Circular RNA CDR1as Promotes Gastric Cancer Growth via miR-299-3p/TGIF1 Axis

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Abstract

Background Gastric cancer (GC) is a common malignancy worldwide. Circular RNA CDR1as has been reported as a crucial regulator in human diseases including cancer. However, its biological roles, mechanisms and clinical values in GC remain largely unknown.

Methods and Results CDR1as levels were surveyed in paired GC and adjacent normal tissues, paired blood samples from GC patients and healthy controls by RT-qPCR. Its clinical values were evaluated by ROC analysis, survival analysis and correlations with clinic pathological features. Cell transfection was performed to manipulate gene expression. In vitro CCK8 and colony formation assays and in vivo xenograft mouse model were employed to determine CDR1as effects on GC growth. CDR1as-miRNA and miRNA-mRNA interactions were predicted by bioinformatics analysis and further verified by RIP, dual-luciferase reporter gene assays, RT-qPCR, western blot and functional rescue experiments. Our results showed that CDR1as level was significantly downregulated in GC tissues and correlated with nerve invasion and poor prognosis. GC patients presented higher plasma CDR1as level than healthy controls. Functionally, knockdown of CDR1as inhibited GC cell proliferation and viability while its overexpression promoted GC growth in vitro and in vivo. The proliferation-related proteins PCNA and Cyclin D1 and apoptosis-related proteins Bax, Bcl-2, Caspase-3 and Caspase-9 were regulated. Mechanistically, CDR1as acted as a miR-299-3p sponge to relieve its suppressive effects and upregulate TGIF1 expression to promote GC growth.

Conclusions CDR1as may be considered as a potential diagnostic and prognostic biomarker for GC. CDR1as/miR-299-3p/TGIF1 axis promotes GC growth *in vitro* and *in vivo*.

Introduction

Gastric cancer (GC) ranks as the fifth most common malignancies and the third leading cause for cancer-related death worldwide [1]. *H. Pylori* infection, environmental factors including diet and exogenous chemicals, and genetic abnormalities are main risk factors of gastric tumorigenesis [2]. Since GC lacks specific symptoms at early stage, most patients are at intermediate or terminal stage with poor prognosis when first diagnosed [3]. Additionally, tissue biopsy remains a gold standard for GC diagnosis which is invasive and cannot be used as a common test. Traditional surgery resection, chemotherapy and radiation therapy are still unsatisfactory due to limited efficacy and severe systemic cytotoxicity and drug resistance [4, 5]. Thus, exploring the underlying molecular mechanisms of GC progression and developing new biomarkers are important for improving prognosis of GC patients.

Circular RNAs (circRNAs), a novel type of noncoding RNAs (ncRNAs) with covalently closed loop structure, have been identified in various cancers, including GC [6]. They have high stability, abundance and conservation, and are widely distributed in human serum, plasma, and other body fluids [7–9]. Consequently, circRNAs are easily detectable with non-invasive methods. CircRNAs have tissue- and developmental-stage-specific expression pattern, which can reflect the alterations of certain physiological

and pathological processes [10, 11]. Many circRNAs have been discovered to be aberrantly expressed in GC and play crucial roles in various biological processes such as cell proliferation, apoptosis, migration and invasion through sponging miRNAs and interacting with proteins [12]. Thus, circRNAs are considered as promising biomarkers for GC. CDR1as (ciRS-7) is one of the most extensively investigated circRNAs. It is transcribed from CDR1 gene but driven by the promoter of LINC00632 and could be cleaved by miR-671 in an Ago2-slicer-dependent manner [13, 14]. Previous studies showed that CDR1as plays crucial roles in multiple physiological and pathological processes including stem cell differentiation [15], insulin secretion [16], neuropsychiatric disorders [17], osteoarthritis [18], and cancer [19]. Most studies focus on the role of CDR1as as a miR-7 sponges since it harbors 63 conserved binding sites for miR-7 and could suppress the functions of miR-7 [20–22]. However, the biological roles, mechanisms of action, and clinical values of CDR1as in GC are not well characterized.

In this study, we attempted to determine CDR1as expression in GC patients, investigate the biological roles and mechanisms of CDR1as in GC progression, and uncover its clinical value for GC diagnosis and prognosis.

Materials And Methods

Clinical specimens

A total of 87 paired GC tissues and corresponding adjacent normal tissues (5 cm away from the tumor edge) were obtained from Department of General Surgery, the Affiliated People's Hospital of Jiangsu University. None of patients received the preoperative chemotherapy and radiotherapy. All tissues were frozen in liquid nitrogen and stored at -80°C until needed. Plasma samples from 68 preoperative GC patients and 68 age- and gender-matched healthy donors were collected from Department of Clinical laboratory, the Affiliated People's Hospital of Jiangsu University. Intravenous blood was collected in EDTA-K2 anti-coagulant tubes and centrifuged at 1,000g for 10 min at 4°C. The upper plasma was transferred into a new tube for centrifugation at 3,000g for 15min at 4°C, and then aliquoted and stored at -80°C until needed. All participants signed written informed consent and this study was approved by the Institutional Ethical Committee of Jiangsu University.

