

Exosome-delivered miRNA-339 represses the progression of gastric cancer via targeting ZNF689 before chemoradiotherapy

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ABSTRACT

Background: MicroRNAs (miRNAs) have crucial roles in human cancers. Many studies have certified that miRNAs are implicated in tumor progression via exosomes. Nevertheless, whether miRNA-339 is derived by exosomes and its effects in gastric cancer (GC) presents obscure. Therefore, our study attempted to clarify the functional role and molecular mechanism of miRNA-339 in GC. **Materials and Methods:** In this research, the potential of miRNA-339 in GC was verified through miRNA-339 elevation with cell function assays. Bioinformatics analysis together with mechanical assay was implemented for assessing the regulatory relation between miRNA-339 and zinc finger protein 689 (ZNF689). Moreover, the existence of exosomes was determined via transmission electron microscopy together with nanoparticle tracking analysis. **Results:** miRNA-339 presented significant down-regulation in GC. miRNA-339 elevation suppressed GC cell proliferation, invasion along with migration while elevated GC cell apoptosis. miRNA-339 targeted ZNF689 3'UTR to repress ZNF689 expression, thereby hindering GC progression. Finally, miRNA-339 was majorly incorporated into exosomes to hinder GC progression. **Conclusion:** In summary, exosome-delivered miRNA-339 may act to be a tumor repressor in GC by targeting ZNF689, which might be an underlying therapeutic target for GC.

INTRODUCTION

Gastric cancer (GC) belongs to a type of the most frequent cancers in the world, with a high incidence together with death rate, ranking fourth among cancer-associated deaths ⁽¹⁾. The relevant risk factors for GC contain age, immoderate salt and nitrates intake as well as inadequate fruit and vegetable intake ⁽²⁾. Despite the continuous improvements of clinical diagnosis and treatment have been achieved, most GC patients are still diagnosed with middle to late stage due to lacking early specific symptoms ⁽³⁾. Investigating the molecular mechanism underlying GC development is therefore crucial. MicroRNAs (miRNAs) belong to single-stranded RNA molecules with 18–25 nucleotides long, and can be extensively discovered in eukaryotic organisms ⁽⁴⁾. miRNAs can adhere to 3' untranslated regions (3'UTRs) of mRNAs, thereby degrading target mRNA as well as hindering post-transcriptional translation of mRNA ⁽⁵⁾. Emerging evidence has revealed that miRNAs take part in a diverse biological processes, containing cell proliferation, apoptosis, as well as migration ⁽⁶⁾. MiRNA-339 has been documented to be linked to the development of cancers, and has emerged to be a research hotspot in cancers. Recent research has discovered that lowly expressed miRNA-339-5p is linked to metastases and poor prognosis in GC ⁽⁷⁾. MiR

-339-5p inhibits pancreatic cancer cell invasion and migration in vitro by direct control of ZNF689 ⁽⁸⁾. Overexpression of miRNA-339 dramatically also reduces hepatocellular cancer metastasis ⁽⁹⁾. Hence, miRNA-339 might be a promising target for cancer therapy. Further research is still needed to determine the precise molecular pathways via which miRNA-339 controls important downstream proteins. Exosomes belong to extracellular membrane-derived vesicles, approximately 30-200 nm in diameter, can be liberated into the extracellular environment upon multivesicular bodies (MVBs) fusing with the plasma membrane ⁽¹⁰⁾. Recently, numerous researches have pointed out that exosomes have a key potential in various physiological processes via intercellularly exchanging biological molecules containing proteins, lipids, mRNAs, as well as miRNAs ⁽¹¹⁾. At the same time, due to their unique properties containing immuno compatibility, low toxicity, and nano-scale size, exosomes can also be designed to be nanocapsules for delivering target drugs as well as nucleic acids ⁽¹²⁾. It is worth noting that exosome-delivered miRNA has demonstrated huge potential in cancer treatment ⁽¹³⁾. It is yet unknown, nevertheless, how exosomal miRNA and stomach cancer are related. In this study, we intended to probe the potential together with mechanism of miRNA-339 in GC cells, as well as

investigate whether miRNA-339 can be transmitted via exosomes to affect GC progression. Our study suggests that miRNA-339 may serve as a promising therapeutic target for GC therapy, as well as provide new perspectives for exosome-delivered miRNA therapy in GC.

MATERIAL AND METHODS

Clinical data

From January 2021 to December 2021, 106 GC patients who accepted therapy in our hospital were collected. During the operation, tissues infected with GC together with the adjacent normal tissues were gathered from patients and kept at -80 °C for testing.

Inclusion criteria: (1) Patients were diagnosed with GC through pathological examination and met the 8th edition of TNM staging criteria. (2) The patient had not received cancer therapy before the study. (3) Patients were informed of the purpose of this study as well as signed consent forms. Exclusion criteria: (1) Patients with other tumor types. (2) Patients who could not cooperate with follow-up. (3) Patients were expected to survive for lower than 1 month.

Cell culture

Three GC cells AGS, HGC-27, and MKN74 cells and human gastric epithelial cell GES-1 were provided from Procell (Wuhan, China), and kept in Ham's F-12 medium and RPMI-1640 medium (Gibco, USA), respectively, at 37 °C under 5% CO₂. Both mediums included 10% fetal bovine serum (FBS, Gibco, USA).

Cell transfection

miRNA-339 mimics/inhibitor as well as negative controls was provided by GenePharma (Shanghai, China). ZNF689 overexpressed plasmid (pcDNA-ZNF689) together with its control (pcDNA) were obtained by GeneChem (Shanghai, China). Cell transfection was implemented transfection by help of the Lipofectamine 3000 (Invitrogen, USA). The transfection solution was allowed to stand for 20 minutes before being added to each well. Cells were taken 72 hours after transfection for further research.

RT-qPCR

A TRIzol kit (Invitrogen, USA) could be implemented for extracting total RNA. A TaqMan Reverse transcription kit (Invitrogen, USA) could be taken to perform reverse transcription. A PrimeScript RT Master Mix kit (Takara, Japan) was implemented for amplification of the PCR. 2^{-ΔΔCt} was implemented for calculating gene expression. The primer sequence of genes was indicated in table 1.

Colony formation

A 6-well plate was taken to plant with 600 cells

per well and cultivated for 10 days. Then, cell colonies were fixed by 10% formaldehyde and were received staining by help of 0.1% crystal violet. The colonies were measured with an Olympus inverted microscope (Japan) and identified as having more than 50 cells per colony.

Table 1. Primer sequence of genes.

