full-length sequence and the circCASP9-mutant
151 (MUT) plasmid (pmirGLO-Dual-luciferase) without miR-589-5p binding sites were obtained from
152 Gene Create (Wuhan, China; Fig. 8e). MicroRNA-589-5p mimics and miR-NC were purchased from
153 Sangon Biotech (Shanghai, China). The plasmids and miR-589-5p mimics, or miR-NC were co-
154 transfected into MGC-803 cells, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and
155 incubated for 48 h. The results were assessed using Dual-Luciferase Assay Kits (Beyotime).

156 **Statistical analysis**

157 Data were analyzed using R version 3.6.1 (<http://www.R-project.org>) and GraphPad Prism version 7
158 (<https://www.graphpad.com/>) [24]. Groups were compared using two-tailed Student t-tests or
159 analyses of variance (ANOVA). A heatmap and volcano plot were generated using the “pheatmap”
160 package in R to visually display differentially expressed circRNAs (DECs) and DEMs. Venn
161 diagrams were constructed using the Venn package in R to visualize overlapping circRNAs and
162 mRNAs. Values with $p < 0.05$ were considered as statistically significant.

163 **Results**

164 **Identification of TCs, TMIs, and TMs**

165 Our circRNA microarray contained 170,417 circRNAs. We acquired 484 DECs from our microarray
166 (filtered by $|\log FC| > 2$ and $\text{adj.} p < 0.05$), comprising 349 downregulated and 135 upregulated
167 circRNAs (Fig. 1a and b). Two overlapping circRNAs were identified from our microarray and the
168 GSE89143 and GSE83521 datasets (Fig. 1c and d). The circRNAs hsa_circ_0010039 (circCASP9)
169 and hsa_circ_0006649 (circDLG5) were downregulated (Table 2 and Fig. 1e-h).

170 Four binding miRNAs (miR-589-5p, miR-574-5p, miR-510-5p, and miR-668-3p) downstream of
171 the DECs were predicted from the overlapping miRNAs in StarBase, circBank, and CircInteractome
172 (Table 3, Fig. 2a–f). Among the overlapping miRNAs identified using expression data from the
173 StarBase database, miR-574-5p, miR-510-5p, and miR-668-3p were not significantly upregulated,
174 owing to a negative correlation between expression levels of the circRNA and corresponding
175 miRNA. We therefore excluded miR-574-5p, miR-510-5p, and miR-668-3p and the upstream
176 circDLG5. The expression of miR-589-5p was significantly upregulated in GC tissues according to
177 StarBase. Thus, miR-589-5p was regarded as a TMI, and circCASP9 as a TC.

178 We obtained 3,069 downregulated and 2,916 upregulated DEMs from the microarray and the
179 GSE33651 ($\log FC > 1$ and $\text{FDR} < 0.05$) dataset (Fig. 3a, b). Finally, 52 mRNAs bound to miR-589-

180 5p were obtained from the intersection of DEMs in StarBase, miRDB, and TargetScan (Fig. 3c, d).
181 The 52 overlapping mRNAs were identified as TMs.

182 **Construction of a ceRNA network**

183 We constructed a circRNA–miRNA–mRNA ceRNA network comprising one downregulated
184 circRNA, one upregulated miRNA, and 52 downregulated mRNAs. The circCASP9 was the ceRNA
185 of miRNA-589-5p, and miRNA-589-5p targeted the 52 mRNAs. The members of the ceRNA
186 network formed 52 regulated axes (Fig. 4).

187 **Functional enrichment analysis of TMs**

188 The 52 TMs were most significantly enriched in GO-BP terms associated with the cellular response
189 to osmotic stress, negative regulation of muscle adaptation, and cellular hyperosmotic response; GO-
190 CC terms were related to integral components of the presynaptic active zone membrane, intrinsic
191 components of the presynaptic active zone membrane, and the presynapse; GO-MF terms were
192 related to calmodulin-dependent protein kinase activity, sodium channel regulator activity, and
193 histone acetyltransferase activity ($p < 0.01$; Fig. 5a-c). The three most significant KEGG pathways
194 involving these genes were the phospholipase D signaling, adrenergic signaling in cardiomyocytes,
195 and Wnt signaling pathways ($p < 0.01$; Fig. 5d).