Cell lines and culture conditions

Human GC cell lines HGC-27, SGC-7901, BGC-823, MGC-803, AGS and MKN-45 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human normal gastric mucosa epithelial cell line GES-1 was obtained from Gefan Biological Technology (Shanghai, China). MKN-45, MGC-803 cells were cultured in high-glucose DMEM (Gibco). HGC-27 and SGC-7901 cells were maintained in RPMI-1640 (Bioind, Israel). AGS cells were propagated in DMEM/F-12 Medium (Bioind, Israel). All cells were cultured in media supplemented with 10% fetal bovine serum (FBS; Bovogen, Australia) at 37°C in a water-saturated incubator with 5% CO₂ atmosphere.

Exosomes isolation

Exosomes from the human plasma and culture supernatants were isolated using ExoQuick precipitation solution (SBI, Mountain View, CA, USA) according to the manufacturer's protocols. In brief, 250µl plasma were mixed with 63µl ExoQuick solutions and incubated at 4°C overnight. The exosome pellets were collected after centrifugation at 1,500g for 30min and then suspended in 50µl sterile PBS for further use. A total volume of 50ml culture supernatants from cells that had grown in exosome-depleted medium were collected. After centrifugation at 1,000g for 30min using 100KDa MWCO, the concentrated solutions were mixed with ExoQuick-TC Exosome Precipitation Solution at 5:1 ratio and precipitated at 4°C overnight. The exosome pellets were resuspended in 200µl sterile PBS after centrifugation at 1,500g for 30min. The exosomes dissolved in PBS were aliquoted and stored at -80°C for further analysis.

Nuclear-cytoplasmic fractionation, RNA extraction and RT-qPCR

Cytoplasmic RNA of cells was isolated with RNeasy Mini Kit (Qiagen, Germany). The remaining nuclear RNA of cells and total RNA from tissue samples and cells were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA from whole plasma and plasma derived exosomes was isolated and purified using miRNeasy Serum/Plasma kit (Qiagen, Germany). The RNA concentration and purity were determined by NanoDrop 2000 (Thermo Fisher Scientific, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) for mRNAs and circRNAs was conducted with HiScript 1st Strand cDNA Synthesis Kit and AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). MiRNAs were transcribed with miScript II RT Kit and detected with miScript SYBR Green PCR Kit (Qiagen, Germany). The ABI StepOnePlus Real-Time PCR Systems (Thermo Fisher Scientific, MA, USA) was used for all quantitative analysis. β -actin was used to normalize mRNAs and circRNAs while U6 was for miRNAs input. The expression levels were presented by $-\Delta Ct$ method and relative expression levels were calculated by $2^{-\Delta\Delta Ct}$ method. PCR products were separated by 1.5% agarose gels and examined by UV irradiation. Sanger sequencing was used to confirm CDR1as junction. The primers for miRNAs and U6 were purchased from Qiagen. CircRNAs and mRNAs primer sequences were listed in Supplementary Table S1.

Gene silencing and overexpression

Two different siRNAs targeting the backspliced junction of CDR1as or TGIF1 were designed and synthesized to knock down its expression (GenePharma, Shanghai, China). MiRNA mimics (miR-876-5p, miR-3167, miR-299-3p, miR-203a) were purchased for miRNA overexpression (GenePharma, Shanghai, China). Cells (2×10^5 /well) were seeded and cultured in 6-well plate overnight before these oligonucleotides were transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in

serum-free medium at concentration of 25nM for 6h and changed with complete medium. Cells were harvested after 48h post transfection for RNA analysis and functional experiments or 72h for protein analysis. The sequences of oligonucleotides were listed in Supplementary Table S2. CDR1as stable overexpression cell lines were constructed by using lentivirus expression vector system (Hanbio Biotechnology, Shanghai, China). MKN-45, AGS, BGC-823 and SGC-7901 cells were transduced with prepared lentivirus HBLV-CDR1as-GFP-PURO or HBLV-GFP-PURO (MOI=30) and selected with 2µg/ml puromycin (Invitrogen, Carlsbad, CA, USA) for 15 days. The fluorescence intensity of GFP and efficiency of CDR1as overexpression were evaluated by fluorescence microscope and RT-qPCR.

