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Circ-EIF4G3 promotes the development of gastric cancer by sponging miR-335

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ABSTRACT

Background: Circular RNAs (circRNAs), a new group of endogenous non-coding RNAs, plays a crucial role in various types of carcinomas. However, there is still limited information on the involvement of circular RNAs in the setting of gastric cancer (GC). In the present study, we aimed to investigate circ-EIF4G3 status in clinical GC patient samples and explored the malignant biological behaviors.

Materials: The expression of circ-EIF4G3 was determined by quantitative real time polymerase chain reaction (qRT-PCR). Microarray was performed to detect si-circ-EIF4G3 and unprocessed BGC-823 cells to find a cluster of differently expressed microRNAs (miRNAs) and bioinformatic tools including circinteractome, GO, NHGR1_GWAS, KEGG analyses were used in follow-up analysis. Luciferase reporter, RNA pull down and fluorescence in situ hybridization (FISH) assays were employed to explore the interaction between circ-EIF4G3 and miR-335. SiRNA-mediated knockdown of circ-EIF4G3, proliferation, migration and invasion in vitro were used to evaluate the function of circ-EIF4G3.

Results: An increase level in the circ-EIF4G3 expression was associated with higher TNM stage and lymphatic metastasis. In vitro assays of the GC cell lines AGS and BGC-823 demonstrated that knockdown of circ-EIF4G3 inhibited cell proliferation, invasion and migration significantly. In addition, circ-EIF4G3 was identified as a sponge of miR-335, further promoting the proliferation, invasion and migration of GC cells.

Conclusion: Our study demonstrates that circ-EIF4G3 promotes the proliferation, invasion and migration of gastric cancer via sponging miR-335.

1. Introduction

Gastric cancer (GC) is still the third leading cause of death related to cancer all over the world [1]. Great progress has been made in diagnosis and treatment recently, but the overall survival hasn't been significantly improved. The reasons for this are the advanced stages of diagnosis and the lack of sensitive and specific molecular markers for early detection. There is a knowledge gap in understanding the relationship between carcinogenic mechanisms and manifestations.

Circular RNAs (circRNAs), a large type of non-coding RNAs that exists universally in the cytoplasm of eukaryotic cells. CircRNAs have become research hotspots in recent years for its specific role at the post-transcriptional level [2,3]. Different from long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), circRNAs form special closed loop structures lack of the 5′ end cap and the 3′ end of poly (A) tail and have a higher degree of stability and sequence conservation among mammalian cells [4]. Recently, circRNAs have been demonstrated involved in the development of several types of diseases, such as atherosclerosis, nervous system disorders and various carcinomas [5–7]. Growing evidence has shown that circRNAs have huge diagnostic and

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therapeutic potentials in GC [8–10]. Recent evidence indicated circRNAs function as miRNA 'sponges' that naturally sequester and competitively suppress miRNA activity [11,12]. However, the role of circRNAs in the tumorigenesis of GC is not well understood.

Previous study utilized human circRNA microarray analysis to screen circRNA expression profiles in cancer tissues, and discovered a significantly up-regulated circRNA named circ-EIF4G3 [13]. In the present study, we aimed to investigate circ-EIF4G3 status in clinical GC patient samples and explored the malignant biological behaviors including cell proliferation, invasion and migration. In addition, we used microarray to detect si-circ-EIF4G3 and unprocessed BGC-823 cells, finding a cluster of differently expressed miRNAs, which may have a binding site for circ-EIF4G3 and subsequently conducted a series of data prediction and analysis.

2. Materials and methods

2.1. Patients and clinical tissue samples

The 64 GC tissues and corresponding non-tumorous tissue samples were collected from GC patients and all tissue samples came from the Department of General Surgery, Nanjing First Hospital, Nanjing, China. All patients underwent no initial-radiotherapy or -chemotherapy before enrollment. Their tissue samples were stored in RNA-fixer Reagent immediately after removal from the stomachs and stored in the refrigerator at −80 °C until analysis. Paired adjacent non-tumor tissues were confirmed to be negative for tumor cells by pathological analysis and localized 5 cm from the edge of the GC site. Prior to recruitment, each patient signed a written informed consent form and the study protocol was approved by the local medical ethics committee.

2.2. Cell line and transfection

AGS and BGC-823 cells were transfected with 100 nM si-circ-EIF4G3 or si-NC (si- Normal control) using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The target sequences for circ-EIF4G3 siRNA were as follows: siRNA-1: 5′- GACTCTCTCAAAATTCCT AGA-3′; siRNA-2 : 5′- TGGACTCTCTCAAAATTCCTA-3′; siRNA-3: 5′-CTGGACTCTCTCAAAATTCCT-3′.After 48 h, knockdown of circ-EIF4G3 was confirmed via quantitative real-time PCR (qRT-PCR).

2.3. RNA isolation, reverse transcription, and qRT-PCR

The steps were guided by MIQE guidelines [14]. Total RNA was extracted by using TRIzol reagent following the manufacturer's instructions. The specific methods are described in the previous article [15]. Circ-EIF4G3 expression level was determined by qRT-PCR using the following primer pair: 5′−CCTACCCCATCCCCTTATTC -3′ (Forward, or F) and 5′- ACCGTGCTGTAGACTGCTGAG -3′ (reverse, or R) following the standard steps.

