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MiR-1271 Inhibits Ovarian Cancer Growth by Targeting Cyclin G1

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AG **Xiaogang Liu**
BC **Lihong Ma**
DE **Qinhua Rao**
EF **Yuhui Mao**
BC **Yuhong Xin**
DE **Huiqin Xu**
BC **Changju Li**
CD **Xiaoyan Wang**

Department of Gynaecology and Obstetrics, People's Hospital of Yuxi, Yuxi, Yunnan, P.R. China

Corresponding Author: Xiaogang Liu, e-mail: bagebageliu@163.com
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Background: Ovarian cancer is the most lethal gynecological malignant cancer in the female genital system. The dysfunction of miRNA contributes to ovarian cancer development.

Material/Methods: The miR-1271 level in ovarian cancer tissues and cells was assayed by qRT-PCR. The miR-1271 expression in cells was overexpressed by miRNA-mimic transfection and reduced by miRNA-antisense-oligonucleotide (ASO) transfection. Cell proliferation was analyzed by an MTT assay. The targeted genes were predicted by a bioinformatics algorithm and confirmed by a dual luciferase reporter assay. The protein level was assayed by Western blotting.

Results: The ovarian cancer tissue and cell lines showed low levels of miR-1271. Low levels of miR-1271 in ovarian cancer tissues were correlated with a low rate of patient survival, and the overexpression of miR-1271 inhibited the proliferation of ovarian cancer cells. The 3' UTR of cyclin G1 (CCNG1) was targeted by miR-1271.

Conclusions: Low levels of miR-1271 in ovarian cancer tissues promoted cancer cell growth. MiR-1271 may be a new predictor of prognosis in ovarian cancer. MiR-1271 exerted its role by targeting CCNG1.

MeSH Keywords: **Cell Growth Processes • MicroRNAs • Ovarian Neoplasms**

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Background

Ovarian cancer is the most lethal malignant gynecological cancer in the female genital system[1]. The management of ovarian cancer generally consists of surgery followed by chemotherapy [2–5]. Although surgery and platinum-based chemotherapy currently provide the cornerstone of standard ovarian cancer management pathways, most of these patients ultimately develop chemoresistance and recurrence [6]. New therapeutic strategies are urgently required to improve outcomes. To develop an effective ovarian cancer treatment, the elucidation of the molecular pathogenesis of ovarian cancer is required.

MiRNAs are endogenous non-coding, single-stranded small regulatory RNA molecules of approximately 22 nucleotides in length [7,8]. MiRNAs deregulation or dysfunction affects cancer development [9–14]. A number of miRNAs have been identified as highly up- or down-regulated in ovarian cancer [15–21]. However, their roles in the pathogenesis of ovarian cancer remain unclear, and other potential miRNAs involved in the pathogenesis of ovarian cancer remain unknown.

A previous study revealed that a 10-microRNA signature was able to distinguish human ovarian cancer tissues from normal tissues with 97% sensitivity and 92% specificity, including miR-1271[22]. Another study showed that miR-1271 acts as tumor suppressor in oral squamous cell carcinoma and inhibits oral squamous cell carcinoma growth and metastasis by targeting ALK [23].

In this study, we investigated the function of miR-1271 in ovarian cancer, and found that miR-1271 plays an important role in the pathogenesis of ovarian cancer. Our data may provide a potential therapeutic target in ovarian cancer.

Material and Methods

Patients and specimens

Surgical specimens from 18 ovarian cancer patients were obtained postoperatively in 2010 from the Department of Gynecology and Obstetrics at the People's Hospital of Yuxi. Every patient provided written informed consent for use of their tissues. The clinical information was listed in the Supplementary Table 1. The ethics of this study were approved by Yuxi Hospital (Yuxi, China). Diagnoses were made pathologically and/or cytologically. The specimens were histologically evaluated by senior pathologists according to the classification criteria of the World Health Organization. The tissues were collected before chemotherapy and radiotherapy, and then were frozen and stored at –80°C until qRT-PCR assay.

Cell culture and transfection

Human ovarian cancer cell line SKOV3, HO 8910 cell lines, and the HEK293 cell line were purchased from the Cell Bank of the Sichuan University (Chengdu, China). DMEM medium (Hyclone, South Logan, UT, USA) was used to culture the SKOV3, HO 8910, and HEK293 cells, being supplemented as previously described with 2 mM L-glutamine, 10% fetal bovine serum (Hyclone, South Logan, UT, USA), and 100 µg/mL penicillin/streptomycin (Bio Light, Shanghai, China) [24].