Dual-luciferase reporter gene assay

The recombinant dual-luciferase reporter plasmids were synthesized by Genscript (Nanjing, China) which were constructed by inserting full-length of CDR1as cDNA into pmirGLO Dual-Luciferase miRNA target expression vectors (Promega) at 5' PmeI and 3' NheI sites. MGC-803 (4×10^4 /well) were seeded in 24-well plates overnight before 400ng luciferase reporter vector and 40nM miRNA mimics were co-transfected into cells. After 24h, the cells were harvested and analyzed for firefly and Renilla luciferase activity using Dual-Glo Luciferase Assay Kit (Promega) and GloMax 20/20 Luminometer (Promega) following the manufacturer's instructions.

RNA immunoprecipitation (RIP) assay

RIP assay was performed with Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, USA). In brief, 6×10^7 MGC-803 cells were lysed in 300µl complete RNA lysis buffer. Then, each 100µl cell lysates were added to 900µl RIP immunoprecipitation buffer containing magnetic beads conjugated with 5µg negative control mouse IgG, positive control anti-snRNP70 antibody or human anti-AGO2 antibody (Mouse, Millipore, Billerica, USA) and rotated overnight at 4°C. After incubation with proteinase K buffer at 55°C for 30min, the immunoprecipitated RNA was purified and analyzed with RT-qPCR.

CCK8 assay

Cell viability was evaluated by CCK-8 Cell Counting Kit (Vazyme, Nanjing, China). 4×10^3 cells in 100µl complete medium were cultured in triplicate 96-well plates for 1 to 5 days respectively. Then, 10µl CCK-8 solution was added to each well and incubation at 37°C for 2h. The absorbance at 450nm was measured with an automatic microplate reader (BioTEK, USA).

Colony formation assay

Cell proliferation ability was assessed by colony formation assay. 2×10^3 cells were seeded and cultured in 6-well plates for 10-14 days. The medium was changed at an interval of 2-3 days. Colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

Western blot

Western blot was performed with the standard protocol. Cells were harvested and lysed in RIPA buffer supplemented with proteinase inhibitors on ice. Equal amount of proteins were separated by 12% SDS-PAGE gel. After electrophoresis, separated proteins were transferred onto PVDF membrane (Millipore, USA), blocked in 5% (W/V) non-fat milk and then incubated with primary antibodies at 4°C overnight. The sources of primary antibodies were as follows: rabbit anti- β -actin antibody (1:5000; Abclonal, Wuhan, China), rabbit anti-Bax antibody (1:1000; Cell Signaling, Boston, MA), Mouse anti-Bcl-2 antibody (1:200; Cell Signaling, Boston, MA), rabbit anti-activated-Caspase-3 antibody (1:500; Bioworld, USA), mouse anti-Caspase-9 antibody (1:600; Cell Signaling, Boston, MA), rabbit anti-PCNA antibody (1:800, Bioworld, USA), rabbit anti-Cyclin D1 antibody (1:500; Bioworld, USA), mouse anti-TGIF1 antibody (1:800; Santa Cruz, USA). After incubation with goat anti-rabbit IgG secondary antibody (1:2000; Invitrogen, USA) and goat anti-mouse IgG secondary antibody (1:1000; Invitrogen, USA), the protein band signals were visualized with enhanced chemiluminescence system (ImageQuant LAS4000mini, GE, Japan). The densitometry readings of bands were analyzed by Image J software and normalized to β -actin expression.

Tumor model

Male BALB/c nu/nu mice (Cavens, Changzhou, China) aged 4-6 weeks were randomly divided into two groups ($n=2$) and received subcutaneous injection of either CDR1as stably overexpressing MKN-45 cells or corresponding negative control cells (5.0×10^6 cells in 200 μ l PBS per mouse). After two weeks, all mice were sacrificed. All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Use Ethics Committee of Jiangsu University.

HE staining and Immunohistochemistry

The subcutaneous tumors from nude mice were fixed by 4% paraformaldehyde and made to paraffin-embedded tissues sections. For HE staining, sections were deparaffinized in xylene, rehydrated through graded ethanol, and stained with hematoxylin-eosin. For immunohistochemistry, SABC kit (Boster, China) was used. After deparaffinization and rehydration, sections were performed heat-induced antigen retrieval in citrate buffer (10 mM, PH 6.0) and exposed to 3% hydrogenous peroxidase for 10min to suppress endogenous peroxidase activity. Then, slides were blocked with 5% BSA, incubated with primary antibody at 4°C overnight, secondary antibody at 37°C for 20min and SABC at 37°C for 30min, and finally stained

with DAB and hematoxylin for microscopic observation. The primary antibody was rabbit anti-Bax antibody (1:100; Cell Signaling, USA).