2.4. Cell counting kit-8 proliferation assay

Cell proliferation was determined using the CCK8 assay according to the instructions. AGS and BGC-823 cells were transfected with si-NC or 100 nM si-circ-EIF4G3 and seeded in 96-well plates per well and monitored. A microplate reader (MD, USA) at the absorbance of 450 nm was used to evaluate the cell viability every 24 h.

2.5. Cell invasion assays and scratch wound assay

AGS and BGC-823 cells were transfected with 100 nM si-circ-EIF4G3 or si-NC, respectively. The specific steps are described in our previous article [14].

2.6. Nucleus-cytoplasm fractionation

Both nuclear and cytoplasmic RNA from GC cells were isolated using PARIS KIT 50 RXNS (life, AM 1921) according to the protocol. Three replicates were executed. Subsequently, qRT-PCR was applied to detect the abundance of circ-EIF4G3 and EIF4G3 mRNA. 45S and 7SL were used as markers to show the efficient nuclear/cytoplasmic RNA isolation.

2.7. RNA fluorescence in situ hybridization (RNA FISH)

Circ-EIF4G3 FISH probe was synthesized by Ribo Bio Technology Co Ltd (Guangzhou, China) and carried out according to the protocol. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), and then permeabilized on ice for 5 min in PBS containing 0.5% Triton X-100. Subsequently, cells were hybridized overnight with 20 μM Cy3 labeled RNA of circRNAs FISH probe mix in a moist chamber at 37 °C. After that, the cells were stained with 6-diamidino-2-phenylindole (DAPI) for 10 min at RT. Confocal microscopy was used to visualize the presence of circ-EIF4G3.

2.8. MiRNA microarray

Total RNA of si-circ-EIF4G3 and unprocessed BGC823 cells was extracted from plasma with the Trizol reagent (Invitrogen) and purified with mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). The cDNA labeled with a fluorescent dye (Cy3-dCTP) was produced by Eberwine's linear RNA amplification method. The amplified cRNA was purified using the RNA Clean-up Kit (MN). Data summary, standardization and quality control of miRNA array data were analyzed by using GeneSpring software V13.0 (Agilent). For selecting differently expressed genes, we used a threshold of ≥ 2 and $\leq$-2 fold changes and a Penjamini-Hochberg corrected p-value of 0.05. The data was Log2 transformed using the Adjust Data function of CLUSTER 3.0 software and centered on the median value, and then further analyzed using hierarchical clustering with average linkage. Finally, we used Java Treeview (Stanford University School of Medicine, Stanford, California, USA) for tree visualization.

2.9. Biotin-coupled probe RNA pull down assay

To pull down the miRNA by circRNA, AGS and BGC-823 with transfected with miR-335 mimics were lysed and incubated with biotin-coupled probe of circ-EIF4G3 which was pre-bound on magnetic beads. For 2 h, target RNA was pulled by the RNeasy Mini Kit (QIAGEN, Germany). Biotin-coupled probe of miR-335 were processed through the same protocol.

2.10. Dual-Luciferase reporter assay

The binding site of circ-EIF4G3-Wild, circ-EIF4G3-Mut were inserted into pGL3 promoter vector (Realgene, Nanjing, China) in luciferase reporter assay. AGS and BGC-823 cells were collected and measured following the manufacturer's instructions by using the Dual Luciferase Assay (Promega, Madison, WI, USA) and the luciferase activity was detected by a microplate reader.

2.11. Bioinformatic and statistical analysis

The bioinformatic tools including circinteractome, GO, NHGR1 GWAS, and KEGG analyses were used. Comparison of continuous data was analyzed using an independent *t*-test between the two groups, whereas categorical data was analyzed by the chi-square test. A receiver operating characteristic (ROC) curve was performed to evaluate its diagnostic value. All statistical analyses were performed using SPSS for Windows v.17.0 (SPSS, Chicago, IL, USA). For all results, $P < 0.05$ was

