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Enhanced expression of circular RNA circ-DCAF6 predicts adverse prognosis and promotes cell progression via sponging miR-1231 and miR-1256 in gastric cancer

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ABSTRACT

Circular RNAs (circRNAs) have been reported as essential regulators in various malignancies, including gastric cancer (GC). Previously, circ-DCAF6 was screened as an elevated circRNA in GC patients' tissue samples compared with the normal tissues. To our knowledge, the role of circ-DCAF6 in human cancers is still needed to reveal. The present project is to evaluate the functions and mechanisms of circ-DCAF6 in GC progression. Upregulation of circ-DCAF6 was determined in GC tissue samples and cells by qRT-PCR. Enhanced level of circ-DCAF6 was linked to deeper tumor invasion, positive lymph node metastasis, and higher TNM stages in GC patients. Multivariate analysis further indicated circ-DCAF6 as an independent prognostic indicator. Functionally, CCK-8, clone forming, flow cytometry and transwell assays verified that circ-DCAF6 played as an oncogene in GC cells. Mechanistically, we found the negative correlation between circ-DCAF6 and miR-1231/-1256 in GC specimens. Moreover, luciferase reporter gene assay illustrated that miR-1231 and miR-1256 could be bound to circ-DCAF6. Rescue experiments revealed that circ-DCAF6 facilitated cell growth and invasion via suppressing the levels of miR-1231 and miR-1256. To sum up, circ-DCAF6 acts as an important role in GC tumor progression, and high circ-DCAF6 level may be a useful biomarker for GC.

1. Introduction

Gastric cancer (GC) is a deadly disease with fourth incidence rate (Jemal et al., 2010; Kim and Baik, 2014). The main risk factors of GC are attributed to tobacco smoking, alcohol consumption, dietary habit, and helicobacter pylori infection (Fagoonee and Pellicano, 2019). Currently, GC is treated using a combination of surgery, chemotherapy and radiotherapy. However, GC patients usually have a poor prognosis and a survival rate of < 50% in a period of 5 years (Petrillo et al., 2019). Therefore, it would be highly useful for identification of novel biomarkers involved in GC progression, and improve the early diagnosis and treatment for GC patients.

Circular RNAs (circRNAs) are a novel class of noncoding RNAs with stable circular structures and are generated in the back-splicing of precursor mRNA (Memczak et al., 2013; Cocquerelle et al., 1993; Zhou et al., 2018). Accumulating studies have reported that aberrantly expressed circRNAs were linked to cancer initiation, metastasis, and therapeutic resistance (Xu et al., 2018a; Li et al., 2018; Xing et al., 2018). A great number of publications proved that circRNAs could function as miRNA sponges, bind to proteins, or regulate mRNA splicing. Abnormally expressed circRNA could contribute to aberrant expression of downstream targets which may facilitate tumor progression (Li et al., 2018; He et al., 2018; Xu et al., 2018b). Notably, some cancer-associated circRNAs are identified as oncogenes or antioncogenes in GC including circ-PVT1, hsa_circ_0074362, circ-LARP4, etc. (Kun-Peng et al., 2018; Xie et al., 2018; Zhang et al., 2017a). Additionally, these circRNAs either up or downregulated in cancers are markedly correlates with patients' prognosis.

MiRNAs, a kind of ncRNA of 20–25 nt, play as gene regulators through interacting with the 3'-UTR of their target mRNAs for mediating post-transcriptional gene expression (Bartel, 2004; Huang et al., 2011). In recent years, a lot of studies have showed that miRNAs mediated the progression of human cancers, including GC (Gu et al., 2019; Necula et al., 2019). MiR-1231 could be sponged by LINC00673 and acts as a tumor suppressive role in GC (Zhao et al., 2019). Down-regulation of miR-1256 could contribute to NSCLC cell progression via regulating TCTN1 reported by Liu et al. (Liu et al., 2018a).

Gu et al. screened the differentially expressed circRNAs in GC tumor

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specimens and adjacent nontumor tissues by circRNA microarray. We noticed that circ-DCAF6 was significantly upregulated in the tumorous tissues (Gu et al., 2018). Circ-DCAF6, also known as hsa_circ_0009109, is located at chr1: 167935866–167,944,253 and the spliced of this circRNA is 279 bp. In the current study, we found circ-DCAF6 in GC was associated with aggressive clinicopathologic parameters and adverse prognosis. Upregulated circ-DCAF6 contributes to GC development and progression. Bioinformatics analysis, qRT-PCR as well as dual-luciferase reporter gene test indicated the binding and regulatory relationship of circ-DCAF6 on miR-1231 and miR-1256. To sum up, this work may offer an effective biomarker and treatment target for GC.