Gene name	Forward	Reverse
miRNA-339	5'-ACACTCCAGCTGCGG TCCCTGTCCTCCAGGAG-3'	5'-TGGTGTCGTG GAGTCG-3'
U6	5'-GCTTCGGCAGC ACATATACTAAAAT-3'	5'-CGCTTCACG AATTTGCGTGTGTCAT-3'
ZNF689	5'-TGGAACGAA ACACCGATGACT-3'	5'-CCATTCTTCTT TCTGGTTCTGCT-3'
GAPDH	5'-ACTCCTCCAC CTTGACGC-3'	5'-GCTGTAGCCAA ATTCGTTGTC-3'

5-ethynyl-20-deoxyuridine (EdU) detection of cell proliferation

A Beyotime, China EdU assay kit was used to carry out the EdU incorporation assay. In summary, GC cells were seeded onto 24-well plates, then after two hours of incubation with 10 μM EdU reagent, they were fixed for thirty minutes with 4% formaldehyde. GC cells were photographed using a confocal microscope (Olympus, Japan).

Flow cytometry

Cells were resuspended, and received treatment with 5 μL Annexin V-FITC and 10 μL PI (Beyotime, China), and incubated in darkness. Then, a flow cytometer (BD Biosciences, USA) could be implemented for testing GC cells apoptosis and FlowJo software (TreeStar, USA) was used to analyze.

Transwell cell migration and invasion assay

GC cells migration and invasion were measured by help of Transwell chamber (BD Biosciences). For cell invasion, GC cells suspended in serum-free Medium were put into the above chamber pre-coated with Matrigel. In the meanwhile, medium containing 10% serum was placed in the bottom chamber. Followed by 24 h culture, the invaded cells received immobilization with methanol, stained by 0.1% crystal violet, followed by observation through a microscope. For cell migration, the experiment procedures were the same as cell invasion, except that the upper chamber without Matrigel pre-coating.

Dual-luciferase reporter assay

The binding sequences of ZNF89 3'UTR including miRNA-339 binding sites as well as their mutated types were cloned into pmirGLO vector (Promega, USA) for constructing ZNF89 3'UTR-WT and ZNF89 3'UTR-MUT. Afterwards, these vectors were cotransfected into GC cells together with NC mimics or miRNA-339 mimics. Fluorescence was observed by help of the Dual-Luciferase Reporter Assay System (Promega, USA).

Western blot

RIPA buffer (Beyotime, China) could be implemented for lysing GC cells to extract total protein. Then, proteins were isolated by help of 10% SDS-PAGE followed by shifting onto PVDF membranes. Next, primary antibodies containing anti-ZNF89, anti-TSG101, anti-HSP70, and anti-GAPDH (Abcam, UK) were implemented for incubating membranes overnight at 4 °C after blocking by help of 5% skim milk. At last, membranes were treated with goat anti-mouse IgG (HRP) (Abcam, UK) for 1 h. BeyoECL Moon (Beyotime, China) and a chemiluminescence system (Bio-Rad, USA) were used to detect signals, which were then analyzed using Image Lab Software.

Exosomes isolation and identification

Exosomes were gathered from culture media, followed by isolation via ultracentrifugation. To eliminate cellular debris, the culture media were briefly centrifuged at 800 × g for 15 min and then centrifuged again at 10000 × g for 30 min. Finally, the exosomes underwent separation, pelleting, and two hours of centrifugation at 100,000 × g. In PBS, the exosome was again suspended. The size and total number of exosomes were measured using the nanoparticle tracking analysis (NTA).

Transmission electron microscopy (TEM)

Exosomes could be resuspended, followed by fixation by help of 2% paraformaldehyde. Then, glow-discharged copper grid could be implemented for absorbing exosomes. Next, glow-discharged copper grids could be fixed by help of 3% glutaraldehyde, followed by staining using uranyl acetate. A TEM (JEOL, Japan) was utilized for observation of exosomes.

Exosomes labeling

PKH26 membrane dye (Sigma, USA) was implemented for staining the exosomes. After fixation, exosomes were dyed with DAPI, followed by photographing by help of a confocal laser scanning microscope (Zeiss, Germany).

Statistical analysis

Statistical analysis was implemented by help of GraphPad Prism 8 and SPSS 26.0 software. All assays were implemented in triplicate. Data were exhibited as the mean ± standard deviation (SD), and Student's t-test was implemented for comparing the difference. $P < 0.05$ was considered significant.

RESULTS

Expression and potential of miRNA-339 in GC

We detected miRNA-339 expression in tumor tissues together with adjacent normal tissues of GC patients. It was found that miRNA-339 expression

lower in tumor tissues relative to paracancerous tissues (figure 1A). Analysis of the relation between the clinical features of patients and miRNA-339 expression, it was demonstrated that patients with low miRNA-339 expression possessed tumors ≥ 5 cm, low differentiation, increased lymph node metastasis as well as high TNM staging (table 2). We also examined miRNA-339 expression in GC cells, which was down-regulated in AGS, AGS, and HGC-27 cells when comparing to GES-1 (figure 1B). All these findings implied that miRNA-339 might have a crucial role in GC. Since miRNA-339 was most significantly reduced in Two GC cells. Therefore, these two cell lines were chosen for follow-up study. We transfected two GC cells with miRNA-339 mimics or NC mimics, and RT-qPCR results indicated that miRNA-339 expression in two GC cells was changed via successful transfection (figure 1C). Colony formation together with EdU assays uncovered that miRNA-339 up-regulation impaired GC cells proliferation significantly (figure 1D-1E). Flow cytometry analysis revealed that enhancing miRNA-339 expression could promote apoptosis of two GC cells (figure 1F). In addition, transwell assays unveiled that the invasive together with migratory potentials of two GC cells presented repressed after miRNA-339 elevation significantly (figure 1G-1H). Therefore, these findings suggest that miRNA-339 is down-regulated in GC and could suppress migration and invasion of GC cells.

Table 2. Relationship between miRNA-339 and pathological data of patients with gastric cancer.