196 **Validation of CircCASP9 expression**

197 Based on the bioinformatic findings, we extracted circCASP9 (located at chr1: 15844604–15844890)
198 as the TM for further investigation. Sanger sequencing results showed that the circular structure of
199 circCASP9 was formed by reverse stitching (Fig. 6a). Compared with paired adjacent normal tissues,
200 we discovered less circCASP9 expression in 40 GC tissues (Fig. 6b) and in the MGC-803 and AGS
201 GC cell lines than in the human gastric epithelial cell line GES-1 (Fig. 6c). The findings of FISH
202 assays revealed that circCASP9 was located in the cytoplasm and nuclei of AGS and MGC-803 cells

203 (Fig. 6d). Transfection with si-circCASP9 in MGC-803 and AGS cells resulted in significant
204 silencing efficacy (Fig. 6e, f).

205 **CircCASP9 suppressed GC cell proliferation, colony formation, and DNA synthesis**

206 The CCK-8 results revealed more multiplication capacity in the AGS and MGC-803 cells transfected
207 with si-circCASP9 than in the negative control si-NC group (Fig. 7a and b). Similarly, EdU assays
208 indicated that circCASP9 silencing increased DNA synthesis (Fig. 7c–e). Transfection with si-
209 circCASP9 increased colony-forming capacity (Fig. 7f and g). These results showed that circCASP9
210 inhibits GC cell activity and proliferation.

211 **CircCASP9 inhibited the migration and invasion of GC cells**

212 Silencing circCASP9 improved the migration and invasion ability of AGS and MGC-803 cells
213 compared with that of the negative control si-NC. Therefore, circCASP9 inhibited GC cell migration
214 and invasion (Fig. 8a-d).

215 **CircCASP9 acted as an miR-589-5p sponge**

216 Luciferase activity of circCASP9-WT was obviously decreased by miR-589-5p mimics in MGC-803
217 cells compared with the circCASP9-MUT or miR-589-5p NC groups (Fig. 8e and f).

218 **Discussion**

219 Circular RNAs were originally regarded as non-coding RNAs produced due to splicing errors.
220 However, circRNAs are in fact a large class of endogenous, conserved non-coding RNA produced by
221 back-splicing [25, 26]. The covalent cyclic structure of circRNA is more stable than that of linear
222 RNA [27]. Owing to the discovery of a wide range of biological and pathological functions,
223 circRNAs have become a major focus of recent research [28].

Circular RNAs have crucial regulatory effects on various biological activities, particularly cancer occurrence and progression [29-31]. They sponge miRNAs to modulate gene expression, which is the main pathway through which tumor progression is regulated [32]. For example, circCCDC9 can suppress the development of GC via the miR-6792-3p/CAV1 axis [33], circLMTK2 can lead to GC growth and metastasis by regulating the miR-150-5p/c-Myc axis [34], and circLARP4 can bind with miR-424-5p to inhibit GC development [35]. However, the mechanisms through which circRNAs contribute to GC remain largely unknown.

We created a ceRNA network comprising one circRNA, one miRNA, and 52 mRNAs, to identify core circRNAs in GC and their potential molecular targets. Circular CASP9 was generated by back splicing CASP9 (caspase 9) exon 10. The initiator caspase CASP9 is involved in intrinsic apoptosis as well as macroautophagy/autophagy regulation [36]. To our knowledge, this is the first study to identify a close relationship between circCASP9 and GC.