2. Materials and methods

2.1. Tissue specimens and cell lines

62 patients with GC underwent operation at our hospital were enrolled in this study. The tissue samples were all collected from these patients and stored at −80 °C. Our study had acquired the authorization of the ethics committee of our hospital. Written informed consent had been provided by all participants.

GC cells AGS, BGC823, MGC803, and SGC7901 and control cells (GES1) were purchased from ATCC. All the cells were cultured in DMEM/RPMI-1640 (Invitrogen) contained 10% FBS for supplement at 37 °C in 5% CO_2.

2.2. qRT-PCR and cell transfection

The RNA was extracted from cells or tissues with TRIzol followed by the directions of manufacturer. Afterwards, the isolated RNA was put into reverse transcription and PCR reactions by PrimeScriptTM RT-PCR kit (Takara). GAPDH was utilized as the internal reference to detect the expression level of circ-DCAF6, and U6 as the internal reference to detect miRNAs. The $2^{(-\Delta\Delta Ct)}$ method was used for statistical analysis.

To achieve the elevation of circ-DCAF6 in GC cell lines, a pcDNA 3.1 circRNA mini vector was applied. For silencing the level of circ-DCAF6, siRNAs specifically targeting to the back-spliced site of circ-DCAF6 were obtained from GenePharma (Shanghai, China). The miR-1231 and miR-1256 mimic/inhibitor and miRNA negative controls were purchased by RiboBio (China). Transfection was performed by Lipofectamine 3000 (Invitrogen, CA), followed by the instruction, and the cells were harvested at 48 h after transfection.

2.3. Cell growth determination

Treated cells were plated in 96-well plates (Corning. Shanghai, China) according to 2000 cells per well. The 96-well plates were maintained at 37 °C, 5% CO_2 and saturated humidity for 0 h, 24 h, 48 h, 72 h and 96 h. Each well was supplied with the reagent and then maintained at 37 °C for 2 h. Optical density (OD) value was measured at 450 nm (Synergy HTX, Biotek Instrument, Inc. Beijing, China). A certain number of GC cells were plated in 2.5 cm plates and cultivated for 12d. Then, the colonies were fixed by formaldehyde, stained, and counted.

2.4. Cell apoptosis assay

Flow cytometric analysis was utilized to detect cell apoptosis followed by our previous study (Jiang et al., 2018).

2.5. Transwell experiment

Transwell experiments were carried out using transwell chambers (Corning, USA) pre-coated with or without Matrigel (BD Pharmingen). The protocol was in accordance with the description of our previous study (Jiang et al., 2018).

2.6. Dual-luciferase reporter assays

Circular RNA Interactome database indicated the potential binding site of miR-1231 and miR-1256 in circ-DCAF6 sequence. Circ-DCAF6 Wt and circ-DCAF6 Mut were constructed based on the predicted binding sequences to miRNA-1231 and miRNA-1256. Cells were cotransfected with circ-DCAF6 Wt or circ-DCAF6 Mut and miRNA-1231-NC or miRNA-1231 mimic for 48 h. Cells were also cotransfected with circ-DCAF6 Wt or circ-DCAF6 Mut and miRNA-1256-NC or miRNA-1256 mimic for 48 h. After cell lysis, relative luciferase activity was determined.

2.7. Statistical analysis

SPSS software (IBM, Armonk, NY) was utilized to analyze the data (mean ± SD). Each experiment was repeated and measured three times. Student's *t*-test or One-way ANOVA was applied. Kaplan-Meier method and Cox regression analysis were applied to analyze the survival data. Fisher's exact test was utilized for evaluating the association between circ-DCAF6 expression and patients' parameters. Expression correlation was analyzed with Spearman's correlation analysis. The $P < .05$ meant the existence of statistical significance.

3. Results

3.1. Expression of circ-DCAF6 in GC and its clinical value for the patients with GC

As displayed in Fig. 1A, positive circ-DCAF6 expression was found in human GC tissues than the matched noncancerous samples. Similarly, overexpression of circ-DCAF6 was also identified in the four