Parameters	High miRNA-339 expression (n=53)	Low miRNA-339 expression (n=53)	P
Age			0.85
≥ 60 years old	25	26	
< 60 years old	28	27	
Gender			0.84
Male	30	29	
Female	23	24	
Tumor size			0.002
≥ 5 cm	10	25	
< 5 cm	43	28	
Differentiation			0.010
Low differentiation	14	27	
Middle and high differentiation	39	26	
Lymph node metastasis			0.003
Yes	15	30	
No	38	23	
TNM staging			0.01
Stage I+ II	37	24	
Stage III+ IV	16	29	

(A) RT-qPCR detected miRNA-339 expression in tumor tissues along with adjacent normal tissues of GC patients. (B) RT-qPCR detected miRNA-339 expression in CG cells and human gastric epithelial cell. (C) RT-qPCR analysis of miRNA-339 expression in Two GC cells transfected with miRNA-339 mimics plasmid. (D) Colony formation of two GC cells proliferation after miRNA-339 overexpression. (E)

EdU assays of two GC cells proliferation after miRNA-339 overexpression. (F) Flow cytometry analysis of two GC cells apoptosis after miRNA-339

overexpression. (G-H) Transwell assays of two GC cells migration and invasion after miRNA-339 overexpression. ** $P < 0.01$, *** $P < 0.001$.

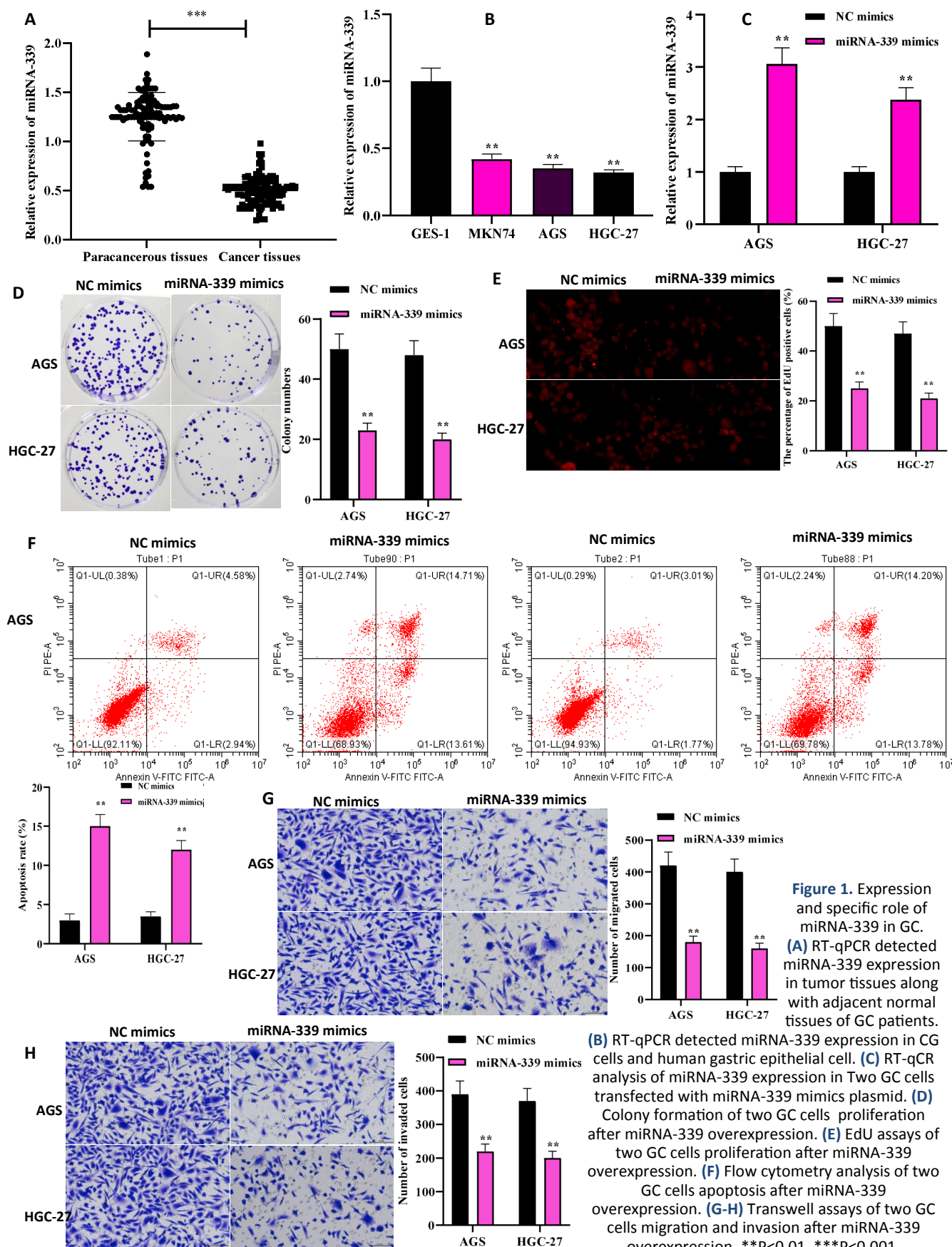


Figure 1. Expression and specific role of miRNA-339 in GC.

(A) RT-qPCR detected miRNA-339 expression in tumor tissues along with adjacent normal tissues of GC patients.

(B) RT-qPCR detected miRNA-339 expression in CG cells and human gastric epithelial cell. (C) RT-qPCR analysis of miRNA-339 expression in two GC cells transfected with miRNA-339 mimics plasmid. (D) Colony formation of two GC cells proliferation after miRNA-339 overexpression. (E) EdU assays of two GC cells proliferation after miRNA-339 overexpression. (F) Flow cytometry analysis of two GC cells apoptosis after miRNA-339 overexpression. (G-H) Transwell assays of two GC cells migration and invasion after miRNA-339 overexpression. ** $P < 0.01$, *** $P < 0.001$.

miRNA-339 targets ZNF689

Previous reference has indicated miRNAs exert functions post-transcriptionally through base-pairing to the mRNA 3'-UTRs to hinder protein synthesis⁽¹⁴⁾. To determine the potential mechanism of miRNA-339, TargetScan (https://www.targetscan.org/vert_80/) was applied for the prediction of mRNAs that bind with miRNA-339. ZNF689 that had a relatively high score was chosen for this study, and the binding sites of ZNF689 3'UTR and miRNA-339 were shown in Figure 2A. The predicted interaction between miRNA-339 and ZNF689 3'UTR was then certified by using a dual-luciferase reporter assay system. The relative luciferase intensity in two GC cells that were transfected with miRNA-339 mimics together with ZNF689 3'UTR-WT was obviously reduced relative to that in two GC cells transfected with miRNA-339 mimics together with ZNF689 3'UTR-MUT (figure 2B). Besides, we discovered that after miRNA-339 overexpression,

ZNF689 mRNA and protein levels in two GC cells were obviously reduced (figure 2C-2D). Subsequently, RT-qPCR was used to assess ZNF689 levels in GC tissues and cells. As displayed in Figure 2E, ZNF689 expression higher in tumor tissues relative to paracancerous tissues. Similarly, three GC cells had the higher level of ZNF689 when comparing with GES-1 cells (figure 2F). Together, these results suggested that miRNA-339 can targets ZNF689 and negatively regulate ZNF689 expression.