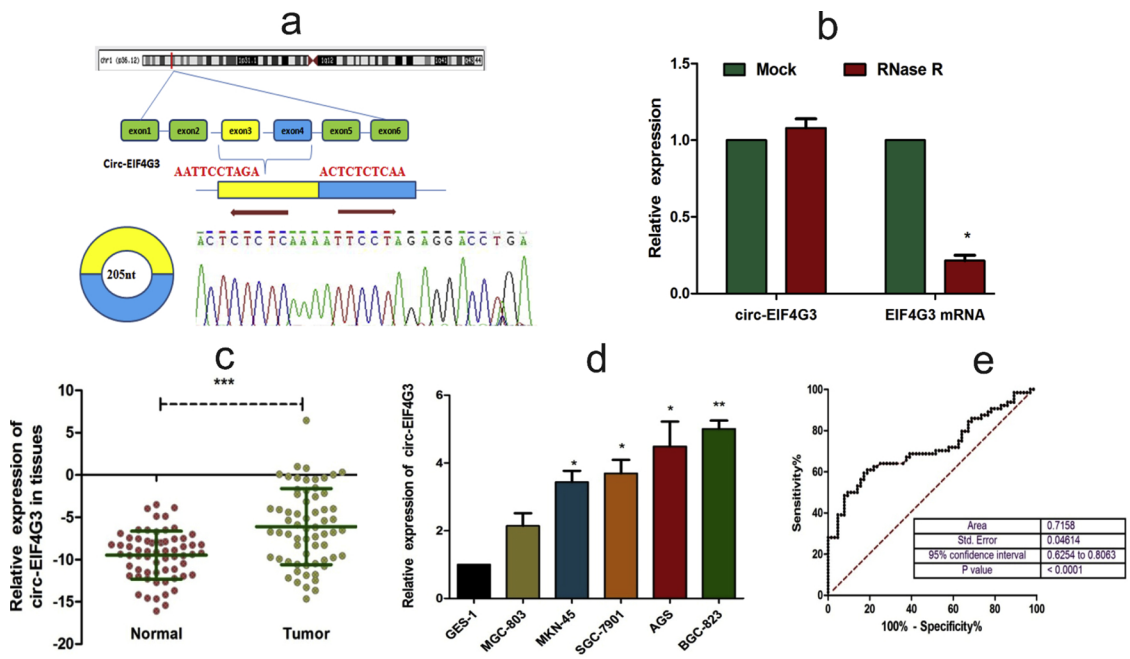

Fig. 1. Expression of circ-EIF4G3 and clinic pathologic information in patients with GC. (a) Schematics showed that circ-EIF4G3 is derived from exons 3–4 of EIF4G3. **(b)** QRT-PCR for the abundance of circ- EIF4G3 and EIF4G3 mRNA in GC cells treated with RNase R. **(c)** The circ-EIF4G3 expression in GC tissues was significantly higher than that of adjacent noncancerous tissues from GC patients. **(d)** The circ-EIF4G3 expressions in AGS, BGC-823, SGC-7901, MKN-45 cells were higher than that in GES-1 significantly. **(e)** The ROC curve was used to evaluate circ-EIF4G3 potential diagnostic value and the area under the ROC curve (AUC) was 0.7158. (* P < 0.05, ** P < 0.01,*** P < 0.001).

considered statistically significant.

3. Results

3.1. Expression of circ-EIF4G3 and clinic pathologic information in patients with GC

A total of 3 hepatocellular carcinoma (HCC) tissues and their matched non-cancerous tissues were collected and screened for dysregulated circRNA using human circRNA microarray and the result showed circ-EIF4G3 was significantly up-regulated in hepatocellular carcinoma. However, its role in the occurrence and development of gastric cancer remains unknown. Therefore, we conducted a series of experiments to explore the biological functions of circ-EIF4G3 in GC. The circ-EIF4G3 was derived from exon3 and exon4 with the splice length of 205 nt (Fig. 1a). Resistance to digestion with RNase R further confirmed that circ-EIF4G3was circular in form (Fig. 1b).

The expression of circ-EIF4G3 level in GC tissues was significantly higher than that of adjacent noncancerous tissues from GC patients (Fig. 1c). The expression of circ-EIF4G3 in 5 GC cell lines was examined by qRT-PCR, revealing that the BGC-823 and AGS cells expressed the highest levels of circ-EIF4G3 expression compared with GES-1 (Fig. 1d). Furthermore, we used the ROC curve to investigate the diagnostic value of circ-EIF4G3 in distinguishing GC tissues from adjacent non-cancerous tissues and the area under the ROC curve (AUC) was 0.7158 (Fig. 1e), with the sensitivity of 60.94% and the specificity of 81.25%. As shown in Table 1, circ-EIF4G3 level was not associated with age, gender, differentiation, or tumor size in patients with GC. However, up-expression of circ-EIF4G3 level was positively associated with TNM stage (P = 0.016) and lymphatic metastasis (P = 0.009).

Table 1
Clinicopathological characteristics and expression of circ-EIF4G3.

Variable	Case	Low expression	High expression	P-value
Age(year)				0.719
≥ 65	35	6	29	
< 65	29	4	25	
Gender				0.240
Female	16	1	15	
Male	48	9	39	
Diameter				0.382
≥3(cm)	40	5	35	
< 3(cm)	24	5	19	
Differentiation				0.878
Low	14	2	12	
middle/ high	50	8	42	
TNM Stage				0.016*
IA-IIB	29	8	21	
IIIA-IV	35	2	33	
Lymphatic metastasis				0.009*
Yes	37	5	32	
No	27	12	15	
CEA				0.386
High	13	1	12	
Normal	51	9	42	
CA242				0.330
High	14	1	13	
Normal	50	9	41	
CA724				0.888
High	18	3	15	
Normal	46	7	39	
CA199				0.520
High	11	1	10	
Normal	53	9	44	

* P < 0.05.