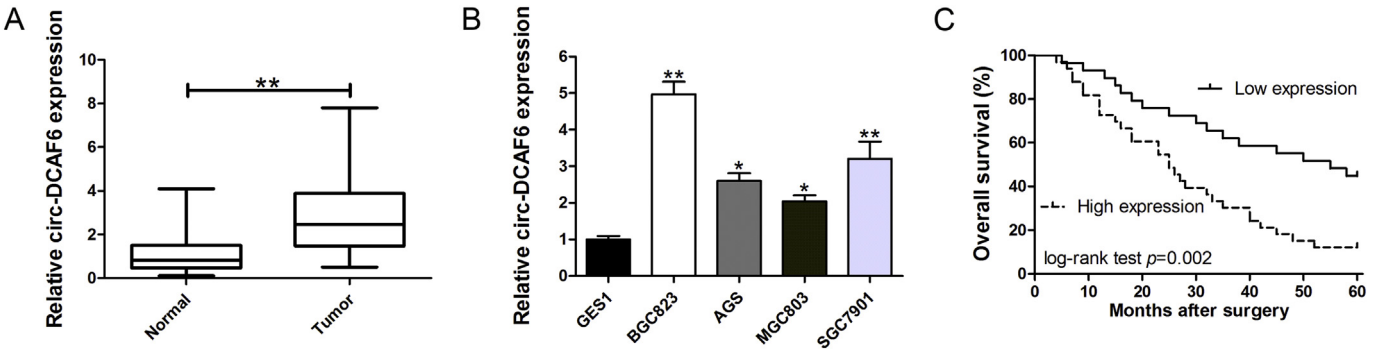

Fig. 1. Relative expression of circ-DCAF6 in GC tissues and cell lines and its clinical significance. (A) Relative expression of circ-DCAF6 in GC tissue samples and their paired non-cancerous tissue samples measured by qRT-PCR. (B) Relative expression of circ-DCAF6 in GC cell lines and normal cell line measured by qRT-PCR. (C) Kaplan-Meier analysis was used to investigate the prognostic role of circ-DCAF6. *$p < .05$, **$p < .01$.

Table 1
Relationship between circ-DCAF6 expression and clinicopathologic features of GC patients.

Clinicopathologic features	Patients	circ-DCAF6 expression		*P*-value
		High	Low	
Gender				0.213
Male	33	15	18	
Female	29	18	11	
Age				0.617
< 60	28	16	12	
≥60	34	17	17	
Tumor size				0.302
< 5 cm	25	11	14	
≥5 cm	37	22	15	
Depth of invasion				0.019
T1 + T2	24	8	16	
T3 + T4	38	25	13	
Lymph node invasion				0.026
Present	49	30	19	
Absent	13	3	10	
TNM stage				0.036
I-II	23	8	15	
III-IV	39	25	14	
Differentiation grade				0.322
Well/moderately	32	15	17	
Poorly/undifferentiated	30	18	12	

recruited GC cells (AGS, BGC823, MGC803, and SGC7901) compared with GES1 (Fig. 1B). To further detect the roles of circ-DCAF6 in clinical practice, we next examined the expression levels of circ-DCAF6 in 62 clinical GC tumor samples and analyzed the correlations of circ-DCAF6 expression to clinical parameters. The high or low circ-DCAF6 expression group was defined by the mean value of circ-DCAF6 expression level. We found that high circ-DCAF6 level was evidently correlated with depth of invasion ($P = .019$), lymph node invasion ($P = .026$) and TNM stages ($P = .036$). Nonetheless, we did not find any associations between circ-DCAF6 expression and tumor size, differentiation grade or other parameters (Table 1). The results indicated that circ-DCAF6 may also related to poor prognosis. Thus, we further analyzed whether circ-DCAF6 expression was linked to patients' prognosis. Kaplan-Meier curves revealed that elevation of circ-DCAF6 in cancerous samples indicated a worse survival rate ($P = .002$, Fig. 1C). The univariate analysis results revealed that high circ-DCAF6 expression ($P = .003$), advanced TNM stages ($P = .006$), and deeper invasion ($P = .004$) were risk factors that affected GC patients' survival, and multivariate analyses showed that high circ-DCAF6 expression is an independent risk factors affecting GC patient overall survival ($P = .033$, Table 2).

3.2. Circ-DCAF6 acts as an oncogene in GC cells

Due to BGC823 and MGC803 exhibited relatively high and low expression of circ-DCAF6, respectively, we chose BGC823 for circ-DCAF6 silencing and MGC803 for circ-DCAF6 overexpression. The transfection efficiencies were confirmed by qRT-PCR (Fig. 2A and B). Functionally, knockdown of circ-DCAF6 resulted to the attenuation of cell proliferation and clone formation in BGC823 cell line. Circ-DCAF6-overexpression MGC803 cells showed strong proliferative, clone-forming capacities compared to the control group (Fig. 2C and D). Furthermore, cell apoptosis was elevated in the circ-DCAF6 depletion groups. Conversely, ectopic expression of circ-DCAF6 markedly attenuated the ability of MGC803 cells to apoptosis (Fig. 2E). In addition, the cells passed through the filter were decreased after silencing of circ-DCAF6. Whereas, ectopic expression of circ-DCAF6 remarkably elevated cell migratory and invasive capacities (Fig. 2F).

