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miR-106a* inhibits the proliferation of esophageal carcinoma cells by targeting CDK2-associated Cullin 1 (CACUL1)

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Abstract

Previous studies suggest that aberrant microRNA expression is common in plenty of cancers. The expression of miR-106a* was decreased in follicular lymphoma, but the expression and functions of miR-106a* in esophageal carcinoma (EC) remain unclear. In this study, we explored the expression and anti-oncogenic roles of miR-106a* in human EC. The expression of miR-106a* is significantly decreased in EC tissues and EC cell lines. Overexpression of miR-106a* suppressed EC cell proliferation, clonogenicity, G1/S transition, and induced apoptosis in vitro, but inhibition of miR-106a* facilitated cell proliferation, clonogenicity, G1/S transition. Luciferase reporter assay results showed that CDK2-associated Cullin 1 (CACUL1) was a direct target of miR-106a* in EC cells. Moreover, silencing CACUL1 resulted in the same biologic effects of miR-106a* overexpression in EC cells, which included suppressed EC cell proliferation, clonogenicity, and blocked G1/S transition through CDK2 pathway by inhibiting cell cycle regulators (Cyclin A, Cyclin E). Our data indicate that miR-106a* might play an anti-oncogenic role in EC by regulating CACUL1 expression, which suggest miR-106a* as a new potential diagnostic and therapeutic target for EC.

Key words: miR-106a*, esophageal carcinoma, proliferation, CDK2-associated Cullin 1, cell cycle.

Introduction

Esophageal carcinoma (EC) is one of the eight most common cancers worldwide and of the six most common causes of cancer death (1). EC includes two histological types, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) (2). The highest incidence rates are in Africa and East Asia, while the lowest rates are in the Central America (3,4). Although incidence of EC has been reduced in recent decades, 5-year survival and mortality have not changed significantly. The disease poses challenges in early diagnosis and prognosis, as well as for delivery and selection of optimal therapy modality. There is clearly need for better understanding of molecular characteristics of the disease, for identification of new molecular markers. Therefore, elucidating the potential molecular mechanism of EC development would help us understand the progression and the therapy.

MicroRNAs (miRNAs) are endogenous single-stranded non-coding RNA molecules ranging from 21 to 25 nucleotides in length which regulate gene expression by perfect or imperfect paring with target mRNAs, therefore inhibiting the translation and/or degrading the mRNAs (5,6). Plenty of studies indicate that miRNAs may function as tumor oncogenes or suppressors by regulating downstream target genes (7-9). miRNAs have been reported to participate in various important cellular processes, such as proliferation, survival, apoptosis, differentiation, tumor suppression and development (10,11). EC is a multifactorial disease caused by the complicated interaction between environmental factors and multiple genes. miRNA is correlated with changes in phenotypes of diseases (12). Some studies indicate that loss and gain of miRNAs may contribute to EC progression and the occurrence of malignant phenotypes. It is reported that some specific miRNAs were aberrantly expressed in EC and participated in biological processes by targeting different genes (13). miR-31 was reported to suppress EC by targeting p21 (14). The expression of miR-145 in ESCC stopped proliferation and invasion of tumor (15). miR-183 suppresses apoptosis and promotes proliferation in EC by targeting PDCD4 (16). Recent studies show that miR-106a* were decreased in follicular lymphoma, but there is no report on its role in EC (17).

In our study, we explored the anti-oncogenic role of miR-106a* in EC. The expression of miR-106a* in EC tissues was examined to disclose its role in the progression of the disease. Some analysis revealed that the expression of CDK2-associated Cullin 1 (CACUL1) was associated with miR-106a*. The findings of this study indicated the anti-oncogenic role of miR-106a* in EC by targeting CACUL1, particularly in the proliferation processes.