3.2. Altered proliferation, invasion and migration of GC cells by circ-EIF4G3 expression level

AGS and BGC-823 cells were transfected with si-circ-EIF4G3 or si-NC and circ-EIF4G3 expression was effectively knocked down in GC cells (Fig. 2a). The siRNA-3 was selected with the best knockdown efficiency and chosen for subsequent experiments. CCK-8 assay results showed that knockdown of circ-EIF4G3 inhibited cell proliferation significantly (Fig. 2b and c). The effects of circ-EIF4G3 on the invasive

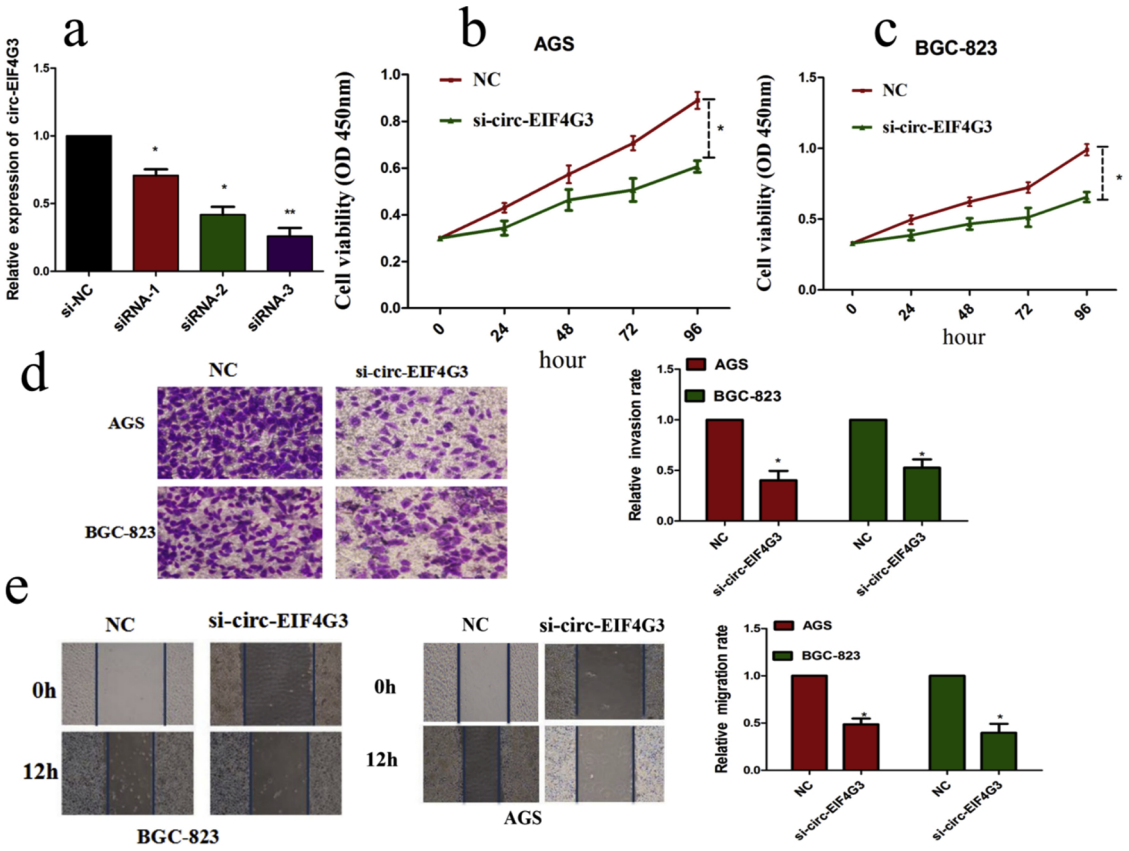

Fig. 2. Altered proliferation, invasion and migration of GC cells by circ-EIF4G3 expression level. (a) GC cells were transfected with si-circ-EIF4G3 or si-NC and circ-EIF4G3 expression was effectively knocked down. **(b)** Knockdown of circ-EIF4G3 inhibited cell proliferation significantly in AGS cells. **(c)** Knockdown of circ-EIF4G3 inhibited cell proliferation significantly in BGC-823 cells. **(d)** Knockdown of circ-EIF4G3 inhibited AGS and BGC-823 cell invasion significantly. **(e)** Inhibition of circ-EIF4G3 produced a lower scratch closure rate than that treated with si-NC in GC cells. (* $P < 0.05$, ** $P < 0.01$).

behavior of GC cell lines were assessed, demonstrating that suppression of circ-EIF4G3 in GC cells with si- circ-EIF4G3 decreased invasion in the Matrigel substrate significantly (Fig. 2d). The results of a scratch-wound assay in the confluent monolayer of the cultured GC cell lines demonstrated that suppression of circ-EIF4G3 exhibited a lower scratch closure rate (Fig. 2e) compared with control cells treated with si-NC, respectively.

3.3. Circ-EIF4G3 could serve as a sponge for miR-335

Nuclear and cytoplasmic separation experiment (Fig. 3a) and in situ RNA hybridization (Fig. 3b) showed that circ-EIF4G3 was mainly abundant in the cytoplasm. We used microarray in si-circ-EIF4G3 and unprocessed cells to identify cluster of differently expressed miRNAs. The top 10 up regulated and 10 down regulated differently expressed miRNAs were shown in Table 2. GO analyses suggested that these

Table 2
Differently expressed miRNAs in si-circ-EIF4G3 group and unprocessed cells.