3.3. MiR-1231 and miR-1256 could be sponged and regulated by circ-DCAF6

To reveal the underlying mechanisms of circ-DCAF6 in GC, we focused on the post-transcriptional regulation of circ-DCAF6. By searching the online database, the complementary pairing between the sequences of circ-DCAF6 and some miRNAs (miR-507, miR-557, miR-616, miR-766, miR-1178, miR-1231, and miR-1256) were observed. We found that knockdown of circ-DCAF6 increased, while overexpression of circ-DCAF6 decreased miR-1231 and miR-1256 levels. The other miRNAs were almost not affected (Fig. 3A). The diagrammatic sketch of the binding sites for circ-DCAF6 and miR-1231/miR-1256 is displayed in Fig. 3B. To further verify the correlation of circ-DCAF6 and miR-1231/1256 in clinical samples, we detected their expression in clinical GC tissues. Interestingly, we found that the expression of circ-DCAF6 was significantly negatively correlated with miR-1231 and miR-1256 (Fig. 3C). The luciferase reporter experiment unraveled that overexpression of miR-1231 or miR-1256 significantly downregulated the luciferase activity of wild-type vector, but had no effects on the mutant ones, indicating that both miR-1231 and miR-1256 could interact with circ-DCAF6 in BGC823 and MGC803 cells (Fig. 3D and E).

3.4. Circ-DCAF6 exerts oncogenic properties by downregulating miR-1231 and miR-1256

Rescue assay was carried out on the two selected GC cells to detect whether the biological functions of circ-DCAF6 are dependent on its inhibition of miR-1231/−1256. qRT-PCR analysis indicated that knockdown of circ-DCAF6 significantly upregulated miR-1231 and miR-1256 expression in BGC823 cells. Nonetheless, this elevation was rescued in the si-circ-DCAF6 and miR-1231/1256 inhibitor co-transfected groups (Fig. 4A). Additionally, enforced expression of circ-DCAF6 attenuated miR-1231 and miR-1256 expression in MGC803 cells. Whereas, upregulating of circ-DCAF6 and miR-1231 or miR-1256 could almost totally restore miR-1231 and miR-1256 expression, respectively (Fig. 4B). Functionally, cell proliferation and invasion attenuation caused by circ-DCAF6 suppression were partially rescued by miR-1231 and miR-1256 inhibitors in BGC823 cells. Moreover, in the

Table 2
Univariate and multivariate analysis of prognostic indicators for overall survival in GC patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Overall survival						
Gender (male vs. female)	0.943	0.525–1.692	0.843			
Age (≥60 vs. < 60)	0.980	0.544–1.766	0.945			
Tumor size (≥3 cm vs. < 3 cm)	1.128	0.617–2.062	0.695			
Depth of invasion (T3 + T4 vs. T1 + T2)	2.575	1.342–4.941	0.004	1.564	0.742–3.296	0.240
Lymph node invasion (positive vs. negative)	1.322	0.615–2.841	0.474			
TNM stage (III-IV vs. I-II)	2.536	1.303–4.937	0.006	1.956	0.947–4.039	0.070
Differentiation grade (poorly/undifferentiated vs. well/moderately)	1.371	0.764–2.463	0.290			
circ-DCAF6 expression (high vs. low)	2.569	1.379–4.785	0.003	2.061	1.059–4.015	0.033