We confirmed that circCASP9 is downregulated in GC tissue samples and cell lines. This was consistent with the results from our circRNA microarray. The results of a series of *in vitro* functional assays to determine the underlying molecular mechanisms of circCASP9, showed that it suppressed GC cell proliferation, colony formation, DNA synthesis, migration, and invasion. Furthermore, we focused on miR-589-5p as the target miRNA of circCASP9. The consequences indicated that circCASP9 might be associated with GC tumorigenesis and development, by sponging miR-589-5p. Circular RNAs typically inhibit mRNA expression by binding directly to target miRNAs in the cytoplasm. The findings of FISH assays showed that circCASP9 was located in the cytoplasm and nuclei.

We found using the ceRNA network that miR-589-5p, which promotes GC metastasis via regulating the PIK3K/AKT pathway [37], binds to circCASP9. This ceRNA network could provide

247 insights into circRNA–miRNA interactions and valuable information for future studies of miRNAs in
248 GC.

249 We identified 52 downstream genes that were predicted to bind to mir-589-5p. Gene ontology
250 analysis revealed that these genes were significantly related to the following GO terms: cellular
251 response to osmotic stress, integral component of presynaptic active zone membrane, and
252 calmodulin-dependent protein kinase activity. The results of KEGG analysis revealed a relationship
253 with 12 signaling pathways, including those associated with tumor-related processes. The
254 phospholipase D signaling pathway affects tumorigenesis [38], the activated Wnt signaling pathway
255 promotes GC cell proliferation, metastasis, and invasion [39, 40], the activated calcium signaling
256 pathway can inhibit non-small lung cancer cell proliferation and promote cell apoptosis [41], and
257 choline metabolism is associated with cancer tumorigenesis and might be a therapeutic target [42].

258 We constructed a novel ceRNA network for GC and identified circCASP9 as a core circRNA.
259 We confirmed circCASP9 expression in GC tissues and cell lines, and that it regulates the growth,
260 migration, and invasion of GC cells. Moreover, circCASP9 acted as a sponge of miR-589-5p in GC
261 cells. However, this study has several limitations. Firstly, the association between circCASP9
262 expression and prognosis could not be clinically validated because of insufficient clinical samples
263 with adequate follow-up. Furthermore, the mechanism(s) underlying the effects of circCASP9 in GC
264 should be investigated *in vivo* and *in vitro*.

265 **Conclusions**

266 Our findings indicate that circCASP9, a core circRNA identified using a circRNA–miRNA–mRNA
267 network, plays significant roles in GC cell growth and invasion. Therefore, circCASP9 might be a
268 useful target for further investigation into the occurrence and development of GC and for the design
269 of individualized therapeutic strategies.

270	Abbreviations
271	BP Biological process
272	CC Cellular component
273	CCK-8 counting kit-8
274	ceRNA Competing endogenous RNA
275	circRNAs circular RNA
276	DAPI 4',6-diamidino-2-phenyl indole
277	DECs Differentially expressed circRNAs
278	DEMs Differentially expressed mRNAs
279	EdU Ethynyl Deoxyuridine
280	FBS Fetal bovine serum
281	FISH Fluorescence in situ hybridization
282	GC Gastric cancer
283	GEO Gene Expression Omnibus
284	GO Gene ontology
285	KEGG Kyoto Encyclopedia of Genes and Genomes
286	MF Molecular function
287	miRNAs microRNAs
288	MUT mutant
289	qRT-PCR quantitative reverse transcription polymerase chain reaction
290	si-circCASP9 small interfering RNA
291	si-NC normal control small interfering RNA
292	TCs Target circRNAs
293	TMI Target miRNAs
294	TMs Target mRNAs
295	WT wild-type

296 **Declarations**

297 **Acknowledgements**

298 Not applicable.

299 **Authors' Contributions**

300 CQ and HZ contributed to research concept, design, experiments, and manuscript writing. XG and
301 HL analyzed the data and edited the figures and tables. AC collected tissues. ZW supervised the
302 study. All authors read and approved the final manuscript.