Real-time quantitative PCR

Real-time quantitative PCR for miR-1271 was performed with standard protocols using an Applied Biosystems 7500 HT sequence Detection System by the Shengong Company (Shanghai). The expression of miR-1271 was evaluated using a mirVanaTM qRT-PCR miRNA Detection Kit (Ambion, USA). The primers were designed and synthesized by the Shengong Company (Shanghai). U6 was used as an internal control.

MTT assay for cells proliferation

For the MTT assay, 5×10^3 cells per well were seeded in triplicate with complete growth medium in a 96-well plate. The number of cells was counted for 5 days via the previously described MTT assay (Promega, Fitchburg, WI, USA) [25–27]. The data were measured using a microtiter plate reader with a 570-nm filter (Promega, Fitchburg, WI, USA).

MiRNA mimics, miRNA ASO and transfection

MiR-1271 mimic, miR-1271 ASO, and negative control were purchased from Qiagen (Venlo, Limburg, The Netherlands). The miRNA ASO, miRNA mimic, and negative control (NC) were transfected according to the manufacturer's instructions at 50 nM with Lipofectamine 2000 (Invitrogen, Canada) transfection reagent. After 48 h, the cells were collected [28].

MicroRNAs targets prediction by algorithm

The online software, TargetScanHuman (*http://www.targetscan.org/vert_61/*) [29–32] was used to identify the potential targets of miR-1271.

Luciferase reporter assay

The 3'UTR fragments of CCNG1 containing putative binding sites for miR-1271 were cloned into pMIR-Report construct (Ambion, Austin, TX). The primers (Biomart, Shanghai, China) were constructed according to previous reports [33,34]. Mutant 3'UTR of CCNG1, which carried a mutated sequence in the complementary site for the seed region of miR-1271, were generated

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Supplementary Table 1. Characteristics of ovarian cancer patients.

No	Age	Type	Stage	Follow-up time (days)	Status
1	56	Epithelial ovarian cancer/serous	III	460	Dead
2	59	Epithelial ovarian cancer/serous	III	479	Alive
3	57	Epithelial ovarian cancer/serous	III	640	Alive
4	36	Epithelial ovarian cancer/serous	IV	2000	Alive
5	39	Epithelial ovarian cancer/serous	III	1560	Dead
6	45	Epithelial ovarian cancer/serous	III	2000	Alive
7	57	Epithelial ovarian cancer/serous	III	2000	Alive
8	45	Epithelial ovarian cancer/serous	IV	2000	Alive
9	59	Epithelial ovarian cancer/serous	IV	120	Dead
10	75	Epithelial ovarian cancer/serous	IV	345	Dead
11	60	Epithelial ovarian cancer/serous	IV	479	Dead
12	58	Epithelial ovarian cancer/serous	IV	421	Dead
13	69	Epithelial ovarian cancer/serous	III	563	Dead
14	59	Epithelial ovarian cancer/serous	IV	743	Dead
15	36	Epithelial ovarian cancer/serous	III	1345	Dead
16	39	Epithelial ovarian cancer/serous	IV	1834	Dead
17	45	Epithelial ovarian cancer/serous	III	2000	Alive
18	44	Epithelial ovarian cancer/serous	III	2000	Alive

using the fusion PCR method. Luciferase reporter assay was performed in HEK293 cells as described previously [12].

Western blot and antibodies

Tumor tissues were collected, lysed, and blotted as previously described [11]. Membranes were blocked with blocking solution (5% skim milk in TBST), and 30 min later, membranes were incubated with the primary antibody, followed by incubation with the appropriate HRP-conjugated antibody. The CCNG1 antibody (anti-CCNG1) was purchased from Santa Cruz Biotechnology, Inc. The densitometry of the Western blotting results was measured using ImageJ software.

Statistical analysis

The data are the mean ± s.d. from 3 independent experiments. The patient survival was evaluated using Kaplan-Meier analysis. When only 2 groups were compared, the difference between them was analyzed using a 2-tailed Student's t test. However, when 3 or more groups were compared, the difference between them was analyzed using ANOVA. Statistically significant differences in the expression of miR-1271 between matched pairs were detected by the Wilcoxon matched-pairs signed rank test. SPSS software (version 17.0) was used to perform statistical analyses, in which $P<0.05$ was significant.

