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MiR-1182 inhibited metastasis and proliferation of ovarian cancer by targeting hTERT

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Abstract. – OBJECTIVE: To investigate the potential effect of miR-1182 on the development of ovarian cancer and the relevant mechanism.

PATIENTS AND METHODS: The expression levels of miR-1182 were detected in ovarian cancer tissues and cells (SKOV3) comparing with corresponding adjacent normal tissues and normal ovarian cell (IOSE80). Luciferase assay was performed to evaluate the interaction between miR-1182 and hTERT. The effects of the miR-1182/hTERT axis on SKOV3 cells were determined by subsequent experiments including cell proliferation, expression level of hTERT, the invasion and metastasis detection.

RESULTS: miR-1182 was found repressed in ovarian cancer tissues and we got the same results at the cellular level. In order to research potential target of miR-1182, we checked it in three publicly available algorithms, TargetScan, miRDB and microRNA. We found that hTERT is a direct target of miR-1182, and luciferase assays confirmed our hypothesis. The subsequent experiments showed that decreased expression of hTERT resulting from the up-regulation of miR-1182 could decelerate cell proliferation, invasion, and metastasis in ovarian cancer cells.

CONCLUSIONS: Our research discovers the suppressor function of miR-1182 in ovarian cancer by targeting hTERT, revealing that miR-1182/hTERT axis may be a potential therapeutic target for the treatment of ovarian cancer.

Key Words:
miR-1182, Eukaryotic release factor, G1 to S phase transition 1 (hTERT), Ovarian cancer.

Introduction

Ovarian cancer is one of the most common gynecologic malignant tumors, whose mortality rate ranks first in gynecological malignant tumors, making it the major tumor that seriously affects the life and health of women[1]. Ovarian cancer has strong malignant biological behaviors, and the intraperitoneal planting metastasis occurs easily[2]. Due to the hidden early symptoms of ovarian cancer, it is not easy to be found, and most patients have been in the late stage when diagnosed, leading to poor prognosis[3,4]. The average 5-year survival rate of patients with advanced ovarian cancer is only about 15-20% after appropriate treatment[5]. The etiology and pathogenesis of ovarian cancer remain unclear yet, so the investigation of the pathogenesis of ovarian cancer on the gene level has always been the emphasis in ovarian cancer research.

MicroRNA (miRNA) is a type of small molecule non coding RNA that binds to three prime untranslated region (3'-UTR) of the target messenger RNA (mRNA) to achieve post-transcriptional level control of gene expression. Increasingly more studies have shown that miRNA may be closely related to the occurrence and development of tumors, and may be involved in the regulation of the biological characteristics of tumor cells, such as proliferation, apoptosis, invasion and metastasis, playing a similar role to tumor suppressor gene or oncogene, which suggests that miRNA may become a target for the cancer treatment[6]. Studies have shown that the abnormality in miRNA expression level is related to the histological subtypes of ovarian cancer, tumor staging and differentiation degree, primary or metastatic tumor, primary onset or relapse, BRCA gene mutation, epigenetic changes, etc., which are the important factors determining the prognosis of patients with ovarian cancer[7-10]. Therefore, miRNA in ovarian cancer has also become one of the research hotspots. MicroRNA 1182 (miR-1182) is an important component of the microRNA regulatory network. Lately, it has been widely certified that miR-1182 exert critical regulatory effects on many diseases[11,12]. However, the effects of miR-1182 on the ovarian cancer

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have not been researched yet. This experiment aimed to detect the functions of miR-1182 axis in the occurrence and progression of OC, so as to provide new ideas and theoretical basis for the clinical treatment and prevention of OC.

Patients and Methods

Ovarian Cancer Cases and Cells

This study included 50 patients definitely diagnosed with OC by pathology undergoing a surgical procedure in Zhongnan Hospital of Wuhan University (Wuhan, China). Preoperative chemotherapy or radiotherapy treatment was forbidden. The liquid nitrogen was used to freeze OC tissues, and corresponding adjacent normal tissues were kept at -70°C refrigerator. The adjacent normal tissues underwent biological biopsy to get rid of cancer cells. This study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China). Signed written informed consents were obtained from all participants before the study. The human ovarian cancer line SKOV3 together with normal ovarian cell line IOSE80 were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) complemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 IU/mL penicillin (Thermo Fisher Scientific, Inc. Waltham, MA, USA) in 5% CO_2 cell culture incubator.