(A) TargetScan predicted binding sites of ZNF689 3'UTR and miRNA-339. (B) The dual-luciferase reporter assay examined the binding of ZNF689 3'UTR and miRNA-339. (C-D) RT-qPCR together with western blot analyses of ZNF689 mRNA and protein levels in two GC cells after miRNA-339 elevation. (E) RT-qPCR of ZNF689 expression in CG cells and human gastric epithelial cell. (F) RT-qPCR of ZNF689 expression in CG cells and human gastric epithelial cell. **P<0.01, ***P<0.001.

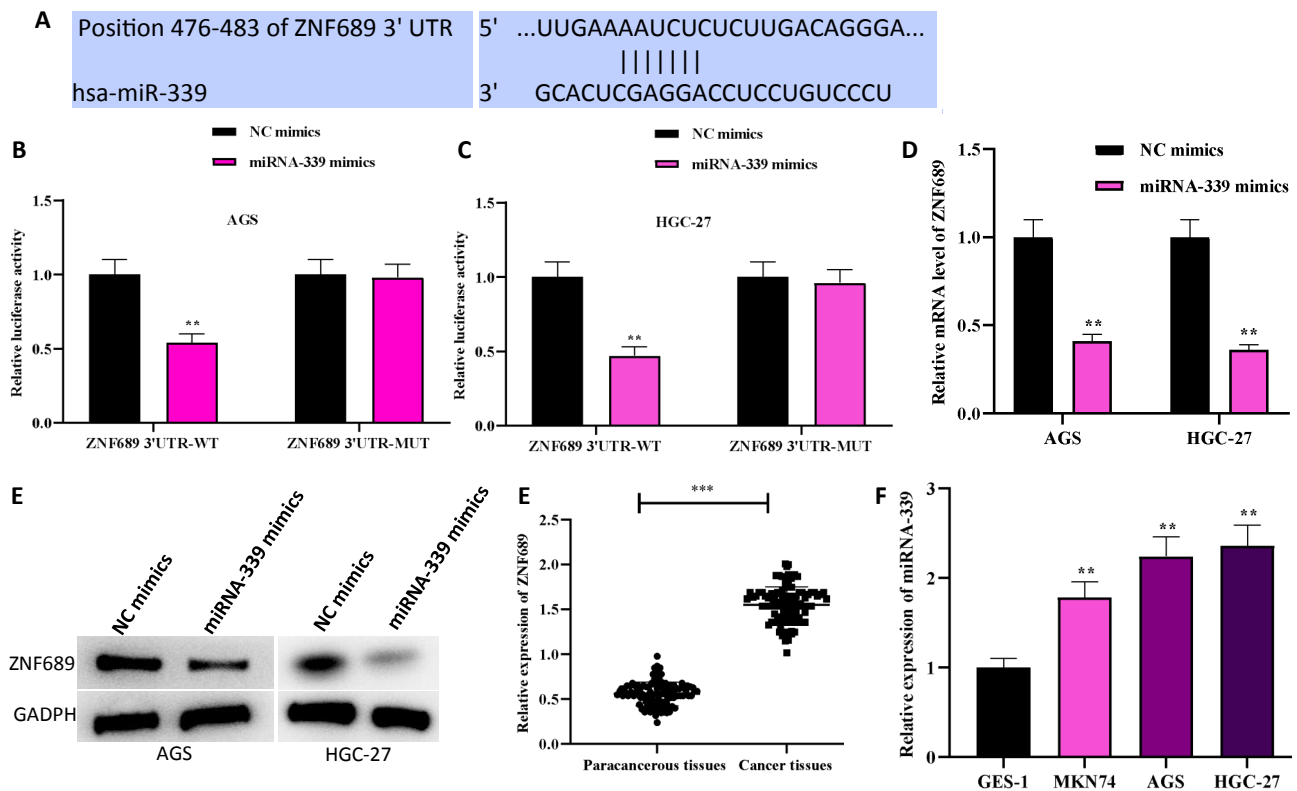


Figure 2. MiRNA-339 targets ZNF689. (A) TargetScan predicted binding sites of ZNF689 3'UTR and miRNA-339. (B) The dual-luciferase reporter assay examined the binding of ZNF689 3'UTR and miRNA-339. (C-D) RT-qPCR together with western blot analyses of ZNF689 mRNA and protein levels in two GC cells after miRNA-339 elevation. (E) RT-qPCR of ZNF689 expression in CG cells and human gastric epithelial cell. (F) RT-qPCR of ZNF689 expression in CG cells and human gastric epithelial cell. **P<0.01, ***P<0.001.

ZNF689 up-regulation rescues the impacts of miRNA-339 elevation

Rescue assays were implemented for exploring the relation between miRNA-339 and ZNF689 together with their impacts. We transfected ZNF689 elevation plasmid into two GC cells and discovered that pcDNA-ZNF689 significantly increased ZNF689 expression (figure 3A-3B). In addition, ZNF689

elevation reversed the lessened proliferation, migration, invasion and elevated apoptosis caused by miRNA-339 overexpression in two GC cells (figure 3C-3G). Taken together, these findings indicated that miRNA-339 controlled ZNF689 expression, hence inhibiting the advancement of GC.

(A-B) RT-qPCR and western blot analyses of ZNF689 expression in two GC cells transfected with

pcDNA-ZNF689 plasmid. (C-D) Colony formation and EdU assays of two GC cells proliferation which were transfected with miRNA-339 mimics together with pcDNA-ZNF689 plasmids. (E) Flow cytometry analysis of Two GC cells apoptosis which were transfected with miRNA-339 mimics together with

pcDNA-ZNF689 plasmids. (F-G) Transwell assays of Two GC cells migration and invasion which were transfected with miRNA-339 mimics together with pcDNA-ZNF689 plasmids. ** $P < 0.01$, compared with NC mimics group. ## $P < 0.01$, compared with miRNA-339 mimics group.