Top 10 up regulated miRNAs	P_value	Top 10 down regulated miRNAs	P_value
hsa-miR-4750-3p	0.001628956	hsa-miR-3937	2.57141E-06
hsa-miR-381-5p	0.002041915	hsa-miR-3178	9.6459E-06
hsa-miR-1307-5p	0.002041915	hsa-miR-4783-5p	2.22501E-05
hsa-miR-6084	0.004079893	hsa-miR-518e-3p	3.53496E-05
hsa-miR-5188	0.006113939	hsa-miR-548an	4.62678E-05
hsa-miR-335	**0.006321386**	hsa-miR-3939	5.07728E-05
hsa-miR-5007-5p	0.006427851	hsa-miR-215-5p	5.34694E-05
hsa-miR-4634	0.00712949	hsa-miR-1247-5p	5.46723E-05
hsa-miR-5739	0.008144061	hsa-miR-4529-5p	0.000111825
hsa-miR-6134	0.009436694	hsa-miR-192-5p	0.000136111

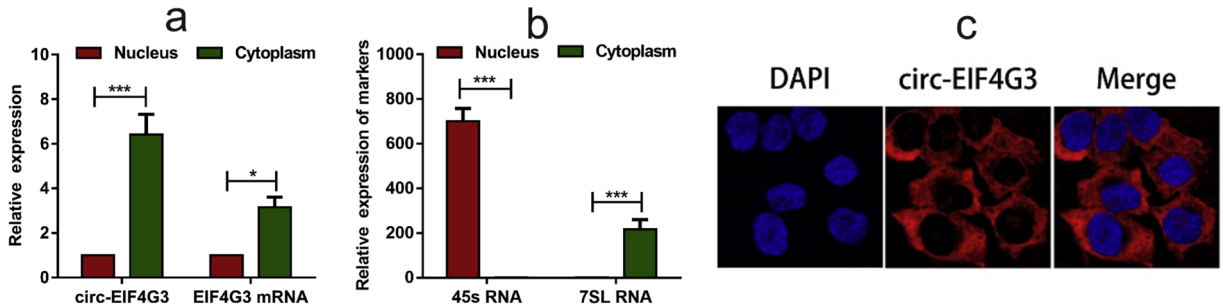

Fig. 3. Circ-EIF4G3 could serve as a sponge for miRNAs. (a) circ-EIF4G3 was predominantly localized in the cytoplasm. **(b)** FISH experiment demonstrated that circ-EIF4G3 was mainly localized in the cytoplasm. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

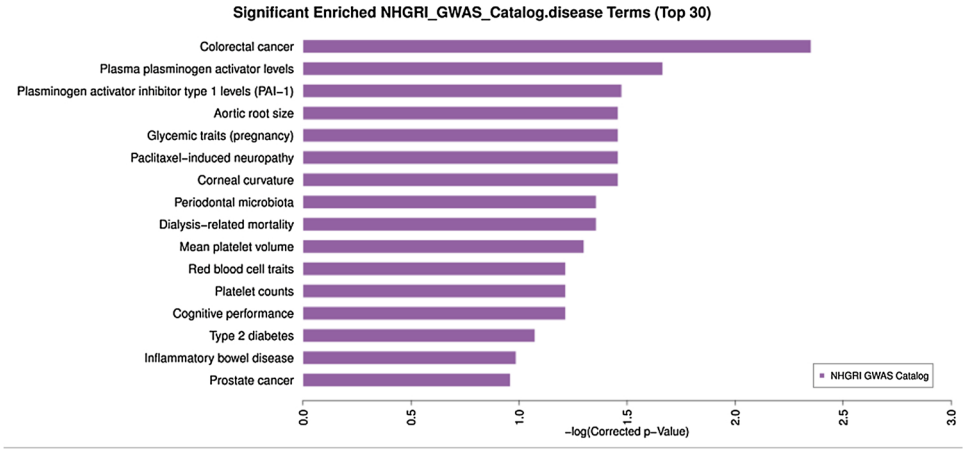

Fig. 4. GO analysis of circ-EIF4G3 interaction with miRNAs.