Statistical analysis

All statistical analysis were performed by SPSS 21.0 (IBM, Chicago, IL, USA) and GraphPad Prism version 5.0 software (LaJolla, CA, USA). CDR1as expression levels between paired tissues, plasma and exosomes in plasma samples were compared by Student's t-test or Mann-Whitney U test. The associations between CDR1as and clinicopathological factors were analyzed by Pearson χ^2 test or Fisher's exact test. ROC curves were established to evaluate the diagnostic value of CDR1as for GC. The survival curve was drawn by Kaplan-Meier method and analyzed by log-rank test. Differences between experimental groups were assessed by Student's t-test or one-way ANOVA. For all results, $P < 0.05$ was considered statistically significant.

Results

The prognostic and diagnostic value of CDR1as in GC tissues and circulating blood

We first measured CDR1as level in 87 paired human GC tissues and adjacent normal tissues by RT-qPCR. Sanger sequencing confirmed the head-to-tail splicing of CDR1as (Supplementary Fig. S1a). As shown in Fig. 1a, 73.6% (64/87) GC patients exhibited lower levels of CDR1as in GC tissues than that in matched adjacent normal tissues. We then examined CDR1as expression in six GC cell lines (MKN-45, SGC-7901, MGC-803, BGC-823, AGS and HGC-27). Compared with normal gastric mucosa epithelial cell line GES-1, GC cells all showed lower expression of CDR1as, which was consistent with that observed in GC tissues (Supplementary Fig. S1b, c). The diagnostic value of CDR1as in distinguishing GC tissues and adjacent normal tissues was determined by ROC analysis. The area under ROC curve (AUC) was 0.771, with a sensitivity of 77.0% and a specificity of 73.6% under the cut off value of -2.85 (Fig. 1b). Then, all the GC patients were divided into higher and lower groups (based on the cut off value) and the correlation between CDR1as expression in GC tissues and clinical pathological parameters was assessed. As shown in Table 1, CDR1as level was not associated with age, gender, tumor size, differentiation or metastasis but related to nerve invasion. GC patients with nerve invasion expressed relatively higher levels of CDR1as than those without nerve invasion (Fig. 1c). Kaplan-Meier survival analysis combined with log-rank tests showed that GC patients with lower CDR1as expression had shorter post-operative survival time (Fig. 1d). These results suggested that CDR1as was downregulated in GC tissues and cells and may serve as a potential diagnostic or prognostic biomarker for GC.

We next investigated CDR1as values as circulating biomarker for GC. A total of 68 paired plasma samples from pre-operative GC patients and age- and gender-matched healthy controls were measured. CDR1as level was significantly higher in the plasma of GC patients than that in healthy controls (Fig. 1e).

The corresponding plasma exosomes samples were also examined. Compared with healthy controls, GC patients had relatively higher levels of CDR1as. However, the difference was not statistically significant (Supplementary Fig. S1d). To further verify this result, we measured CDR1as level in exosomes from four GC cell lines. The results showed that exosomes from BGC-823, SGC-7901 and HGC-27 cells had relatively higher levels of CDR1as than that from GES-1 cells (Supplementary Fig. S1e). Furthermore, CDR1as level in exosomes from GC cells was greater than that in their producing cells (Supplementary Fig. S1f), suggesting that CDR1as may be selectively sorted into exosomes and released into extracellular medium. Additionally, correlation analysis showed that plasma CDR1as level was not associated any of the clinical pathological features (Supplementary Table S3). ROC curve analysis showed that the AUC value was 0.652, with a sensitivity of 89.93% and a specificity of 46.15% under the cut off value of -7.585 (Fig. 1f), revealing that plasma CDR1as has the potential to be a good diagnostic biomarker for GC.

Table 1

The correlation between CDR1as expression level in cancer tissues and clinicopathological parameters of GC patients

Features	Number	Tissue-CDR1as		<i>P</i> value
		High(<i>n</i> =23)	Low(<i>n</i> =64)	
Age				
<60	17	4	13	1.000
≥60	70	19	51	
Gender				
Male	63	18	45	0.464
Female	24	5	19	
Tumor size				
<5cm	43	11	32	0.858
≥5cm	44	12	32	
Differentiation ^a				
Poor	47	14	33	0.675
Moderate	37	8	29	
Lymphatic metastasis				
N0	27	6	21	0.803
N1	12	3	9	
N2	21	5	16	
N3	27	9	18	
Distant metastasis ^a				
Absent	82	22	60	0.671
Present	2	0	2	
Vascular invasion				
Absent	75	20	55	1.000
Present	12	3	9	
Nerve invasion				
Absent	72	15	57	0.020*
Present	15	8	7	
TNM stage ^a				

I	6	2	4	0.275
II	23	3	20	
III	53	17	36	
IV	2	0	2	
Invasion depth				
T1	2	1	1	0.328
T2	6	1	5	
T3	3	2	1	
T4	76	19	57	
^a Missing 3 cases, * $P < 0.05$				