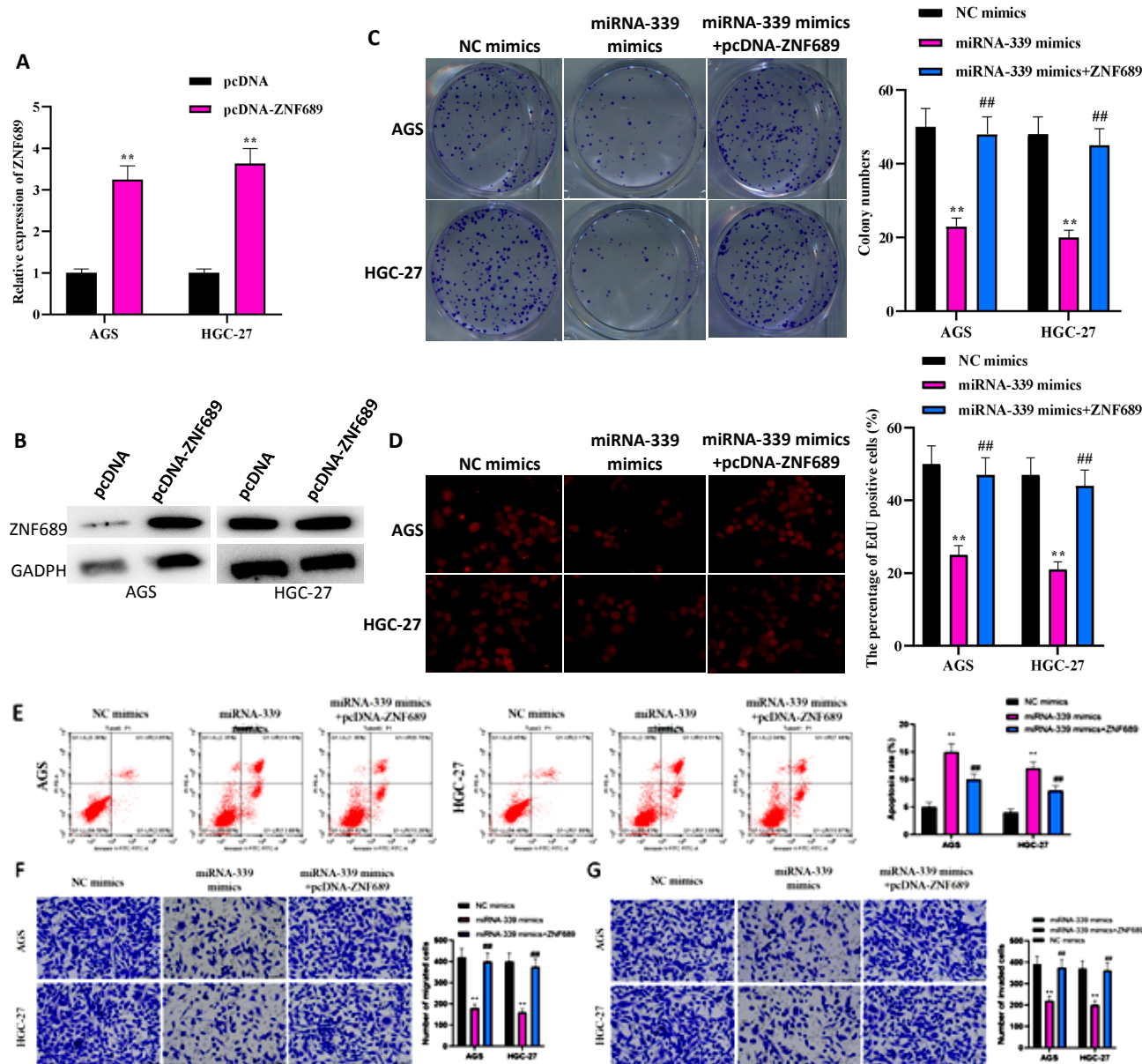


Figure 3. ZNF689 overexpression rescues the impacts of miRNA-339 elevation. (A-B) RT-qPCR and western blot analyses of ZNF689 expression in two GC cells transfected with pcDNA-ZNF689 plasmid. (C-D) Colony formation and EdU assays of two GC cells proliferation which were transfected with miRNA-339 mimics together with pcDNA-ZNF689 plasmids. (E) Flow cytometry analysis of Two GC cells apoptosis which were transfected with miRNA-339 mimics together with pcDNA-ZNF689 plasmids. (F-G) Transwell assays of Two GC cells migration and invasion which were transfected with miRNA-339 mimics together with pcDNA-ZNF689 plasmids. ** $P < 0.01$, compared with NC mimics group. ## $P < 0.01$, compared with miRNA-339 mimics group.

Exosomes are the major carriers of extracellular miRNA-339

Literatures have revealed that miRNAs are enriched in exosomes⁽¹⁵⁾. miRNAs have a crucial role in cancer progression via exosomes⁽¹⁶⁾. To analyze whether extracellular miRNA-339 hinders GC progression by exosomes, the presence form of extracellular miRNA-339 was tested. Exosomes were

confirmed by TEM to be present in the growth media of two GC cells. (figure 4A). The size and quantity distributions of exosomes were shown by NTA (figure 4B). Exosome indicators TSG101 and HSP70 were found to be abundant in exosomes as opposed to cell extracts without exosomes, as shown by Western blot analysis (figure 4C). RT-qPCR results suggested that miRNA-339 expression in exosomes

was equivalent to that in cell culture medium (figure 4D), which reflected that extracellular miRNA-339 was majorly incorporated into exosomes. MiRNA-339 levels in GES-1 exosomes were higher relative two GC exosomes (figure 4E). We further measured whether GC cells could absorb exosomes. Exosomes derived from GES-1 were all found in the cytoplasm of GC cells, as shown in figure 4F. Exosomes labeled with PKH26 aggregated around the nucleus in the form of dots or clots, indicating that GC recipient cells were able to effectively absorb the exosomes secreted by GES-1 cells. We then silenced miRNA-339 expression in GES-1 cells (figure 4G), and RT-qPCR results displayed that miRNA-339 expression in recipient cells presented elevated upon co-incubating with exosomes from GES-1 cells, whereas this impact could be lessened upon treating with exosomes from miRNA-339-silenced GES-1 cells (figure 4H).

(A) The amount of exosomes in the growth media of two GC cells was assessed by TEM. (B) NTA quantified the distributions of exosome sizes and numbers. (C) Western blot examination of exosome markers in both exosome-containing and exosome-free cell extracts. (D) RT-qPCR results of miRNA-339 expression in exosomes as well as cell culture medium. (E) RT-qPCR showed miRNA-339 expression in GC cells-derived exosomes and GES-1 cell-derived exosomes. (F) GES-1 cells-derived exosomes were dyed with PKH26 and cultivated with GC cells. (G) RT-qPCR analysis of miRNA-339 expression in GES-1 cells transfected with miRNA-339 inhibitor plasmid. (H) RT-qPCR results of relative miRNA-339 expression in GC cells when co-incubating with GES-1 cells-derived exosomes or miRNA-339-silenced GES-1 cells. ** $P < 0.01$. ## $P < 0.01$, compared with GES-1 exosomes group.