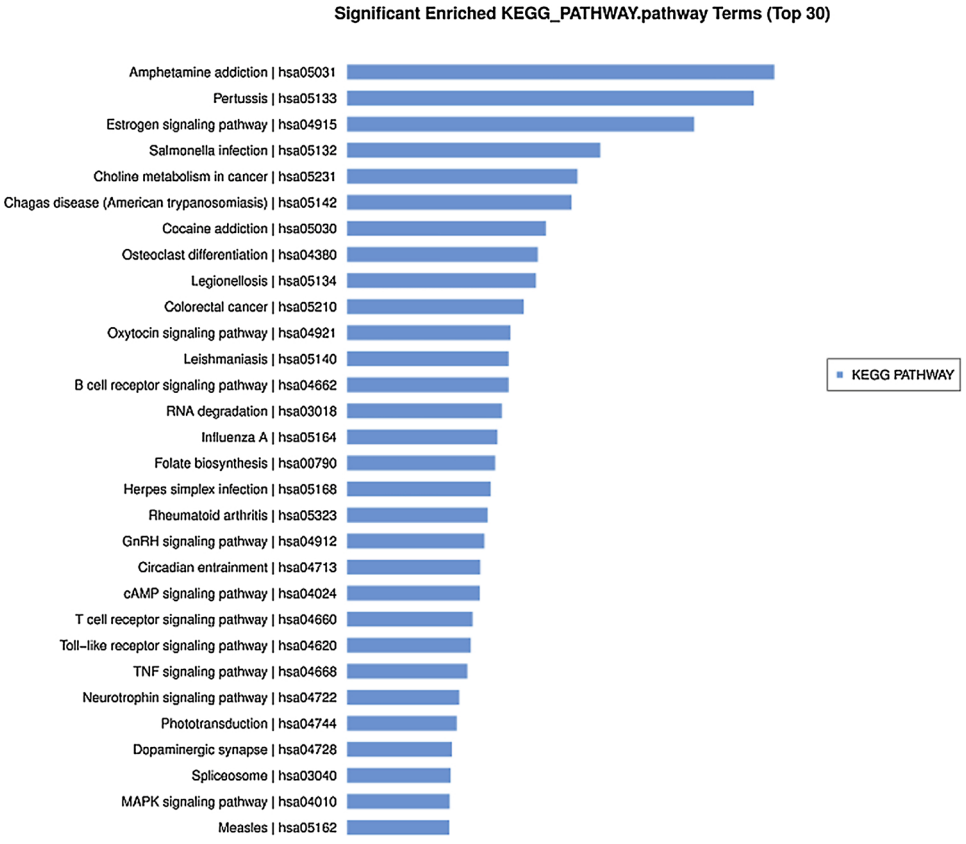

Fig. 5. Top 30 classes of possible diseases of circ-EIF4G3 interaction with miRNAs via NHGR1 GWAS analysis method.

differently expressed miRNAs binding to circ-EIF4G3 were relevant to cellular components, molecular functions, and critical signaling pathways (Fig. 4). The significant enriched NHGRI_GWAS_Catalog disease Terms showed that some terms including colorectal cancer, plasma plasminogen activator levels, plasminogen activator mimics type 1 levels (PAI-1), paclitaxel-induced neuropathy, corneal curvature were the most enriched ones (Fig. 5). Signaling pathways regulating pluripotency of stem cells, adrenergic signaling in cardiomyocytes, cardiac muscle contraction, salivary secretion, pancreatic secretion were the fifth enriched terms of the significant enriched KEGG Pathway terms in miRNA assay (Fig. 6).

Given that circRNAs could bind to different miRNAs and regulate downstream genes, we found that circ-EIF4G3 possessed a complementary sequence to miR-335 seed region by bioinformatics analysis through Circinteractome database (https://circinteractome.nia.nih.gov/). Moreover, miR-335 was down-expressed based on the results of miRNAs microarray. To confirm the website prediction, the biotin-coupled probe pull-down assay was performed and the results showed miR-335 and circ-EIF4G3 was detected in the circ-EIF4G3 pulled-down pellet compared with the control group (Fig. 7a and b). These interactions were confirmed by performing luciferase reporter assays. The results showed that the over expression of miR-335 could significantly reduce the activity of the luciferase expression in circ-EIF4G3-WT (Fig. 7c and d).

To explore whether circ-EIF4G3 participated in GC cell proliferation, invasion and migration through sponging miR-335, the following experiments were performed. The proliferation of cells treated with si-circ-EIF4G3 + miR-335 mimics was all significantly suppressed

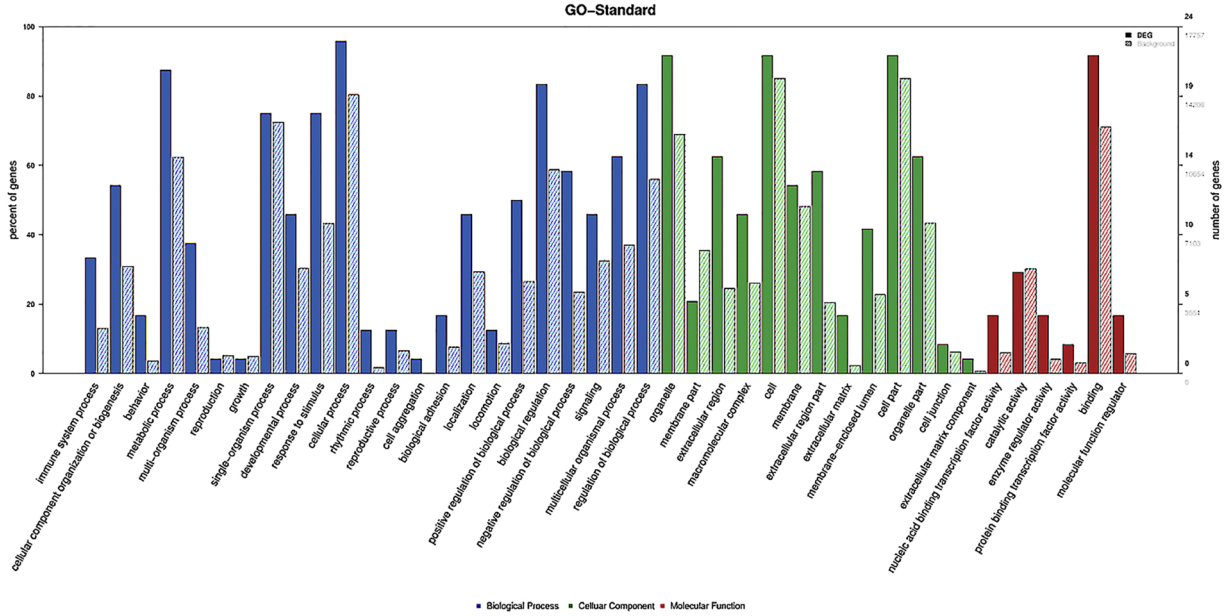

Fig. 6. Top 30 classes of pathway of circ-EIF4G3 interaction with miRNAs via KEGG analysis method.