Results

A low miR-1271 level in ovarian cancer tissues was correlated with a low survival rate.

We assayed the miR-1271 levels in the ovarian cancer tissues and matched tumor-adjacent normal tissues from 18 patients and found that 16 of the 18 tissues showed lower levels of miR-1271 in cancer tissues than in the matched tumor-adjacent normal tissues (Figure 1A). The mean miR-1271 expression level in ovarian cancer tissues was lower than that in normal tissues (Figure 1B). To determine the clinical significance of miR-1271, we investigated whether the level of miR-1271 was associated with the overall patient survival. The median values of all 18 cases were used as the cutoff to separate cases of high and low miR-1271 expression. Those patients with a low expression of miR-1271 showed poor prognoses (Figure 1C).

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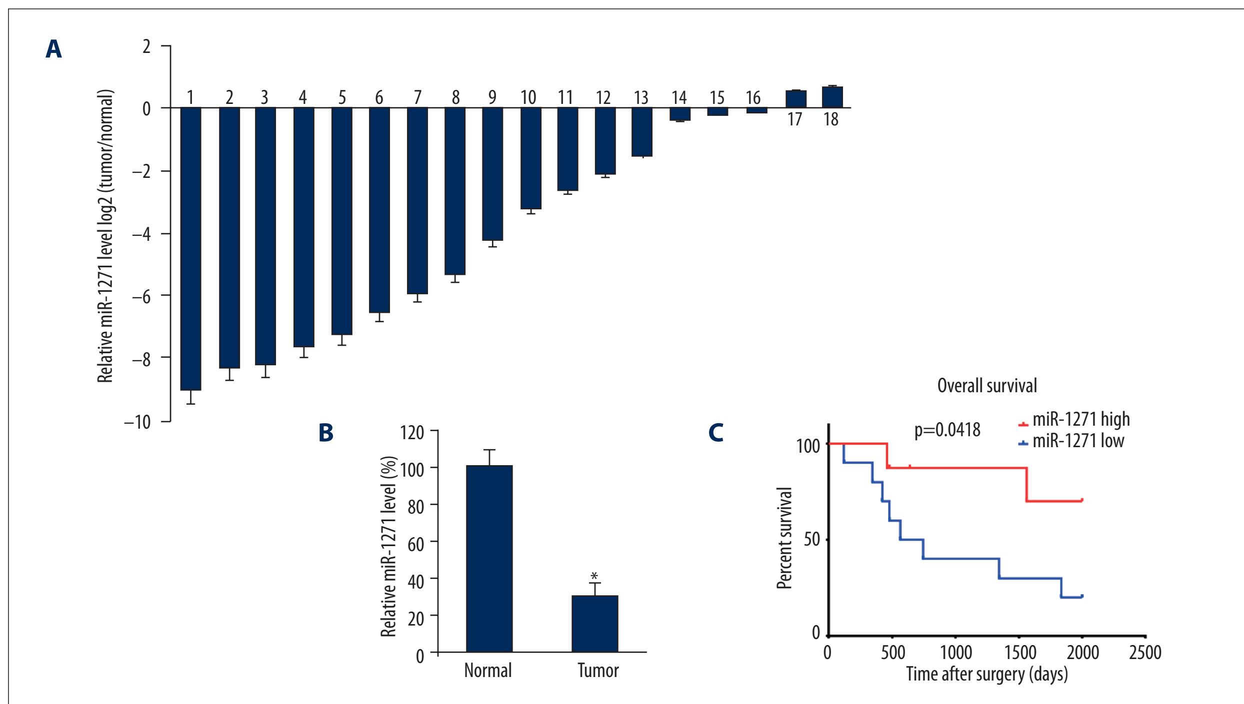

Figure 1. Low miR-1271 level in ovarian cancer tissues were correlated with a low rate of patient survival. The miR-1271 levels in 18 ovarian cancer tissues and matched tumor-adjacent normal tissue specimens were analyzed using qRT-PCR. The difference in the expression of miR-1271 between these tissues was compared (**A**). The mean expression of miR-1271 in ovarian cancer tissues and matched tumor-adjacent normal tissue was calculated. The miR-1271 level in tumor tissues was arbitrarily defined as 100% (**B**). The Kaplan-Meier plot shows the overall survival in post-operative ovarian cancer patients according to the expression of miR-1271. The median values of all 18 cases were used as the cutoff to separate cases of high and low miR-1271 expression (**C**). The qRT-PCR experiments were performed in triplicate, and the data are the mean ±s.d. of 3 separate experiments; * $P<0.05$.