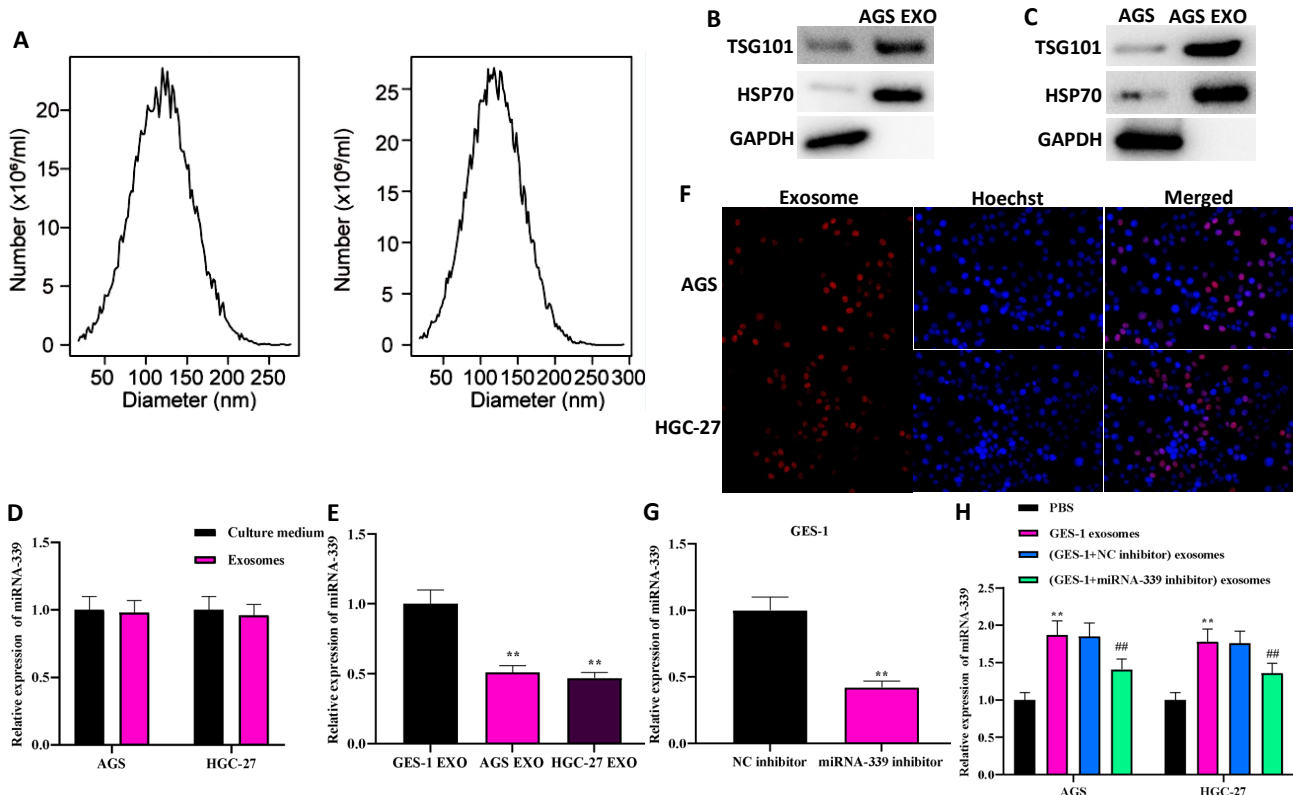


Figure 4. Exosomes are the major carrier of extracellular miRNA-339. (A) The amount of exosomes in the growth media of two GC cells was assessed by TEM. (B) NTA quantified the distributions of exosome sizes and numbers. (C) Western blot examination of exosome markers in both exosome-containing and exosome-free cell extracts. (D) RT-qPCR results of miRNA-339 expression in exosomes as well as cell culture medium. (E) RT-qPCR showed miRNA-339 expression in GC cells-derived exosomes and GES-1 cell-derived exosomes. (F) GES-1 cells-derived exosomes were dyed with PKH26 and cultivated with GC cells. (G) RT-qPCR analysis of miRNA-339 expression in GES-1 cells transfected with miRNA-339 inhibitor plasmid. (H) RT-qPCR results of relative miRNA-339 expression in GC cells when co-incubating with GES-1 cells-derived exosomes or miRNA-339-silenced GES-1 cells. ** $P < 0.01$. ## $P < 0.01$, compared with GES-1 exosomes group.

miRNA-339 delivered by exosomes inhibits GC progression

We further examined whether miRNA-339 transmitted by exosomes could hinder GC progression. It was discovered that after treating with the EXO-miRNA-339, the decreased cell proliferation was found by Colony formation and EdU

(figure 5A-B). Similarly, flow cytometry results showed that migration and invasion were repressed, while cell apoptosis was promoted in two GC cells (figure 5C-E). All of these findings pointed to the extracellular miRNA-339's inhibition of GC development via exosomes.

(A) Colony formation results of GC cells

proliferation when co-incubating with GES-1 cells-derived exosomes. (B) EdU results of GC cells proliferation when co-incubating with GES-1 cells-derived exosomes. (C) Flow cytometry results of GC cells apoptosis when co-incubating with GES-1 cells-derived exosomes. (D-E) Transwell results of GC cells migration and invasion when co-incubating with GES-1 cells-derived exosomes. **P<0.01.

GW4869-treated GES-1 cells have no effect on GC progression

Finally, to explore whether exosomal miRNA-339 has a deterministic role, GW4869 was utilized to inhibit the production of exosomes in GES-1 cells. It was reported that GW4869 appeared to reduce the quantity of exosomes that GES-1 cells released. (figure 6A-6B). Cell function assays also revealed that co-incubating with the culture medium used for culti-

vation of GW4869-treated GES-1 cells possessed no obvious repressive impact on the proliferation (figure 6C-D), migration (figure 6F), as well as invasion of two GC cells (figure 6G), and possessed no significant promoting impact on apoptosis of GW4869-treated GES-1 cells (figure 6E).