compared with that treated with si-circ-EIF4G3 in GC cells (Fig. 8a and b). The invasion and migration ability of cells treated with si-circ-EIF4G3 + miR-335 mimics was consistent with the results of proliferation (Fig. 8c and d). Above all, these results illustrate that circ-EIF4G3 takes part in the progression of gastric cancer by sponging miR-335.

4. Discussion

CircRNAs were identified as the products of transcription errors earlier and until 2012. As the development of high-throughput sequencing technologies and bioinformatics, Salzman did a systematic and comprehensive study on circRNAs about that their general characteristics of gene expression programs in human cells, making them very popular in non-coding RNA fields [16]. A lot of researches have demonstrated the potential clinical applications of several circRNAs in GC diagnosis and prognosis, while the number of unknown functions of circular RNA is very large and the existing research is only a last resort [17,18]. Thus, more research on functional consequences of circRNA, especially the circRNA biogenesis in GC is needed to be further explored.

In the present study, we confirmed that circ-EIF4G3 was a closed loop structures without the 5′ end cap and the 3′ end of poly (A) tail and had a higher degree of stability. This study indicated that circ-EIF4G3 exhibited an increased level in GC tissues compared with that in adjacent noncancerous tissues and was positively associated with TNM stage and lymphatic metastasis. In vitro cell experiment showed suppression of circ-EIF4G3 inhibited the proliferation, invasive and migration significantly. These results revealed circ-EIF4G3 might be involved in the procession of gastric cancer.

Growing evidence indicates circRNAs play critical roles in regulating gene expression by sequestering the miRNAs. For example,

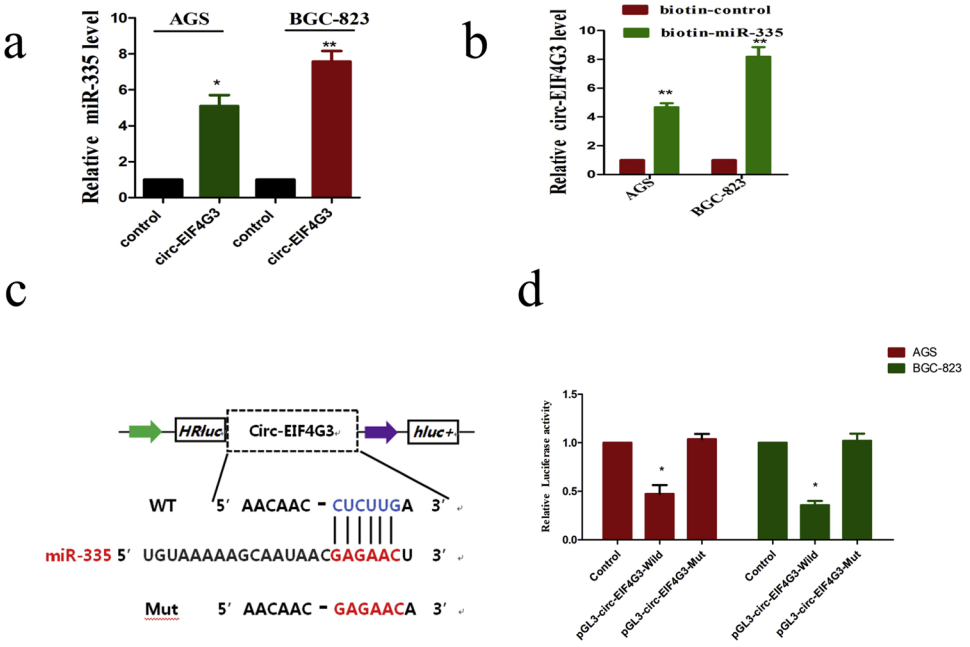

Fig. 7. Circ-EIF4G3 could serve as a sponge for miR-335. (a) The biotin-coupled probe pull-down assay was performed and the results showed miR-335 was detected in the circ-EIF4G3 pulled-down pellet compared with the control group. **(b)** Circ-EIF4G3 was detected in the biotin-miR-335 vector compared with the control group. **(c)** The sequence of pGL3- circ-EIF4G3-Mut/WT and miR-335 was shown. **(d)** MiR-335 mimics induced a decrease in relative luciferase expression in pGL3- circ-EIF4G3-Wild compared with the control. (* $P < 0.05$, ** $P < 0.01$).