The overexpression of miR-1271 inhibited ovarian cancer cell growth

We also studied the role of miR-1271 in ovarian cancer cell lines. We assayed the miR-1271 levels in SKOV3 and HO8910 cells, while the miR-1271 levels in normal ovarian and HEK293 cells were used as controls. SKOV3 and HO8910 cells showed lower miR-1271 levels (Figure 2A). We overexpressed miR-1271 using miR-1271-mimic transfection, and the transfection efficiency was evaluated by qRT-PCR (Figure 2B). Then, an MTT analysis was used to analyze the cellular proliferation, and we found that the transfection of miR-1271 mimic inhibited the proliferation of SKOV3 and HO8910 cells (Figure 2C).

The suppression of miR-1271 promoted ovarian cancer cell growth

Next, we suppressed miR-1271 levels via the transfection of miR-1271 ASO. Then, 48 h post-transfection, the miR-1271 levels in SKOV3 and HO8910 cells were assayed by qRT-PCR, and we found that miR-1271 ASO inhibited the miR-1271 levels in SKOV3 and HO8910 cells (Figure 3A). Similarly, an MTT analysis was performed to analyze cellular proliferation following miR-1271 ASO transfection, revealing that the transfection of miR-1271 promoted cell proliferation (Figure 3B).

CCNG1 is a targeted gene of miR-1271

To investigate the mechanisms of miR-1271, a bioinformatics algorithm was used to predict many potential target genes of miR-1271 (data not shown), including CCNG1. CCNG1 is a cell-cycle-regulatory protein that is frequently overexpressed in malignant tissues [35]. Due to the importance of CCNG1 in cancers, the CCNG1 was chosen. The miR-1271 and CCNG1 binding sites are shown in Figure 4A. The intact and mutated 3'UTRs of CCNG1 were cloned into a luciferase reporter plasmid that was used for its co-transfection with miR-1271 into HEK293 cells. miR-1271 decreased the luciferase activity of the wild-type 3' UTR reporter, while that of the mutated reporter was not significantly affected (Figure 4B). Western blotting was performed to confirm the relationship between miR-1271 and CCNG1 at the protein level. SKOV3 cells were transfected with miR-1271 mimic; after 48 h, the CCNG1 protein level was assayed by Western blotting. The CCNG1 protein level was inhibited 48 h after miR-1271-mimic transfection, indicating that CCNG1 was targeted by miR-1271 (Figure 4C).

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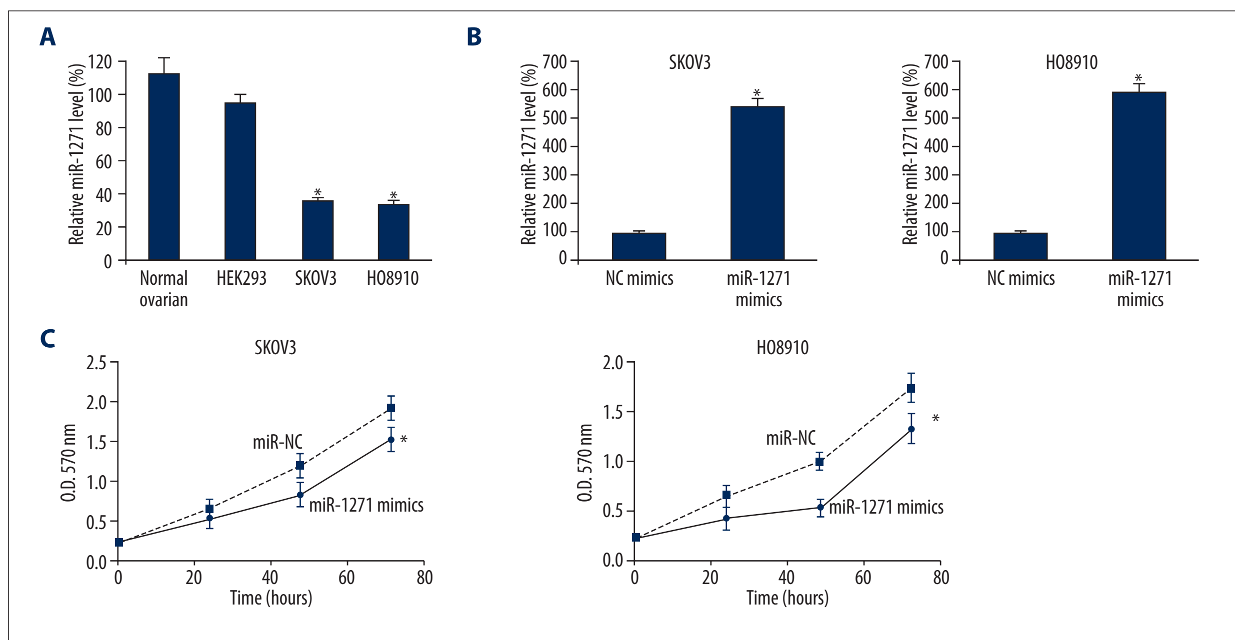