CDR1as promoted GC growth *in vitro* and *in vivo*

To investigate the biological effects of CDR1as on GC growth, we first constructed stable CDR1as overexpression GC cell lines by using lentivirus-mediated transfection. The transduced GC cells (AGS, MKN-45, SGC-7901 and BGC-823) showed high intensities of green fluorescence (Fig. 2a; Supplementary Fig. S2a). RT-qPCR results indicated that CDR1as expression in experimental group was mostly over 100-fold higher than that in control group (Fig. 2b; Supplementary Fig. S2b), suggesting the successful construction of stable CDR1as overexpression GC cell lines. CCK8 assays showed that CDR1as overexpressing cells (especially MKN-45 cells) had higher cell viability than control cells (Fig. 2c; Supplementary Fig. S2c). Colony formation assays also showed that CDR1as overexpressing GC cells had enhanced proliferation ability (Fig. 2d; Supplementary Fig. S2d). The expression levels of proliferation-related proteins PCNA and Cyclin D1 were significantly increased in CDR1as overexpressing GC cells. Moreover, the expression levels of pro-apoptosis proteins Bax, caspase-3 and caspase-9 were downregulated while that of anti-apoptosis protein Bcl-2 was upregulated in CDR1as overexpressing GC cells (Fig. 2e).

To further clarify the role of CDR1as in GC cell growth, we conducted CDR1as knockdown in two GC cell lines (HGC-27 and MGC-803) with relatively higher level of CDR1as. Two siRNAs targeting the junction sites of CDR1as were designed and the knockdown efficiency was verified by RT-qPCR (Supplementary Fig. S3a). The results revealed that CDR1as knockdown markedly inhibited the proliferation of GC cells and exhibited opposite effects on proliferation and apoptosis associated proteins expression (Supplementary Fig. S3b-d). Additionally, we constructed subcutaneous xenograft tumor models. CDR1as overexpressing MKN-45 cells (OE-CDR1as) and corresponding negative control cells (OE-NC) were injected into right armpit of nude mice. Increased tumor sizes were observed in OE-CDR1as group compared with those in OE-NC group (Fig. 2f). HE staining also showed that more cell division could be

found in OE-CDR1as tumor (Fig. 2g), suggesting that CDR1as could promote cell proliferation of GC *in vivo*. IHC staining also confirmed that the expression of Bax was decreased in OE-CDR1as group (Fig. 2g). These results suggested that CDR1as promoted GC growth *in vitro* and *in vivo*.

CDR1as acted as a miRNA sponge for miR-299-3p

Previous studies indicated that CDR1as could serve as a miR-7 sponge to regulate tumor progression [22-24]. Our results of cell fraction analysis showed that CDR1as was preferentially localized in the cytoplasm of GC cells (Fig. 3a). RIP assay revealed that CDR1as could indeed be detected in the immunoprecipitates of miRNA binding protein AGO2 (Fig. 3b). However, correlation analysis showed the expression levels of miR-7 and miR-135a-5p (another reported CDR1as-binding miRNA), both upregulated in GC tissues (Fig. 3c, d), were not significantly correlated with that of CDR1as in GC tissues (Fig. 3e, f). Therefore, there might be new target miRNAs for CDR1as in GC.

Prediction analysis by database starBase v2.0 (<http://starbase.sysu.edu.cn/>) indicated that there were five potential binding sites in CDR1as for other miRNAs in addition to miR-7 (Supplementary Fig. S4). According to the value of biComplex and clipReadNum and the number of targetSites, we selected four miRNAs (miR-3167, miR-299-3p, miR-203a, miR-876-5p) for further validation. The results of dual-luciferase reporter assay presented that the luciferase activities of all reporter genes that contained CDR1as sequence were markedly decreased when co-transfected with these four miRNAs (Fig. 3g). To further identify the main miRNA that CDR1as sponges to regulate GC cell growth, we performed rescue experiments in MGC-803 and MKN-45 cells. Colony formation assays indicated that CDR1as promoted cell proliferation, while miR-876-5p and miR-299-3p, especially miR-299-3p, observably reversed this effect (Fig. 3h, i). In addition, GC cell viability could be enhanced by CDR1as, but was attenuated by miR-299-3p (Fig. 3j). These results suggested that CDR1as may serve as miR-299-3p sponge to regulate GC growth.