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306 **Availability of data and materials**

307 The GSE89143 and GSE83521 datasets can be found at Gene Expression Omnibus
308 (<https://www.ncbi.nlm.nih.gov/geo/>). The circRNA sequence was acquired from circBase
309 (<http://www.circbase.org/>), and the network diagrams for circRNAs are in the Cancer-Specific
310 CircRNA database (<http://gb.whu.edu.cn/>). The miRNA profiles are in StarBase
311 [<http://starbase.sysu.edu.cn/>]. The mRNA datasets are in StarBase (<http://starbase.sysu.edu.cn/>),
312 MiRDB (<http://www.mirdb.org/>), and TargetScan (<http://www.targetscan.org/>). All data generated or
313 analyzed during the current study are available from the corresponding author on reasonable request.

314 **Ethics approval and consent to participate**

315 The Ethics Committee of Chongqing Medical University approved the present study. All participants
316 provided written informed consent before the study started.

317 **Consent for publication**

318 Not applicable.

319 **Competing interests**

320 The authors declare that they have no competing interests.

321 **Authors' information**

322 Chuan Qin and Han Zhang have contributed equally to this work and share first authorship.

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Figures

Figure 1

Identification of differentially expressed circRNAs. a Heatmap of 135 downregulated and 349 upregulated circRNAs in our microarray dataset. b Volcano map of 484 DECs in our circRNA microarray dataset. Red, blue, and black regions indicate upregulated, downregulated and not significantly different circRNAs, respectively ($|\log FC| > 2$ and $\text{adj. } p < 0.05$). c, d Venn diagram of downregulated and upregulated circRNAs in GSE83521, GSE89143, and our circRNA microarray datasets. Colors represent different circRNA microarray datasets. e, f Expression of circCASP9 and circDLG5 in circRNA microarray datasets g, h.

Network diagrams of circCASP9 and circDLG5 from CSCD. Red, blue, and green areas show miRNA binding sites, RNA conjunct proteins, and open reading frames (ORF), respectively.

Figure 2

Identification of differentially expressed miRNAs. a, b Venn diagram of predicted miRNAs in StarBase, circBank, and CircInteractome. e–h Expression of miRNA-589-5P, miRNA-510-5P, miRNA-574-5P miRNA-668-3P in StarBase.

Figure 3

Identification of TMs. a, b Heatmap for top 50 DEMs, and volcano map for 3,069 downregulated and 2,916 upregulated mRNAs in GSE33651 dataset ($|\logFC| > 1$ and $\text{adj.p} < 0.05$). c Heatmap for 52 TMs obtained from overlap of predicted mRNAs from three databases and DEMs. D Venn diagram of 52 TMs of miRNA-589-5p.

Figure 6

Characterization and expression of circCASP9. **a** Head-to-tail circular connection of circCASP9 verified by Sanger sequencing. **b** Expression of circCASP9 in 40 paired GC and paracancerous tissues, and **c** AGS, MGC-803, MNK45, and GES-1 cells. **d** Cellular localization of circCASP9 in AGS and MGC-803 cells. **e**, **f** Silencing efficacy of si-circCASP9 in AGS and MGC-803 cells. * $p < 0.05$, ** $p < 0.01$.

Figure 7

Circular CASP9 suppresses cell proliferation, colony formation, and DNA synthesis. a, b CCK-8 findings of the AGS and MGC-803 cells transfected with si-NC and si-circCASP9. c, d EDU assays of the AGS and MGC-803 cells transfected with si-NC or si-circCASP9. f, g Colony formation assays of the AGS and MGC-803 cells transfected with si-NC or si-circCASP9. * $p < 0.05$, ** $p < 0.01$.

Figure 8

Cell migration and invasion were suppressed by circCASP9 and circCASP9 acted as a sponge of miR-589-5p in GC cells. **a** Migration and **b** invasion of the AGS and MGC-803 cells transfected with si-NC or si-circCASP9. **c** Schematic representation of the potential binding sites of miR-589-5p with circCASP9. **d** Luciferase activity of WT or Mut circCASP9 after the transfection with miR-589-5p mimic or miR-NC in MGC-803 cells. * $p < 0.05$, ** $p < 0.01$.