GES-1 cells-derived exosomes were treated with DMSO or GW4869. (A) NTA examined the number and size distributions of exosomes. (B) Relative density of exosomes. (C) Colony formation results of GC cells proliferation when co-incubating with exosomes. (D) EdU results of GC cells proliferation when co-incubating with exosomes. (E) Flow cytometry results of GC cells apoptosis when co-incubating with exosomes. (F-G) Transwell results of GC cells migration and invasion when co-incubating with exosomes. **P<0.01.

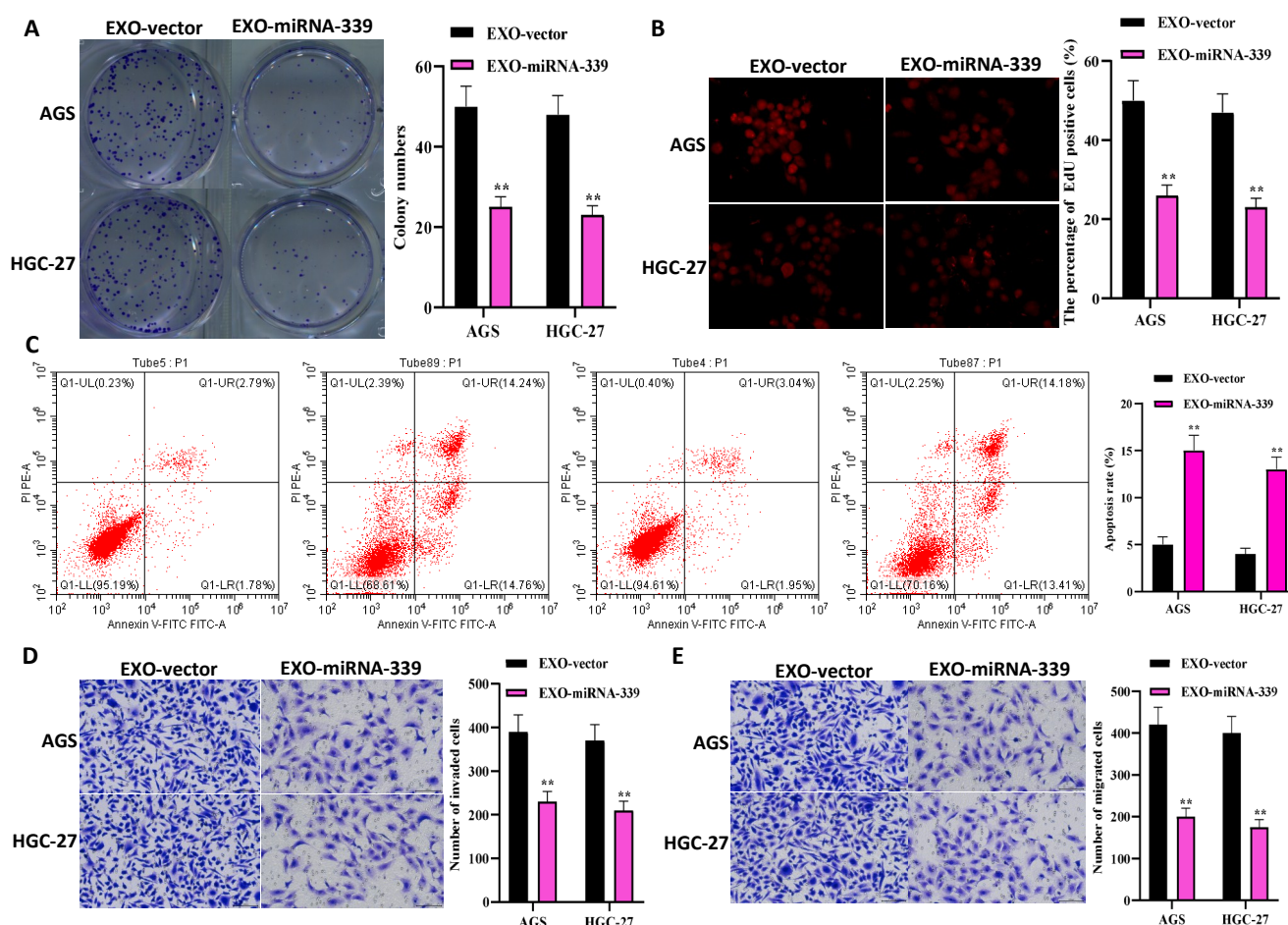


Figure 5. MiRNA-339 delivered by exosomes inhibits GC progression. (A) Colony formation results of GC cells proliferation when co-incubating with GES-1 cells-derived exosomes. (B) EdU results of GC cells proliferation when co-incubating with GES-1 cells-derived exosomes. (C) Flow cytometry results of GC cells apoptosis when co-incubating with GES-1 cells-derived exosomes. (D-E) Transwell results of GC cells migration and invasion when co-incubating with GES-1 cells-derived exosomes. **P<0.01.