Materials and methods

Preparation of tissue specimens and cell lines

Human tissue specimens were collected from the

First Affiliated Hospital, College of Medicine, Xi'an Jiaotong University. Informed consent was obtained before specimen collection. The specimen collection was conducted in accordance with the guidelines of the National Institutes of Health. The experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University and followed the guidelines of the declaration of Helsinki. The human esophageal epithelial cells (Het-1A) and human EC cell lines (EC109, EC9706, TE-1, SEG-1, BIC-1) were maintained in the Key Laboratory of Environment and Genes Related to Diseases at Xi'an Jiaotong University College of Medicine. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, NY, USA) containing 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Quantitative real-time PCR analysis

Total RNA was extracted from human tissue specimens or cells using TRIzol reagent according to manufacturer's protocol (Invitrogen). RNA was reverse transcribed using SuperScript First-Strand cDNA System (Invitrogen). qRT-PCR analysis were conducted with Power SYBR Green (Takara). All protocols were performed according to the manufacturer's instructions. qRT-PCR reactions were carried out using an iQ Multicolor qRT-PCR Detection System (Bio-Rad, USA). The results were normalised to the expression ofβ-Actin or U6. The primer sequences were as follows: miR-106a* reverse-transcribed primer: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGTAAGAA-3′; miR-106a* forward: 5′-ATCCAGTGCGTGTCGTG-3′; miR-106a* reverse: 5′-TGCTCTGCAATGTAAGCAC-3′; U6 reverse-transcribed primer: 5′-CGCTTCACGAATTTGCGTGTCAT-3′; U6 forward: 5′-GCTTCGGCAGCACATATACTAAAAT-3′; U6 reverse: 5′-CGCTTCACGAATT TGCGTGTCAT-3′; CACUL1 forward: 5′- GCAGCATATTCAGAAAGTTCAGA-3′; CACUL1 reverse: 5′-CATTTACAGCCTAATGCCTTTACT-3′; β-Actin forward: 5′-TGGCACCCAGCACAATGAA-3′; β-Actin reverse: 5′-CTAAGTCATAGTCCGCCTAGAAGC A-3′.

Luciferase reporter assay

The 5′ untranslated region (5′-UTR) of human CACUL1 mRNA was constructed by synthetic oligonucletides and cloned in between the SacI and XhoI sites of the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, USA). PmirGLO-CACUL1-5′UTR vector was co-transfected with miR-106a* into HEK293 cell lines, with pmirGlO-vector as their control. Next, the cells were harvested and lysed for luciferase assays 24 h after transfection. Dual-Luciferase Assay System was utilized to measure the reporter activity according to the manufacturer's protocol.

Vector construction, miR-106a* inhibitor synthesis, siRNA synthesis and transfection

The miR-106a* expression vector (pre-miR-106a*) were constructed with synthetic oligonucleotides and cloned in between the EcoRI and HindIII sites of the pcDNA6.2-GW/EmGFP vector. The inhibitor of miR-106a* and small interfering RNA (siRNA) targeting CACUL1 were purchased from Gene-Pharma Corporation (SGC, Shanghai, China). The anti-miR-106a* sequence was: 5′-GUAAGAAGUGCUUACAUUGCAG-3′. Scramble siRNA was used as negative control (named anti-control). The anti-control sequence was: 5′-CAGU ACUUUUGUGUAGUACAA-3′. Human CACUL1 siRNA sense: 5′-GGAUGGUGCCAUAGAUCAAUU-3′, antisense: 5′-UUGAUCUAUGGCACCAUCCUU-3′; negative siRNA (NC-siRNA): sense 5′-UUCUCCGAACGUGUCACGUUU-3′, antisense: 5′-ACGUGACACGUUCGGAGAAUU-3′). siRNA oligonucleotide were synthesized and 1μg of siRNA mimic was transfected into cells by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's guidelines, and then diluted to desired transfection concentrations to be added to the plated cells in different experimental procedures.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbr-omide]- based assay was applied to detect the effect of CACUL1 on EC109 cell proliferation. Cells were seeded into 96-well plates (5,000 cells/well in 200 μl medium) and cultured for 24 h. These cells were treated with control vector, miR-106a*, anti-control, anti-miR-106a*, NC-siRNA or CACUL1 siRNA (60 nM) for 24, 48 and 72 h, respectively. At last, 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution was added per well and the cells were cultured for another 4 h at 37 °C. Supernatants were removed and formazan crystals were dissolved in 150 μl of dimethylsulfoxide (Sigma, St Louis, MO, USA). Optical density was determined using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). The results were collected as the mean of more than three independent experiments.