CDR1as upregulated TGIF1, a target of miR-299-3p

We next explored the potential mechanisms for the biological roles of miR-299-3p in GC. In accordance with rescue experiments results, colony formation assays and CCK8 assays proved that miR-299-3p inhibited GC cell proliferation and cell viability markedly (Fig. 4a, b). We then predicted miR-299-3p-mRNAs interactions by using starBase v2.0 database and selected the potential targets according to the coincidence degree (Supplementary Table S4). We mainly focused on oncogenes as miR-299-3p has tumor suppressive roles in GC growth and thus selected 10 candidate targets. After transfection with miR-299-3p mimics in MGC-803 and AGS cells, the expression levels of five genes including ABCE1, AP1G1, TGIF1, PRPS1 and PTP4A1 were significantly decreased (Fig. 4c), suggesting that these genes might be potential targets of miR-299-3p. To identify which gene might be regulated by CDR1as when sponging miR-299-3p, we detected the expression of these five genes in GC cells with CDR1as knockdown and overexpression. Only TGIF1 mRNA level was decreased in HGC-27 and MGC-803 cells with CDR1as

knockdown while increased in AGS and MKN-45 cells with CDR1as overexpression (Fig. 4d, e). Moreover, the upregulation of TGIF mRNA level in CDR1as-overexpressing AGS and MKN-45 cells was reversed by co-transfection with miR-299-3p mimics (Fig. 4i). The similar changes were also observed in TGIF protein level (Fig. 4f-h, j). Taken together, these results suggested that TGIF1 was a target of miR-299-3p and could be regulated by CDR1as.

Knockdown of TGIF1 reversed CDR1as to suppress proliferation of GC cells

In order to identify whether TGIF1 was an important regulator mediating the promotive effects of CDR1as, we knocked down TGIF1 in AGS and MGC-803 cells with two different siRNAs. RT-qPCR and western blot analysis verified the knockdown efficiency (Fig. 5a, b). Colony formation assays and CCK8 assays showed that knockdown of TGIF1 markedly suppressed the proliferation and viability ability of GC cells (Fig. 5d, e). Furthermore, when we knocked down the TGIF1 in CDR1as-overexpressing MKN-45 and AGS cells, the promotive effects of CDR1as on cell proliferation and viability were significantly reversed (Fig. 5f, g), suggesting that TGIF1 mediated the promotive effects of CDR1as on GC cell growth. In addition, western blot analysis of subcutaneous tumor tissues showed that TGIF1 protein level was increased in CDR1as overexpressing group (Fig. 5c). Meanwhile, the expression of PCNA was increased while that of Bax was decreased. When TGIF1 was knocked down in AGS and MGC-803 cells, PCNA protein level was decreased while that of Bax was increased (Fig. 5b). These results revealed that TGIF1 might mediate CDR1as regulating proliferation-related protein PCNA and pro-apoptosis protein Bax.

Discussion

Previous studies have shown that circRNA CDR1as is aberrantly expressed in multiple cancers. It is significantly upregulated in hepatocellular carcinoma (HCC) [22], non-small cell lung cancer (NSCLC) [24], osteosarcoma (OS) [25], esophageal squamous cell carcinoma (ESCC) [26] and pancreatic ductal adenocarcinoma (PDAC) [21] while downregulated in melanoma [19] and ovarian cancer (OC) [27]. In this study, CDR1as was also found to be markedly downregulated in GC tissues and cells. Some studies reported that CDR1as shares the same promoter of LINC00632 and its aberrant expression might result from histone modification especially the balance between the repressive mark H3K27me3 and the activating mark H3K27ac [13, 19]. Moreover, CDR1as has important regulatory effects on tumor cell proliferation, apoptosis, migration and invasion. Through gain- and loss- of function studies in GC cells and subcutaneous xenograft tumor models, we found that it promoted GC growth and regulated proliferation and apoptosis-related genes expression which play an important role in determining the tumor growth and aggressiveness [28]. Generally, most oncogenic ncRNAs are upregulated in tumor tissues and cells compared with normal ones [29, 30]. Here, we found that CDR1as has promotive effects on GC growth while are downregulated in GC tissues and cells. Similarly, hsa_circ_0000096 was reported to be downregulated in GC tissues while its knockdown inhibited cell proliferation and migration in vitro

and *in vivo* [31]. Therefore, there may be complicated regulatory mechanisms on circRNAs expression under pathological circumstance which remain to be uncovered.

CDR1as usually acts as miR-7 sponge to suppress miR-7 activities and upregulate target genes since it has 63 conserved binding sites for miR-7. For example, CDR1as acts as an oncogene in HCC through targeting miR-7 and upregulating CCNE1 and PIK3CD expression [22]. Silencing CDR1as inhibits colorectal cancer (CRC) progression by targeting miR-7 and downregulating EGFR and IGF-1R expression [23]. In this study, we found that CDR1as sponged a new miRNA, miR-299-3p, to promote GC growth. Bioinformatic prediction combined with RIP assay and dual-luciferase reporter gene assay verified the binding ability of CDR1as and miR-299-3p. Cell function assays proved that miR-299-3p suppressed GC cell proliferation. Rescue experiments confirmed that miR-299-3p could reverse the promoting role of CDR1as in GC cell proliferation. MiR-299-3p has been previously reported to act as a tumor suppressor and induce cell apoptosis [32, 33]. Thus, we proposed that CDR1as promoted GC growth by sponging suppressive miR-299-3p.