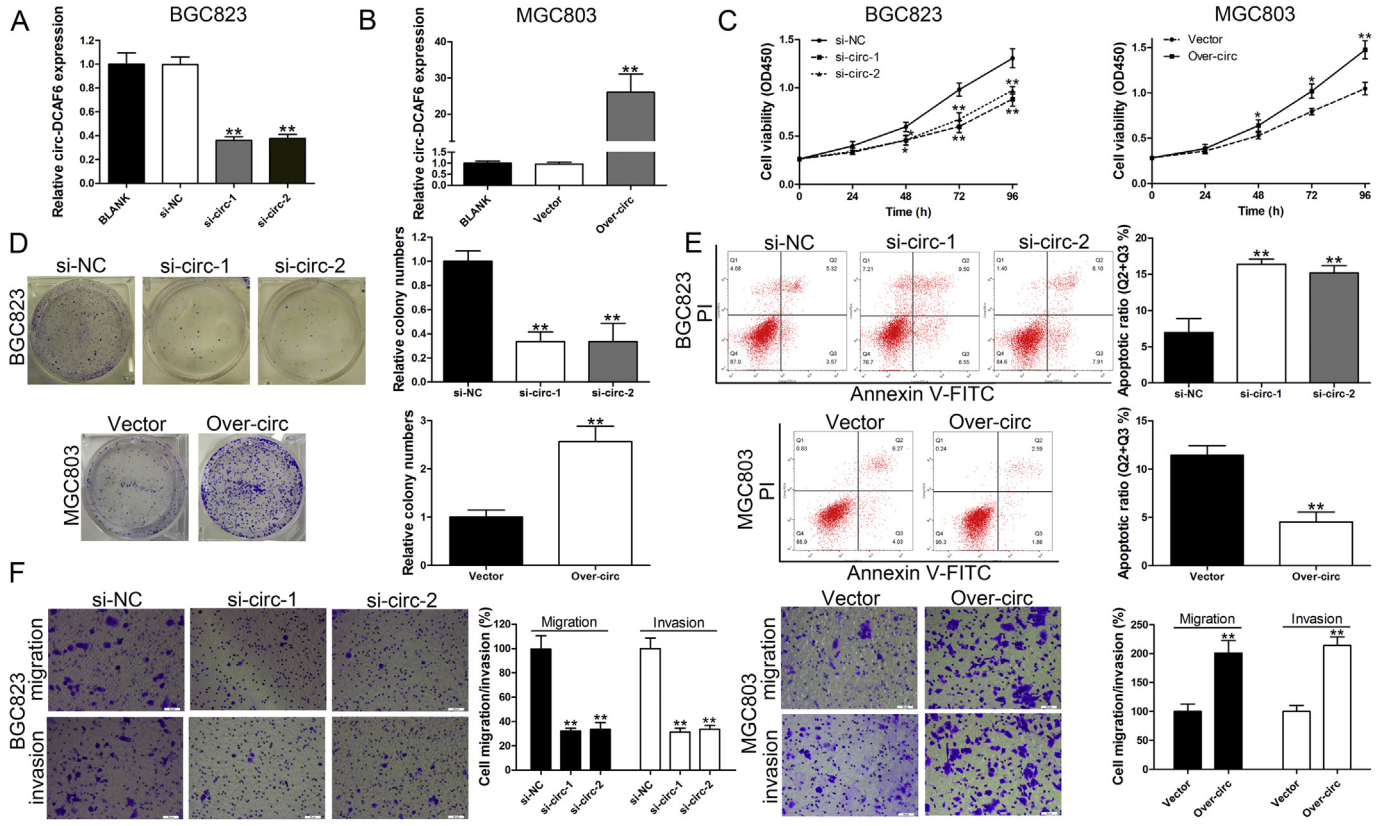

Fig. 2. Circ-DCAF6 promotes GC cell progression. (A) circ-DCAF6 expression was detected after transfection in BGC823 cells by qRT-PCR. (B) circ-DCAF6 expression was detected after transfection in MGC803 cells by qRT-PCR. (C) CCK-8 assays were used to detect cell viability of BGC823 and MGC803 cells after transfection. (D) Colony formation assays were used to detect the clone ability of BGC823 and MGC803 cells after transfection. (E) Flow cytometric analysis was used to detect cell apoptosis of BGC823 and MGC803 cells after transfection. (F) Transwell assays were used to detect cell migration and invasion capacities of BGC823 and MGC803 cells after transfection. $*p < .05$, $**p < .01$.

group of si-circ-DCAF6, miR-1231 inhibitor and miR-1256 inhibitor cotransfected cells, the oncogenic functions were almost totally restored (Fig. 4C and D). MGC803 cell proliferation and invasion promoted by circ-DCAF6 vector were evidently inhibited by miR-1231 or miR-1256 mimics. Furthermore, in the cells cotransfected with circ-DCAF6 vector, miR-1231 and miR-1256 mimics, the oncogenic functions were further suppressed (Fig. 4E and F).