Cell cycle analysis

The EC109 cells were cultured in 12-well plates for 24 h and treated by control vector, miR-106a*, anti-control, anti-miR-106a*, NC-siRNA or CACUL1 siRNA (60 nM) for 48 h. Next, the cells were fixed in 75 % ethanol overnight at 4 °C. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 15 min at room temperature and detected by fluorescence-activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA, USA). Finally, the proportion of cells was calculated in G0/G1, S, and G2/M stages. The procedures were carried out in triplicate. Data obtained were presented as mean ± SEM.

Colony formation assay

Cells were transfected for 6 h. Then, stably transfected cells were seeded at a density of 5,000 per 12-well plate, incubated for 12 days, and stained with 0.5% crystal violet for 30 minutes. Excess dye was rinsed off three times with PBS. The number of colonies was counted by ImageJ.

Apoptosis analysis

Cells were cultured in 12-well plates for 24 h and treated by control vector, miR-106a*, anti-control, anti-

miR-106a*, NC-siRNA or CACUL1 siRNA (60 nM) for 48 h, then harvested and washed thrice with PBS. The cells were stained by 5 μL FITC-Annexin V and 10 μL PI at 250 μg/ml for 15 min at room temperature in the dark. The cells were washed with PBS and detected using flow cytometry. Quantification of apoptosis was determined by counting the number of cells stained by FITC-labeled Annexin V.

Western blot analysis

The human tissues and EC109 cells were lysed in RIPA lysis buffer. Protein were subjected to electrophoresis using 10 % SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 2 h in 5 % non-fat dry milk in TBST (10 mM Tris–HCl and 0.05 % Tween 20), incubated with primary antibodies overnight at 4 °C, and then incubated with secondary antibody for 2 h at room temperature. The primary antibodies included mouse monoclonal anti-CACUL1 (1:1000, GeneTex, USA), mouse monoclonal anti-CDK2 (1:1000, Santa Cruz, CA, USA), mouse monoclonal anti-Cyclin A (1:1000, Neomarker, Fremont, CA, USA), rabbit monoclonal anti-Cyclin E (1:1000, Santa Cruz, CA, USA), and mouse monoclonal anti-β-Actin (1:5000, Santa Cruz, CA, USA). The membranes were then incubated in the dark with ECL (Pierce) for chemiluminescence detection. The luminescent signal was detected by CCD camera, recorded and quantified with Syngene GBox (Syngene, UK).

Statistical analysis

Statistical analysis was performed with SPSS 18.0 software. Student's t test and one-way ANOVA were used according to the data characteristics. The quantitative data were presented as mean ± SEM. $P < 0.05$ was considered statistically significant. Each experiment was repeated at least 3 times independently.

Results

miR-106a* was decreased in human EC tissues and cell lines

To explore the role of miR-106a* in EC, we analyzed the expression of miR-106a* in 52 pairs of EC and adjacent normal tissues by qPCR. There were 88.5% (46/52) of EC tissues with downregulation of miR-106a* compared with the matched normal tissues, the expression of miR-106a* decreased to 56.3% in cancer tissues (Fig. 1A). We analyzed the expression of miR-106a* in 5 EC cell lines (EC109, EC9706, TE-1, SEG-1, BIC-1) and human esophageal epithelial Het-1A cells. The results showed that miR-106a* was downregulated in tumor cell lines, and the expression of miR-106a* was lowest in EC109 cells (Fig. 1B; $P < 0.01$). Therefore, EC109 cells were chosen to be used in the following experiments.

CACUL1 was a target gene of miR-106a* in human EC

Through PicTar, miRBase and TargetScan databases, we found matching bases between miR-106a* and the 5'UTR of CACUL1 (Fig. 2A). The results of Western blot and qRT-PCR showed that CACUL1 mRNA and protein significantly increased in EC tissues compared with their normal tissues (Fig. 2B, C). In order to determine whether miR-106a* regulates CACUL1, we performed a luciferase assay to evaluate the relationship between miR-106a* and CACUL1. We transfected the wild-type CACUL1-5′UTR construct into HEK293 cells in combination with miR-106a* or control. miR-106a* led to a reduction of luciferase activity of CACUL1-5′UTR construct compared to control ($P < 0.01$). After the conserved targeting regions for miR-106a* recognition were mutated, the relative luciferase activity of the reporter gene was restored (Fig. 2D). It indicates that miR-106a* can inhibit CACUL1 expression through its binding sequences at the 5′UTR. After transfected in EC109 cells with miR-106a* mimics, the results showed that miR-106a* expression significantly increased to 21.63 folds compared with control vector (Fig. 2E; $P < 0.01$). The CACUL1 mRNA and protein expression levels obviously decreaed to 0.43 folds and 0.58 folds in transfected cells with pre-miR-106a* vector compared with control vector (Fig. 2F, G; $P < 0.01$).