TG-Interacting Factor 1 (TGIF1) is a transcriptional corepressor that belongs to three-amino loop extension (TALE) family of homeodomain proteins [34]. It can interfere with retinoid X receptor (RXR) binding to DNA or recruit corepressors including histone deacetylases (HDACs) to TGF- β signaling intermediate Smad2 to regulate various biological processes [34, 35]. Previous studies suggested that TGIF1 acts as an oncogene in tumorigenesis. In NSCLC, it promotes the growth and migration of cancer cells through activating β -catenin/TCF signaling pathway [36]. In breast cancer (BRC), TGIF1 promotes Wnt1-driven tumor progression [37]. In this study, TGIF1 also exerted potential oncogenic role on GC growth as its knockdown significantly inhibited GC cell proliferation and viability with downregulation of PCNA and upregulation of Bax. In addition, TGIF1 was identified as a target gene of miR-299-3p. Bioinformatic prediction combined with RT-qPCR and western blot analysis showed that miR-299-3p could bind to TGIF1 and suppress its expression. Furthermore, we found that TGIF1 was a crucial downstream target for CDR1as sponging miR-299-3p to regulate GC growth. CDR1as could upregulate TGIF1 expression *in vitro* and *in vivo*, which could be reversed by rescued with miR-299-3p, suggesting that CDR1as upregulated TGIF1 through inhibiting miR-299-3p effects. The promotive effects of CDR1as on GC cell proliferation and viability were reversed markedly by knockdown of TGIF1, revealing that TGIF1 was important for the role of CDR1as on GC growth. Meanwhile, CDR1as and TGIF1 are both able to positively regulate PCNA and negatively regulated Bax during GC growth. Taken together, CDR1as upregulated oncogene TGIF1 by sponging miR-299-3p to promote GC growth.

CDR1as has shown good potential to be a novel biomarker in multiple cancers. In OS, CDR1as could be used as a diagnostic biomarker with the AUC of 0.857 in OS tissues [25]. In CRC and cholangiocarcinoma (CCA), CDR1as is a promising prognostic biomarker and its overexpression in tumor tissues was associated with poor prognosis [38, 39]. Our study indicated that CDR1as could be a potential prognosis predictor for GC as its downregulation in GC tissues was correlated with shorter post-operative overall survival. Besides, upregulated CDR1as in plasma could serve as a diagnostic biomarker for GC with the AUC of 0.652. Interestingly, CDR1as expression in GC tissues (downregulated) and plasma samples

(upregulated) was not consistent, which was similar to hsa_circ_0006633 and hsa_circ_002059 [40, 41]. Further experiments indicated that CDR1as was relatively enriched in GC cells exosomes compared with its producing cells and normal GES-1 cells exosomes. We speculated that there might be some molecular mechanisms mediating selectively sorting of circRNAs into exosomes and released into the circulation [42]. In addition, we found that high level of CDR1as in GC tissues was related with nerve invasion. Considering that intense neuronal expression of CDR1as has been previously reported [20], the role of CDR1as in nerve invasion of GC needs to be further investigated.

Conclusions

Our findings revealed that CDR1as promoted GC growth through CDR1as/miR-299-3p/TGIF1 axis and could be used as a potential diagnostic and prognostic biomarker for GC.

Abbreviations

GC: gastric cancer; circRNAs: circular RNAs; ncRNAs: noncoding RNAs; GES-1: gastric epithelial cells; AUC: area under ROC curve; RT-qPCR: reverse transcription quantitative polymerase chain reaction; GFP: green fluorescent protein; RIP: RNA immunoprecipitation; FBS: fetal bovine serum; HCC: hepatocellular carcinoma; NSCLC: non-small cell lung cancer; OS: osteosarcoma; ESCC: esophageal squamous cell carcinoma; PDAC: pancreatic ductal adenocarcinoma; OC: ovarian cancer; CRC: colorectal cancer; TGIF1: TG-Interacting Factor 1; TALE: three-amino loop extension; RXR: retinoid X receptor; HDACs: histone deacetylases; BRC: breast cancer; CCA: cholangiocarcinoma

Declarations

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Conflicts of interest

The authors declare that they have no financial conflicts of interest.

Availability of data and material

The data used to support the findings of this study are available from the corresponding author request.