Figure 2. MiR-1271-mimic transfection inhibited the proliferation of ovarian cancer cells. The miR-1271 levels in normal ovarian tissues and in HEK293, SKOV3, and HO8910 cells were assayed by qRT-PCR. The miR-1271 level in normal ovarian tissues was arbitrarily defined as 100% (**A**). The miR-1271 expression level in SKOV3 and HO8910 cells was assayed by qRT-PCR 48 h after miR-1271-mimic transfection. The miR-1271 level in the miR-NC group was arbitrarily defined as 100% (**B**). After miR-1271-mimic transfection, an MTT analysis was performed to analyze the cellular proliferation at the indicated time (**C**). All of the experiments were performed in triplicate, and the data are the mean ±s.d. of 3 separate experiments; * P<0.05.

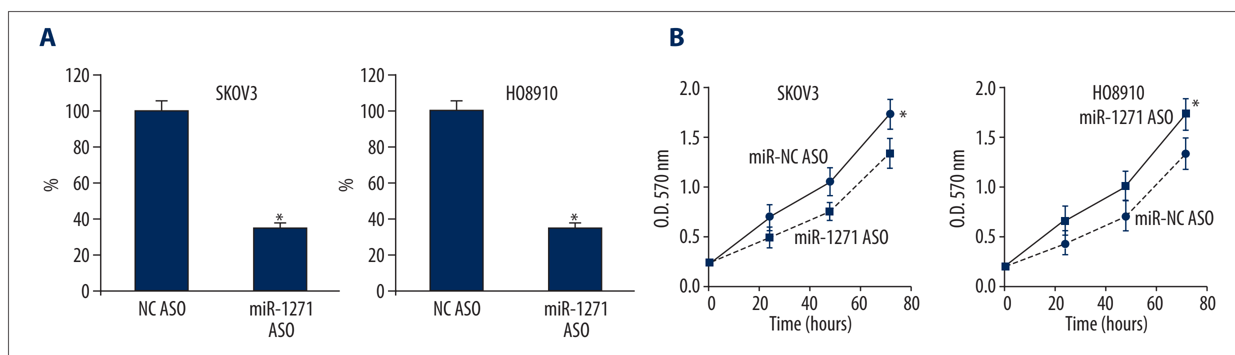

Figure 3. MiR-1271 ASO transfection inhibited the proliferation of ovarian cancer cells. The miR-1271 expression level in SKOV3 and HO8910 cells was assayed by qRT-PCR 48 h after miR-1271-ASO transfection. The miR-1271 level in the miR-NC ASO group was arbitrarily defined as 100% (**A**). After miR-1271 ASO transfection, an MTT analysis was performed to analyze the cellular proliferation of SKOV3 and HO8910 cells at the indicated time (**B**).

Discussion

Increasing evidence suggests that miRNAs are frequently dysregulated in various cancers, including ovarian cancer [36]. In the present study, we found that miR-1271 was down-regulated in ovarian cancer cell lines and tissues. Furthermore, we showed that low levels of miR-1271 in ovarian cancer tissues were correlated with low survival rate of HCC patients. Overexpression of miR-1271 inhibited the proliferation of ovarian cancer cells and suppression of miR-1271 promoted the cell growth. 3' UTR of CCNG1 was bound by miR-1271. Accordingly, we concluded that miR-1271 inhibited ovarian cancer cells growth by targeting CCNG1, and the low level of miR-1271 in ovarian cancer tissues promoted ovarian cancer cells growth, which in turn contributed to the lower survival rate of ovarian cancer patients.