Overexpression of miR-106a* suppressed EC cell proliferation in vitro

MTT assays, cell cycle analysis, colony formation assay, and apoptosis analysis were performed. Our results showed that the overexpression of miR-106a* suppressed the proliferation of EC109 cells at 48 and 72 h after transfection (Fig. 3A; $P < 0.01$). We analyzed the cell cycles using a flow cytometer 48 h after transfection. The population of G1/G0 stage increased sig-

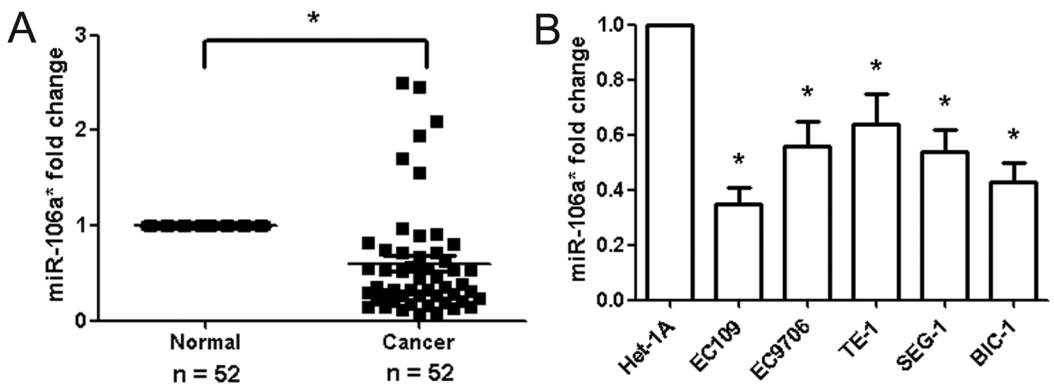

Figure 1. Expression of miR-106a* in Human EC Tissues and Cell Lines. (A) qRT-PCR was performed to examine miR-106a* expression in 52 paired human EC and normal tissues. (B) qRT-PCR was used to analysis of miR-106a* expression in esophageal epithelial cells and EC cell lines.

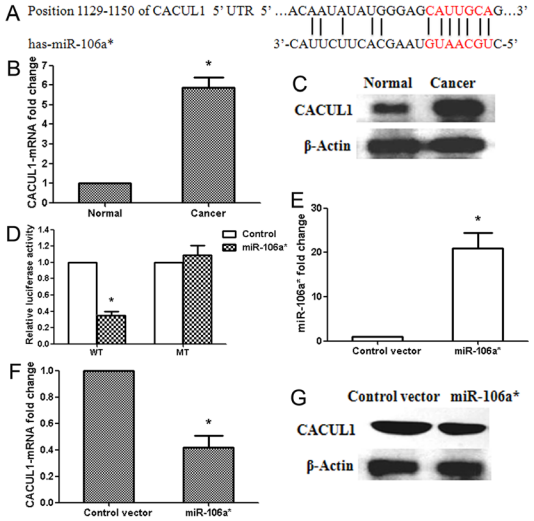

Figure 2. miR-106a* directly targets the CACUL1 gene in EC. (A) Bioinformatics predicted interactions of miR-106a* and their binding sites at the 5′-UTR of CACUL1. (B) CACUL1 mRNA expression in EC and normal tissues. (C) CACUL1 protein in EC and normal tissues were measured by Western blotting. (D) The luciferase reporter plasmid containing wild-type or mutant CACUL1 5′-UTR was cotransfected into HEK293 cells with miR-106a or control. Luciferase activity was determined by the dual luciferase assay. (E) The expression of miR-106a* was determined after transfected cells with pre-miR-106a* vector. (F) The level of CACUL1 mRNA was examined after miR-106a* treatments. (G) The expression of CACUL1 protein was analysed. β-Actin was used as a loading control. *P<0.01, n=3.