4. Discussion

CircRNA field is a hot spot in recent years. With the development of circRNA microarray sequencing technology, many tumor-associated circRNAs were identified including circ_0016760, circ_0030235, and circ_0034642 (Li et al., 2018; Xu et al., 2018b; Yang et al., 2019). Circ-DCAF6 was also screened as an elevated circRNA in GC proved by the previous study (Gu et al., 2018). This circRNA has not been investigated before. In this work, we also found upregulation of circ-DCAF6 exist in GC patients' samples and cell lines. Studies have demonstrated that several dysregulated circRNAs may be used as prognostic indicators (Li et al., 2018; Xing et al., 2018; Xu et al., 2018b). Further correlation analysis in clinical GC samples showed that circ-DCAF6 expression was correlated with depth of invasion ($P = .019$), lymph node invasion ($P = .026$), TNM stage ($P = .036$), and patients' prognosis ($P = .002$). Further univariate and multivariate analyses suggested that high circ-DCAF6 level is a risk factor for GC survival ($P = .033$). Collectively, circ-DCAF6 may be an important factor in predicting the prognosis of GC.

Gain/loss-of-function experimetns suggest that circ-DCAF6 acts as an important role in GC progression. Multiple lines of evidence unveil that circRNAs can function as a ceRNA to regulate gene expression via sponging miRNAs, thus participating in the occurrence and progression of cancers (He et al., 2018; Xu et al., 2018b; Yang et al., 2019). In the present study, overexpression and knockdown of circ-DCAF6 significantly decreased and increased miR-1231 and miR-1256 expression, respectively, indicating that circ-DCAF6 is an upstream negative regulator for miR-1231 and miR-1256. Furthermore, we found that miR-1231 as well as miR-1256 was abundantly absorbed by circ-DCAF6, as determined by dual-luciferase reporter assays.

Various evidence has revealed downregulation of miR-1231 in many tumors, such as papillary thyroid cancer (Pan et al., 2019), glioma (Wang et al., 2018a), and pancreatic cancer (Zheng et al., 2016). Actually, some downstream targets of miR-1231 have been identified. PTPN11 and IFNAR1 were reported to be the downstream targets of miR-1231 (Zheng et al., 2016; Zhou et al., 2012). Additionally, miR-1231 executes a tumor suppressive function in glioma via affecting EGFR/PI3K/AKT signaling pathway (Zhang et al., 2018). In myocardial infarction process, the expression of CACNA2D2 is affected by miR-1231 at posttranscriptional level (Zhang et al., 2017b). For miR-1256, Liu et al. unraveled that miR-1256 attenuates NSCLC cell proliferation and migration via targeting TCTN1 (Liu et al., 2018a). Another study reported that decreased miR-1256 level is linked to advanced TNM stage, positive lymph node metastasis, and poor survival in colorectal cancer patients (Liu et al., 2018b). In this project, rescue experiments uncovered that the oncogenic effect induced by circ-DCAF6 was partly inhibited by miR-1231 and miR-1256, demonstrating that miR-1231 and miR-1256 are imperative mediators of circ-DCAF6. Collectively, the results indicated that circ-DCAF6 is an upstream negative modulator for miR-1231 and miR-1256 and functions as an oncogene through targeting these two miRNAs in GC. Further research is warranted to investigate whether circ-DCAF6/miR-1231/miR-1256 axis