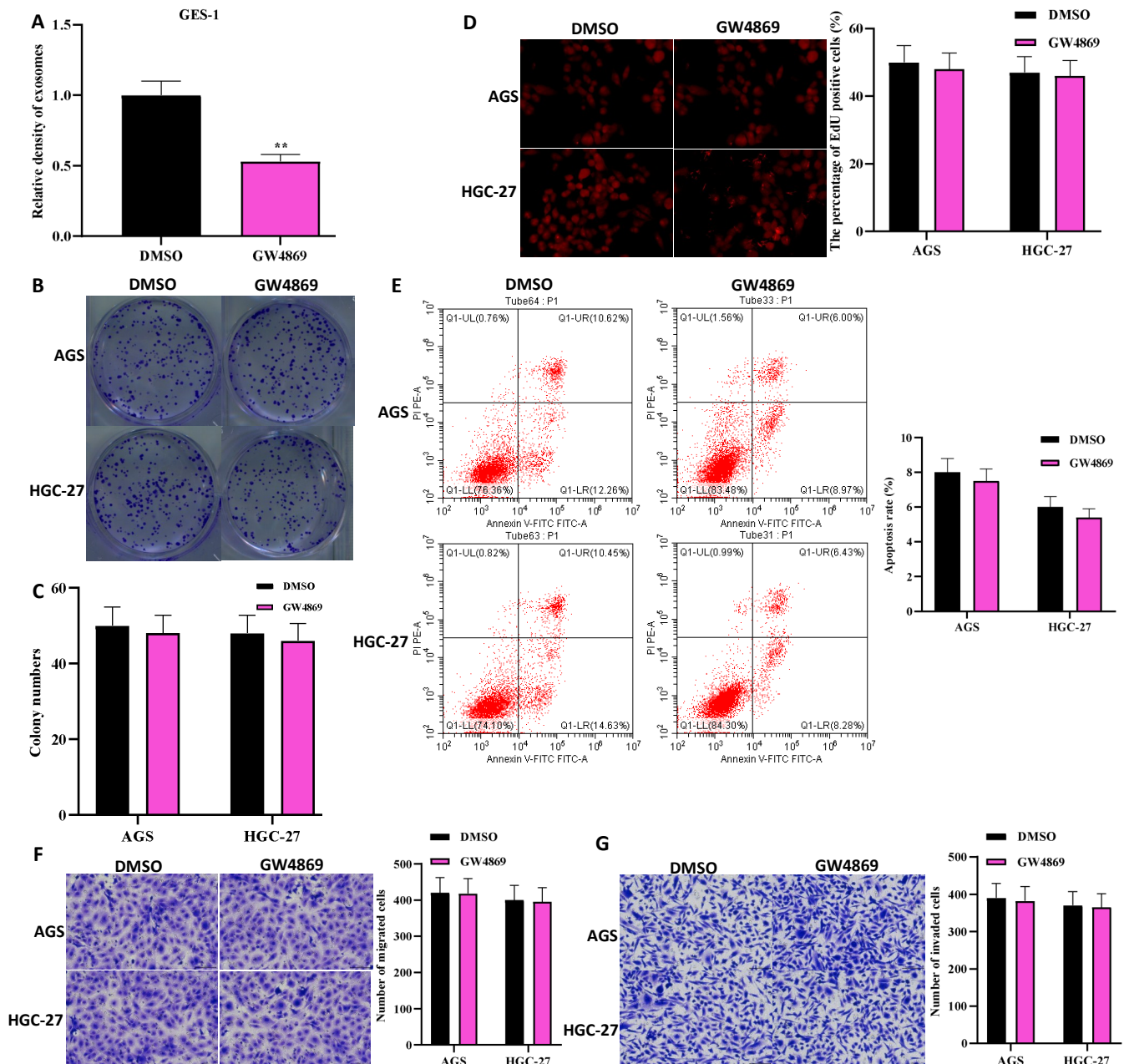


Figure 6. GW4869-treated GES-1 cells have no effect on GC progression. GES-1 cells-derived exosomes were treated with DMSO or GW4869. (A) NTA examined the number and size distributions of exosomes. (B) Relative density of exosomes. (C) Colony formation results of GC cells proliferation when co-incubating with exosomes. (D) EdU results of GC cells proliferation when co-incubating with exosomes. (E) Flow cytometry results of GC cells apoptosis when co-incubating with exosomes. (F-G) Transwell results of GC cells migration and invasion when co-incubating with exosomes. **P<0.01.

DISCUSSION

As RNA sequencing technology advances, an increasing number of miRNAs are found to be differently expressed in malignancies, garnering significant interest from academics (17). Over the years, various studies have unveiled the vital potentials of miRNAs in GC progression. MiR-96-5p accelerated GC cells proliferation by binding to FOXO3 (18). MiR-146b-5p represses the malignant progression of GC via targeting TRAF6 (19). MiR-4317 hinders human GC cell proliferation by regulating ZNF322 (20). Nevertheless, due of miRNAs'

multiformity and tissue specificity, it is unclear exactly what role miRNAs could have in the evolution of GC. In this research, we discovered that miRNA-339 was down-regulated in GC tissues and cells. Besides, we demonstrated that patients harboring low miRNA-339 expression possessed tumors ≥ 5 cm, low differentiation, increased lymph node metastasis, as well as high TNM staging, suggesting that miRNA-339 might be a possible target for GC therapy.

As reported previously, miRNA-339 hinders pancreatic cancer cells invasion and migration (8). Consistently, -function studies showed that

overexpressed miRNA-339 repressed GC cells proliferation, migration, invasion, while promote GC cells apoptosis, further confirming the tumor inhibitory potential of miRNA-339 in cancers. In GC tissues, miRNA-339 expression is low, which can greatly contribute to the development and occurrence of GC. It is still unknown, nevertheless, how miRNA-339 carries out its biological activities at the molecular level.

Numerous literatures have validated that miRNAs take part in biological functions through modulating target genes expression ⁽²¹⁾. Hence, this research adopted bioinformatics prediction as well as luciferase report gene experiment to reveal that miRNA-339 could combine with ZNF689 3'-UTR. Moreover, overexpressed miRNA-339 in GC cells could down-regulate ZNF689 expression. Many literatures have manifested that the abnormal ZNF689 expression is implicated in the development of multiple cancers, containing hepatocellular carcinoma ⁽²²⁾ and GC ⁽²³⁾. Likewise, we discovered an inverse correlation between the level of circRPS5 and the expression level of miR-151a. Our study discovered that ZNF689 was high-expressed in CG, and performed rescue assays to verify that overexpressed ZNF689 could block the suppressive effect of miRNA-339 up-regulation on GC progression. Taken together, these data support that miRNA-339 represses GC progression by regulating ZNF689.

Scholars are highly interested in exosomes because they are a novel way for information to be delivered between cells and because they play a critical role in the progression of cancer ⁽²⁴⁾. Exosome-mediated miRNAs have been found to be crucial in impeding the advancement of GC ⁽²⁵⁾. Therefore, we went on to talk about the relationship between miRNA-339 and exosomes after examining the precise mechanism of miRNA-339 underpinning GC development. As expected, our research uncovered that miRNA-339 could incorporate into exosomes. Upon absorbed by recipient cells, it could pass on the repressive impacts on GC progression. This outcome implied that exosomes packaged with miRNA-339 may be utilized for GC therapy.

CONCLUSION

In summary, miRNA-339 is down-regulated in GC and miRNA-339 elevation represses GC progression through targeting ZNF689 3'UTR. Moreover, we discover that miRNA-339 can be packaged into exosomes and pass on the repressive impact on GC progression, which offer novel sights for GC therapy.

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Conflicts of interests: No potential conflict of interest was reported by the authors.

Ethical consideration: All patients provided their written, voluntarily informed consent. All procedures were carried out in accordance with the guidelines outlined in the Helsinki Declaration and this study was approved by the Ethics Committee of our institution.

Author contribution: Houxiang Jiang conceived and designed the experiments. Linhu Liang, Zhengrong Zhang, Zhengwu Cheng, and Haoran Li contributed significantly to the experiments and arranging data. Zhengrong Zhang and Zhengwu Cheng performed data analyses. Houxiang Jiang and Linhu Liang wrote the draft manuscript. Houxiang Jiang revised the manuscript. All authors read and approved the final manuscript.

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