nificantly, and the population of S and G2/M stage decreased significantly in the miR-106a* overexpression group (Fig. 3B; $P < 0.01$). The colony formation assay results showed that miR-106a* inhibited colony formation compared with control vector-transfected cells (Fig. 3C). Next, we examined apoptosis by Annexin-V/PI staining. The results showed that the proportion of early apoptosis and late apoptosis increased in miR-106a* overexpression group (Fig. 3D; $P < 0.01$). These findings demonstrated that miR-106a* could inhibit the proliferation of EC109 cells in vitro. To further investigate the possible mechanisms of miR-106a* inhibiting cell proliferation, we detected the protein expression of CACUL1 and its downstream pathway regulators after transfection with pre-miR-106a*. It was found that miR-106a* could inhibite the expression of CACUL1 and CDK2 protein. We furtherly investigated the underlying mechanisms of cell-cycle regulation. miR-106a* could diminish the expression of Cyclin A and Cyclin E (Fig. 3E).

Inhibition of miR-106a promoted the proliferation of EC109 cells*

Human EC109 cells were transfected with miR-106a* antisense oligonucleotides (anti-miR-106a*) or control (anti-control). The results of MTT assay showed that anti-miR-106a* promoted the proliferation of EC109 cells (Fig. 4A; $P < 0.01$). The number of the G1/G0 phase cells decreased significantly in the anti-miR-106a* group compared to the anti-control group, meanwhile the number of the S phase cells remarkably increased (Fig. 4B; $P < 0.01$). In addition, anti-miR-106a* promoted cell clone formation compared to the anti-control (Fig. 4C; $P < 0.01$). There was no signifi-

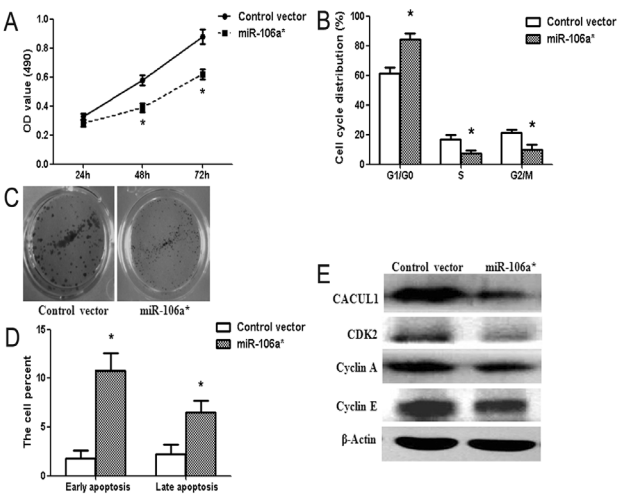

Figure 3. Effect of miR-106a* on EC109 cell progression in vitro. (A) Proliferation of EC109 cells was determined by an MTT assay at 24, 48 and 72 h after transfection with miR-106a*. (B) The results of flow cytometry analysis of the cell cycle in EC109 cells were visualized via PI staining. The data show the percentage of cells in the G1/G0, S and G2/M phases. (C) Results of tablet colony formation after transfection with miR-106a*. (D) The results of apoptosis were visualized using Annexin-V/PI staining. The data show the percentage of early apoptosis and late apoptosis. (E) The expression analysis for CACUL1/CDK2 pathway regulation proteins at 48 hours after transfection with pre-miR-106a* by Western blot analysis. *P<0.01, compared with control vector group, n=3.

cant difference among the proportion of early apoptosis and late apoptosis of EC109 cells in different treatment groups after anti-miR-106a* treatments (Fig. 4D). In addation, the inhibition of miR-106a* upregulated the expression of CACUL1, CDK2, Cyclin A and Cyclin E.