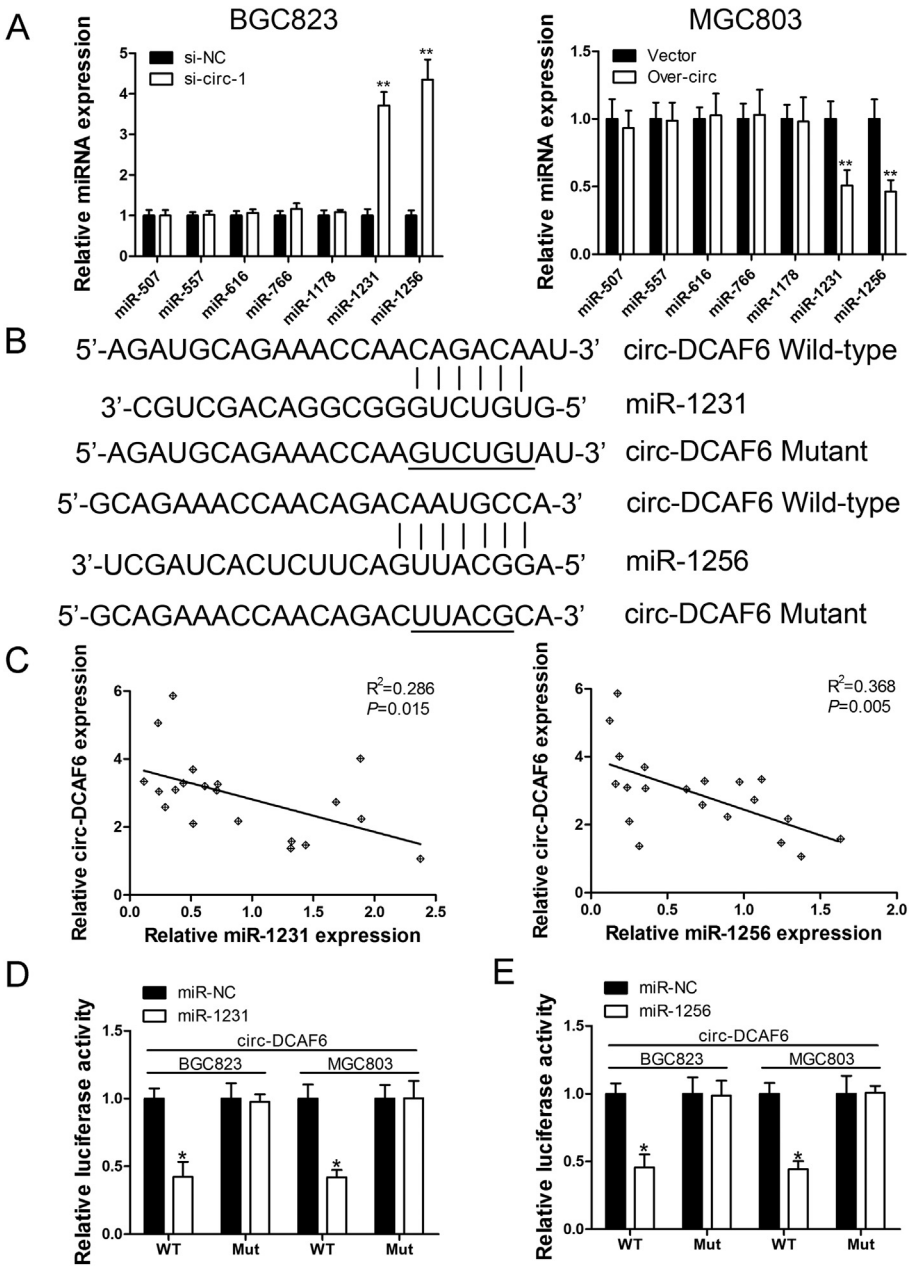

Fig. 3. Circ-DCAF6 sponges miR-1231 and miR-1256 in GC. (A) Relative miRNA expression was detected after transfection in BGC823 and MGC803 cells. (B) Diagrammatic sketch of the binding sites for circ-DCAF6 and miR-1231/miR-1256. (C) Correlation analysis of circ-DCAF6 and miR-1231/miR-1256 in GC patients' tissues. (D) Luciferase reporter assay was conducted to evaluate the interaction ability between miR-1231 and circ-DCAF6. (E) Luciferase reporter assay was conducted to evaluate the interaction ability between miR-1256 and circ-DCAF6. *$p < .05$, **$p < .01$.

also functions in other malignancies and it is still needed to reveal the underlying targets of miR-1231 and miR-1256 in GC. Additionally, whether silencing of circ-DCAF6 or ectopic expression of circ-DCAF6 markedly retarded/enhanced the in vivo tumor growth is needed to reveal.

There are still some limitations in this project. For instance, the molecular mechanism which made circ-DCAF6 upregulation in GC is still unknown. Some recent studies may provide the researchers several clues. CircRNA biogenesis could be modulated by RNA binding proteins (RBPs) though an RBP-driven circularization mechanism (Lyu and Huang, 2017). RBPs bind to the introns near splice sites, which may facilitate the production of circRNAs (Conn et al., 2015). For example, Dudekula et al. reported that has_circ_0000020 has four binding sites for eIF4A3, with much higher frequency than the frequency seen for targeting the body sequence of the circRNA. This finding indicates a preference of eIF4A3 to bind to the has_circ_0000020 junctions (Dudekula et al., 2016). Furthermore, Wang et al. revealed that eIF4A3-induced upregulation of circ-MMP9 could facilitate glioblastoma multiforme cell tumorigenesis (Wang et al., 2018b). Whether circ-DCAF6 has a similar regulatory mechanism is needed to reveal.

In summary, we found circ-DCAF6 plays an essential role GC through sponging and regulating miR-1231 and miR-1256. Targeting of this axis may be an efficacious approach against this lethal disease.

Authors' contributions

Study design: Yu Yang; Conducted the experiments: Ligang Wu; Drafted the manuscript: Ligang Wu; Analyzed the data: Ligang Wu, Deshui Liu; Revised the manuscript: Yu Yang.