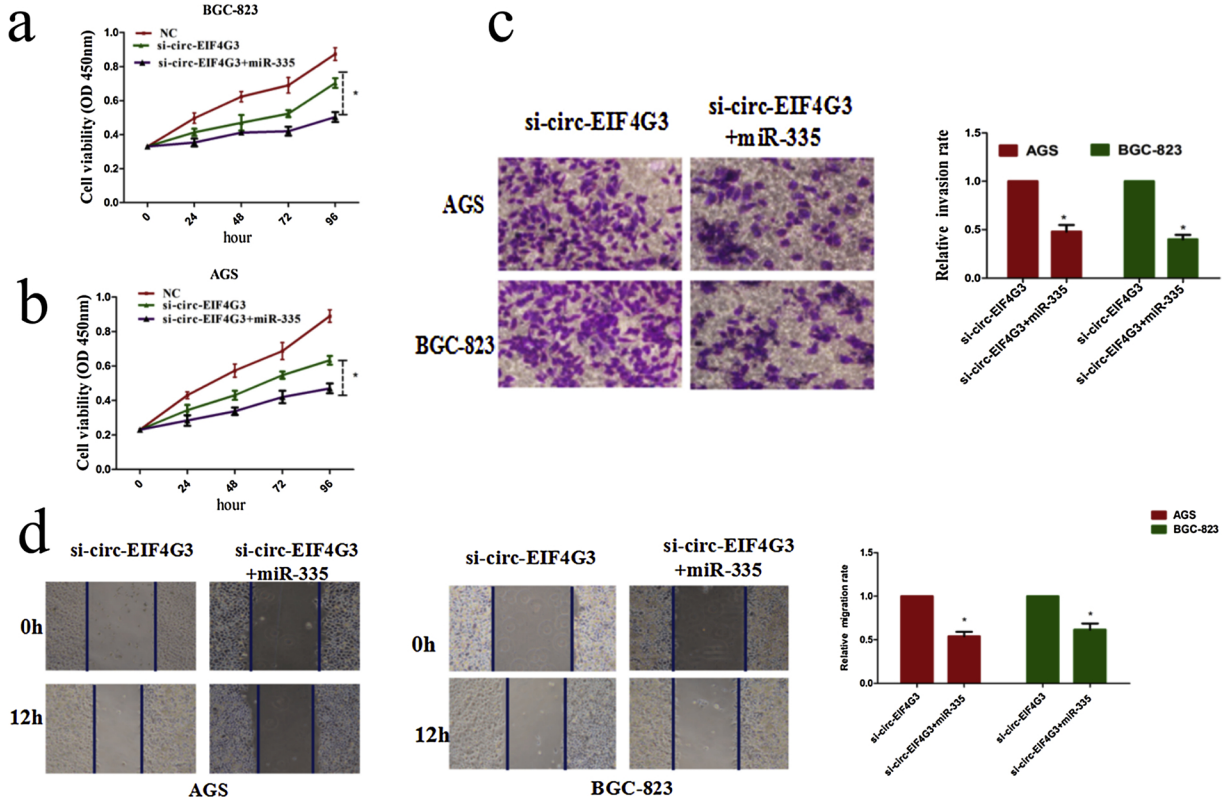

Fig. 8. Circ-EIF4G3 promotes the proliferation, invasion and migration of gastric cancer by sponging miR-335. (a) The proliferation ability of cells treated with si-circ-EIF4G3 + miR-335 mimics were significantly suppressed compared with treated with si-circ-EIF4G3 in AGS cells. **(b)** The proliferation ability of cells treated with si-circ-EIF4G3 + miR-335 mimics were significantly suppressed compared with treated with si-circ-EIF4G3 in BGC-823 cells. **(c)** The invasion ability of cells treated with si-circ-EIF4G3 + miR-335 mimics were significantly suppressed compared with treated with si-circ-EIF4G3 in GC cells. **(d)** The migration ability of cells treated with si-circ-EIF4G3 + miR-335 mimics were significantly suppressed compared with treated with si-circ-EIF4G3 in GC cells. (* $P < 0.05$).

Zhang J et al. reported a new circular RNA_LARP4 (circLARP4) was identified to sponge miR-424 by circRNA expression profile and bioinformatic analysis. The experiment results verified that increased expression of miR-424 or decreased LATS1 expression was relative to pathological stages and poor prognosis of GC patients. Ectopic expression of miR-424 promoted GC cells' proliferation and invasion via targeting LATS1 gene. In addition, circLARP4 was mainly located in the cytoplasm and inhibited biological behaviors of GC cells by sponging miR-424, suggesting that circLARP4 might be a new tumor suppressive factor and a potential biomarker in GC [19]. Given that circ-EIF4G3 was abundant and stable in the cytoplasm, we investigated the ability of circ-EIF4G3 to bind to miRNAs. Results showed that circ-EIF4G3 could serve as a sponge for miR-335. Liang H et al. reported that miR-335-5p mimics could reverse the effects of upregulated DANCR on proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of cervical cancer cells [20]. Therefore, our result is consistent with previous study. As for which target genes are further applied downstream of circ-EIF4G3, we look forward to more in-depth research.

5. Conclusion

Our study demonstrates that circ-EIF4G3 promotes the proliferation, invasion and migration of gastric cancer via sponging miR-335.

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