Silencing of CACUL1 produced similar effects to that of miR-106a overexpression in EC cells*

Then, we silenced CACUL1 expression by RNA interference to determine whether CACUL1 is involved

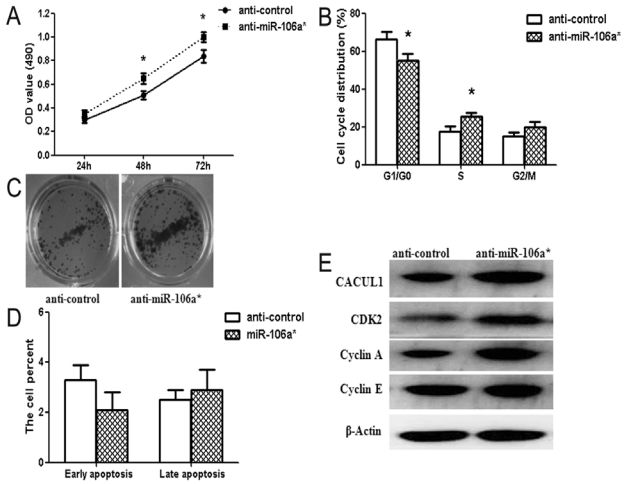

Figure 4. Inhibition of miR-106a* promotes the proliferation of EC109 cells. (A) MTT assay showed that anti-miR-106a* increased the activity of EC109 cells at 48 and 72 h. (B) The results of flow cytometry analysis show the percentage of cells in the G1/G0, S and G2/M phases. (C) The results of colony formation after transfection with anti-miR-106a*. (D) The data show the percentage of early and late apoptosis. (E) The expression analysis for CACUL1/CDK2 pathway regulation proteins after transfection with anti-miR-106a*. *P<0.01, compared with anti-control group, n=3.

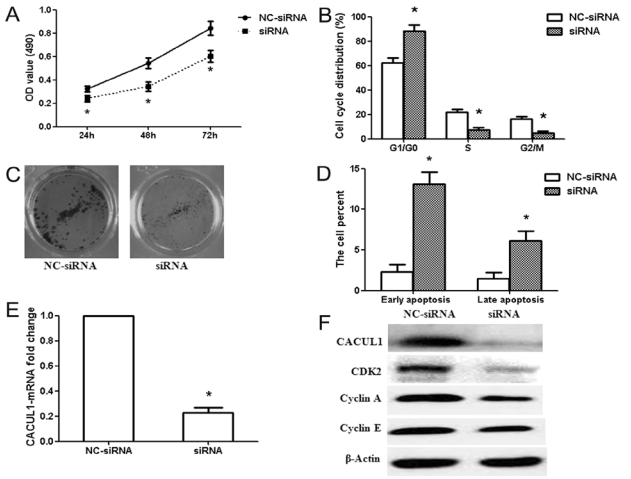

Figure 5. Silencing of CACUL1 could inhibit EC cell proliferation in accordance with miR-106a*. (A) MTT assay showed that CACUL1-siRNA (60 nM) decreased the activity of EC109 cells at 24, 48 and 72 h. (B) The results of flow cytometry analysis show the percentage of cells in the G1/G0, S and G2/M phases. (C) The results of colony formation after transfection. (D) The data show the percentage of early late apoptosis. (E) The CACUL1 mRNA expression was determined after transfection with CACUL1-siRNA. (F) The expression analysis for CACUL1/CDK2 pathway regulation proteins in EC109 cells at 48 h after transfection with CACUL1-siRNA. *P<0.01, compared with NC-siRNA group, n=3.

in the antitumor effects of miR-106a*. As shown in Fig. 5A-D, silencing of CACUL1 resulted in suppressed cell proliferation, induced G1/G0 phase arrest, inhibited colony formation and promoted cell apoptosis which followed the same trend as miR-106a* in human EC109 cells. From mRNA and protein expression levels, CACUL1 can be specifically knocked down to 0.23 folds and 0.16 folds by siRNA in EC109 cells (Fig. 5E, F). In addition, we analyzed the proteins expression in the CDK2 pathway. Our results showed that CACUL1-siRNA could decrease the expression of CDK2, Cyclin A and Cyclin E (Fig. 5F).