Authors' contributions

All authors contributed to the study conception and design. Rong Li, Jiajia Jiang, Junyi Wang, Jie Hou and Dongli Wang consulted literatures and performed the experiment conduction and data analysis. Rong Li drafted the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Institutional Ethical Committee of Jiangsu University and conducted in accordance with ethical standards. Written informed consent was obtained from all patients prior to participation. For the animal study, all the procedures and care were in accordance with Guide for the Care and Use of Laboratory Animals and approved by the Animal Use Ethics Committee of Jiangsu University.

Consent for publication

Not applicable

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Figures

Figure 1

The prognostic and diagnostic value of CDR1as in GC tissues and circulating blood. a RT-qPCR analysis of CDR1as level in GC and matched noncancerous tissues from 87 patients. Data are presented as log₂ fold change and red (green) bar represents upregulation (downregulation). b The ROC curve analysis of CDR1as level in GC tissues. c RT-qPCR analysis of CDR1as level in GC tissues from GC patients with or without nerve invasion. d Kaplan-Meier analysis of the correlation of CDR1as expression level in GC tissues with overall survival of GC patients. e RT-qPCR analysis of CDR1as level in plasma from GC patients and healthy controls. f The ROC curve analysis of CDR1as level in GC plasma. (* $P<0.05$, ** $P<0.01$)

Figure 2

CDR1as promoted GC growth in vitro and in vivo. a GFP intensity in CDR1as stably overexpressing GC cells and corresponding control cells. The scale bar indicates 1000 μ m. b RT-qPCR analysis of CDR1as expression in CDR1as stably overexpressing cells and control cells. c, d CCK8 and colony formation assays of AGS and MKN-45 cells after CDR1as overexpression. The bar graph shows quantitative comparison of the number of colonies. e Western blot analysis of proliferation and apoptosis related

proteins. f The macroscopic appearance and size of dissected tumors. g HE staining and immunostaining of Bax expression of subcutaneous tumor tissues in OE-NC and OE-CDR1as groups. Red arrows indicate cells in division. (*P<0.05, **P<0.01, ***P<0.001)

Figure 3

CDR1as acted as a miRNA sponge for miR-299-3p. a RT-qPCR analysis of CDR1as level in the cytoplasm and nucleus of HGC-27 and MGC-803 cells. β -actin (U3) mRNA was used as positive (negative) control for

cytoplasm (nuclear). b RIP analysis of CDR1as in MGC-803 cells using antibodies against AGO2. The RIP enrichment was measured by RT-qPCR. FOS and U1 are positive control for antibodies against AGO2 and snRNP70 respectively. c, d RT-qPCR analysis of miR-7 and miR-135a-5p levels in GC and GCN tissues. e, f Pearson correlation analysis between the levels of CDR1as and miR-7/miR-135a-5p. g Dual luciferase reporter assay was used to evaluate binding properties between CDR1as and miRNA candidates. Ratio of firefly and Renilla luciferase activities are presented. h, j The colony formation assays and CCK8 assays were performed to evaluate proliferation ability of CDR1as overexpressing cells reversed by miRNA candidates after co-transfection. i The bar graph shows quantitative comparison of colony numbers. (*P<0.05, **P<0.01, ***P<0.001)

Figure 4

CDR1as upregulated TGIF1, a target of miR-299-3p. a, b The colony formation assays and CCK8 assays of MGC-803 and AGS cells transfected with miR-299-3p mimics. c RT-qPCR analysis of 10 potential targets expression levels in MGC-803 and AGS cells after miR-299-3p mimics transfection. d, e RT-qPCR analysis of 5 miR-299-3p targets expression levels in GC cells with CDR1as knockdown and overexpression. f-h, j Western blot analysis of TGIF1 protein level after miR-299-3p mimics transfection,

CDR1as knockdown and overexpression, and CDR1as co-transfection with miR-299-3p mimics. i RT-qPCR analysis of TGIF1 mRNA level in CDR1as overexpressing cells reversed by miR-299-3p after co-transfection. (*P<0.05, **P<0.01, ***P<0.001)

Figure 5

Knockdown of TGIF1 reserved CDR1as to suppress proliferation of GC cells. a RT-qPCR analysis of TGIF1 mRNA level in AGS and MGC-803 cells after transfection with NC and TGIF1 siRNAs. NC represents

negative control. b Western blot analysis for TGIF1, PCNA and Bax protein levels in AGS and MGC-803 cells with TGIF1 knockdown. c Western blot analysis of protein expression for TGIF1, PCNA, Bax in subcutaneous tumor tissues. d, e Colony formation assays and CCK8 assays of MGC-803 and AGS cells after TGIF1 knockdown. f, g Colony formation assays and CCK8 assays of CDR1as overexpressing GC cells co-transfected with TGIF1 siRNAs. (*P<0.05, **P<0.01, ***P<0.001)

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