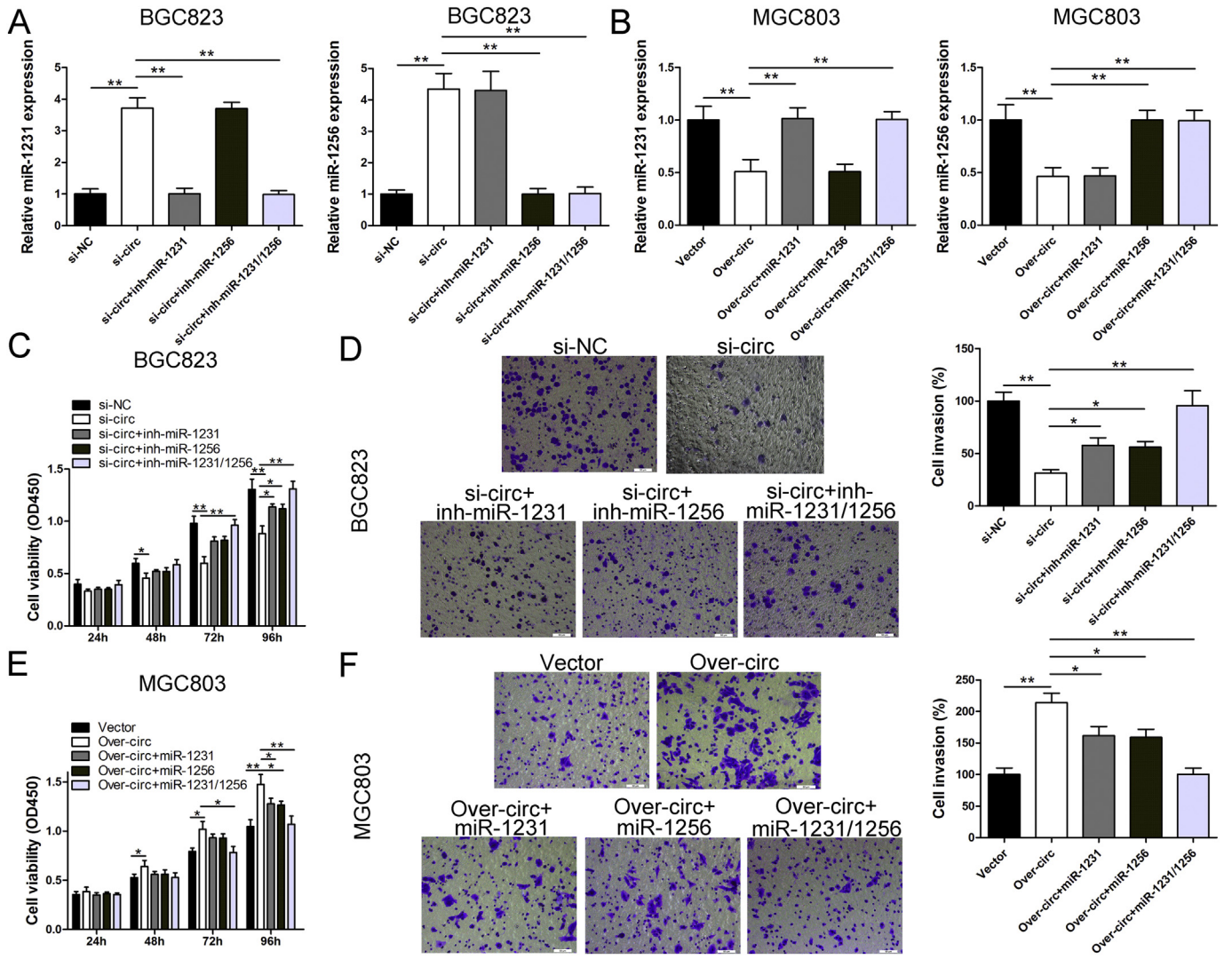

Fig. 4. The oncogenic role of circ-DCAF6 is dependent on its regulation of miR-1231 and miR-1256. (A) qRT-PCR was used to detect miR-1231 and miR-1256 expression after transfection in BGC823 cells. (B) qRT-PCR was used to detect miR-1231 and miR-1256 expression after transfection in MGC803 cells. (C) CCK-8 assays were used to evaluate cell viability after transfection in BGC823 cells. (D) Transwell assays were used to evaluate cell invasive potential after transfection in BGC823 cells. (E) CCK-8 assays were used to evaluate cell viability after transfection in MGC803 cells. (F) Transwell assays were used to evaluate cell invasive potential after transfection in MGC803 cells. $*p < .05$, $**p < .01$.

Declaration of Competing Interest

The authors declare no conflict of interests exist in the present project.

Acknowledgement

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