Discussion

In the last decade, miRNAs have been shown to have important regulatory roles in cancer biology (18,19). It is already reported that miRNAs can regulate cell survival, proliferation, apoptosis and invasion in EC development and progression (15,16,20,21). Therefore, miRNAs are increasingly viewed as a potential diagnostic and therapeutic tool. At present, only a small proportion of identified miRNAs have been investigated to elucidate their important roles in EC. Identifying miRNAs specifically involved in EC will help in developing new targets for diagnosis and therapy. It is reported that the expression of miR-106a* decreased in follicular lymphoma. The aim of the present work is to elucidate the biological functions of miR-106a* in EC. Our results showed that miR-106a* was frequently downregulated in both EC tissues and cell lines. Overexpression of miR-106a* inhibited EC cell proliferation and cell viability by blocking cell-cycle transition in vitro. These data suggested that miR-106a* might act as a tumor suppressor in EC. The role of miRNAs in each different cell line is dependent on the specific target gene of the miRNA (22-24). Therefore, a single miRNA may display an opposite role in a different cell line. Thus, identifying the target gene of miRNA is considered to be critical. We investigated potential targets of miR-106a*. CACUL1 was postulated to be a target of miR-106a* using different databases. As indicated on reporter assaying, miR-106a* repressed the construct with the CACUL1 5′-UTR. Overexpression of miR-106a* inhibited the expression of CACUL1 mRNA and protein; miR-106a* overexpression suppressed CACUL1 5′-UTR luciferase report activity and this effect was abolished by mutation of the miR-106a* seed binding site. The results suggest that miR-106a* may play the critical role of tumor suppressor for cell proliferation partly mediated by repressing CACUL1 expression in EC.

CACUL1 is a novel gene identified in colorectal carcinoma (25). It has been mapped to human chromosome 10q26.1. CACUL1 is highly conserved in mammals and other species, suggesting that it might play a critical role in cell biology. It is reported that the expression of CACUL1 increased significantly in colorectal cancer (25), gastric cancer (26) and lung cancer (27), implicating a causal role of CACUL1 in promoting tumor progression. We analyzed expression of CACUL1 mRNA and protein in EC samples using quantitative real-time PCR and western blot. Interestingly, CACUL1 expression was significantly upregulated in EC samples as compared with normal tissues, which was consistent with previous findings in cancer tissues(25-27). CACUL1 is a novel cell cycle-associated protein capable of promoting cell proliferation (25,28). Although CACUL1 has been reported to play a role in colorectal cancer and lung cancer, but it is still not clear about the biological functions of CACUL1 and the mechanisms in EC.

Although CACUL1 plays potent proliferative role in different steps of oncogenesis, there still exist controversies about regulatory mechanism. CACUL1 is activated by multiple extracellular signals, including growth factors and mitogens, leading to the activation of cyclin-dependent kinase 2 (CDK2), which can regulate cell proliferation and allows progression from the G1 to S phase of the cell cycle (21). siRNA is a popular reverse genetic tool which inhibits gene expression through sequence-specific degradation of a target mRNA (29-31). It is reported that CACUL1 knockdown by RNAi inhibits cell proliferation by blocking G1-to-S cell cycle progression in gastric cancer and lung cancer (26,27). The results showed that miR-106a* induce G1-phase arrest by targeting CACUL1 in EC cells. To further reveal the functions of CACUL1 in EC, we silenced CACUL1 by synthetic CACUL1 siRNA and observed that CACUL1 siRNA-treated EC109 cells displayed significant reduction of CACUL1 mRNA and protein level, consequently resulting in inhibited cell proliferation and colony formation, blocked G1/S transition in EC cells. These findings demonstrated that miR-106a* and its target gene CACUL1 share similar cellular and molecular effects in human EC109 cells.

It is reported that CACUL1 was involved in CDK2 pathway in colorectal cancer and gastric cancer. This pathway has been implicated in promoting tumor cell survival and proliferation. In this study, our results showed that miR-106a* and CACUL1 silencing suppressed EC cell proliferation through inhibiting the

expression of CDK2. Further experiments revealed that CACUL1 expression was positively correlated with cell cycle-related protein CDK2, Cyclin A and Cyclin E in EC cells. These findings are concordance with previous results (25,26). The findings suggested that increased CACUL1 may drive more cells crossing G1/S node and entering into cell cycle via CDK2 pathway in the proliferative progression of EC.

In conclusion, we investigated the role of miR-106a*, its targeted gene CACUL1, and their potential implication in pathologic processes of EC. We found that miR-106a* inhibites the proliferation of EC cells by down-regulating CACUL1 expression. Our findings can be the basis for further analysis of miR-106a* and CACUL1 to develop a new potential diagnostic and therapeutic target for EC screening and treatment.

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