Our data revealed the anti-tumor role of miR-1271 in ovarian cancer, and showed that miR-1271 can be used as a prognosis

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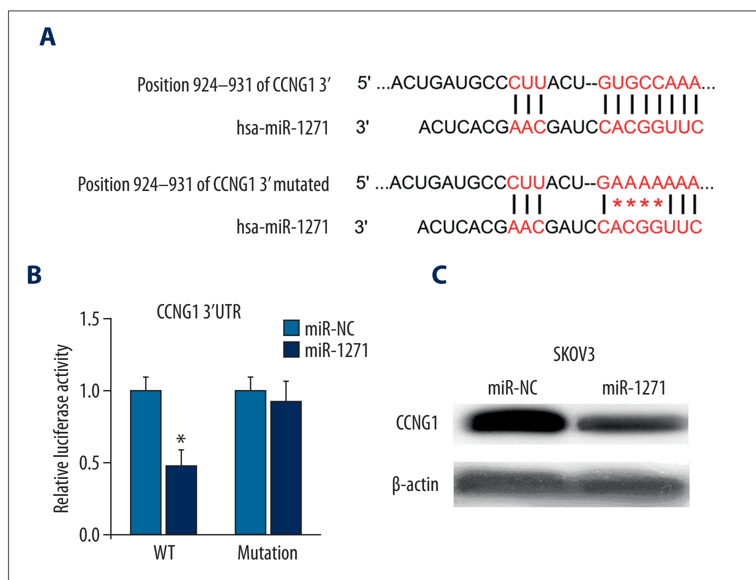

Figure 4. Prediction and confirmation of target genes of miR-1271. TargetScanHuman was used to predict the putative targeted genes; these genes, along with their binding site and the mutated site of miR-1271, are shown (**A**). RL reporter plasmids (RL-control, RL-CCNG1, and RL-mutated CCNG1) and either miR-1271 or miR-NC were co-transfected into SKOV3 cells with a firefly luciferase reporter (pGL control) for normalization. The luciferase activity was measured after 48 h. The ratio of RL activity to firefly luciferase activity in the miR-1271-treated group was then calculated and compared to that in the miR-NC group, which was arbitrarily defined as 100% (**B**). The CCNG1 protein levels were assayed by Western blotting 48 h after miR-1271-mimic transfection into SKOV3 cells (C). The data are the mean ±s.d. of 3 separate experiments; * $P<0.05$

predictor. To the best of our knowledge, this is the first report showing the function of miR-1271 in ovarian cancer.

Because miR-96 shared similar sequences with miR-1271 [37], we guessed that miR-96 may play a similar role in miR-1271. The role of miR-96 in ovarian cancer will be investigated in our next study.

By informatics prediction and reporter gene assay, CCNG1 was identified as the targeted gene of miR-1271. CCNG1 is usually overexpressed in human tumor cells, suggesting CCNG1 is an oncogenic protein [38,39]. Another study suggested a different role of CCNG1, reporting that CCNG1 showed an inhibitory role in hepatocellular carcinoma [35]. In this study, we found that CCNG1 could be inhibited by miR-1271; therefore, we guessed that CCNG1 may be present at high levels in ovarian cancer. We will investigate the role of CCNG1 in ovarian cancer in our next study.

In this study, we examined the role of miR-1271 in ovarian cancer. However, many relevant questions remain that deserve further study. For example, the most frequently de-regulated miRNAs in ovarian cancer are members of the let-7 and miR-200 families, both involved in epithelial-mesenchymal transition (EMT) [40]. The E-cadherin transcriptional repressor, ZEB1, is the EMT-inducing transcriptional factor. ZEB1 represses E-cadherin expression and promotes cancer cell migration and invasion [41–44]. We also found ZEB1 was a target of miR-1271. Thus, we wondered if miR-1271 may be involved in EMT.

Conclusions

Our data show that low levels of miR-1271 promote ovarian cancer cells growth, and that miR-1271 exerts its role through CCNG1. We hope our results will provide useful information and a potential therapeutic target.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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