Luciferase Reporter Assays

In TargetScan, miRDB and microRNA websites, it was found that hTERT is the target gene of miR-1182. The binding sequence of miR-1182 at the 3'-end of hTERT was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA), and the mutated hTERT (Mut-type) and non-mutant hTERT (WT-type) were connected with the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). PGL3-basic vector with mutant hTERT was transfected into SKOV3 cells after lentivirus intervention on the 24-well plate. The same treatment was performed on the pGL3-basic vector connected with the non-mutant hTERT according to steps in the luciferase reporter gene assay kit. Then, the luciferase activity was detected in a multi-function microplate reader.

Transfection

MiR-1182 mimics control and inhibitor were synthesized and transfected to OC cell line to analyze biological function of miR-1182. Then, three groups were established to study the potential relevance between miR-1182 and SKOV3 cell: NC group (negative control), miR-1182 mimics (SKOV3 cell transfected by miR-1182 mimics) and mimics + hTERT (SKOV3 cell transfected by miR-1182 mimics and si-hTERT). All the products and reagents were purchased form RiboBio (Guangzhou, China), and were transfected by using lipofectamin RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

qRT-PCR Analysis

Total RNA was procured by TRIzol Reagent in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the level of hTERT expression and endogenous controlled by GAPDH. TaqMan miRNA assay (Applied Biosystems, Waltham, MA, USA) was used to measure the level of miR-1182 expression normalized to miRNA U6.

Western Blotting

Protein concentration was measured by BCA reagent kit (Merck, Kenilworth, NJ, USA). SKOV3 cell lysates were separated on polyacrylamide gels and electroblotted onto nitrocellulose membranes. Then, the blocking buffer (TBS with 0.05% Tween 20, pH 7.6 with 5% skimmed milk) was used for mounting. After that, the tissue sections were washed and incubated with anti-hTERT antibody or anti-GAPDH antibody (diluted at 1:1,000, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight.

Cell Proliferation

SKOV3 cells were harvested and inoculated into 96-well plates at a density of 2´10^3 cells for 48 h. MTT solution (5 mg/mL, MultiSciences, Hangzhou, China) was appended to each well after 4-h incubation. 150 μL of dimethylsulfoxide (DMSO) was added to each well for dissolving the formazan. 30 min later, the optical density value at the wavelength of 490 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Invasion and Metastasis Assays

Cell invasion and metastasis assays were performed using transwell plates (Corning, Corning, NY, USA) with 8-μm-pore membranes with

Matrigel (for invasion assay) or without Matrigel (for metastasis assay). Briefly, 2´10[4] cells were inoculated into the upper chamber with serum-free medium. In addition, the lower chamber was added with medium containing 10% fetal bovine serum (FBS) as a chemoattractant. At 2 days after incubation, the cells on the top of membrane were cleared by a brush. Subsequently, the membrane was stained by 0.2% crystal violet, and then drenched by 95% ethanol. Cell invasion and metastasis were observed via an inverted microscope.

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by least significant difference. $p < 0.05$ represented that the difference was statistically significant.

Results

MiR-1182 Expression Was Reduced in Both Tissues and Cells of OC

To examine the role of miR-1182 in OC, we used qRT-PCR to detect the expression level of miR-1182 in OC tissues and the adjacent normal tissues. The results showed that the expression of miR-1182 was pretty low in OC tissues compared with the adjacent normal tissues (Figure 1A). Furthermore, we found the same results in OC cell (SKOV3 cell) compared to the normal ovarian cell (IOSE80 cell) ($p < 0.05$) (Figure 1B). As a result, it was believed that miR-1182 might regulate OC progression.

hTERT was a Direct Target of miR-1182 in OC Cell

To research a potential target of miR-1182, we checked it in three publicly available algorithms, TargetScan, miRDB, and microRNA to elucidate the putative and possible targets of miR-1182. Finally, we found the hTERT was checked a supposed target of miR-1182 (Figure 2A). Then, we established luciferase reporter vectors containing the wild- or mutant-type miR-1182 seed sequences of the hTERT 30UTR. The increased expression of miR-1182 with mimics resulted in the decrease of the luciferase activity of the wile-type hTERT 30UTR reporter gene, but it exerted no effect on mutant-type (Figure 2B), suggesting that the expression of hTERT can be regulated by miRNA 1182.

MiR-1182 Suppressed Proliferation of OC Cell

To test the effect of miR-1182 on proliferation of OC cells, MTT assay was used to detect the cell proliferation rate. MTT assay results revealed that the cell proliferation rate of SKOV3 cells was decreased by up-regulating miR-1182 transfected

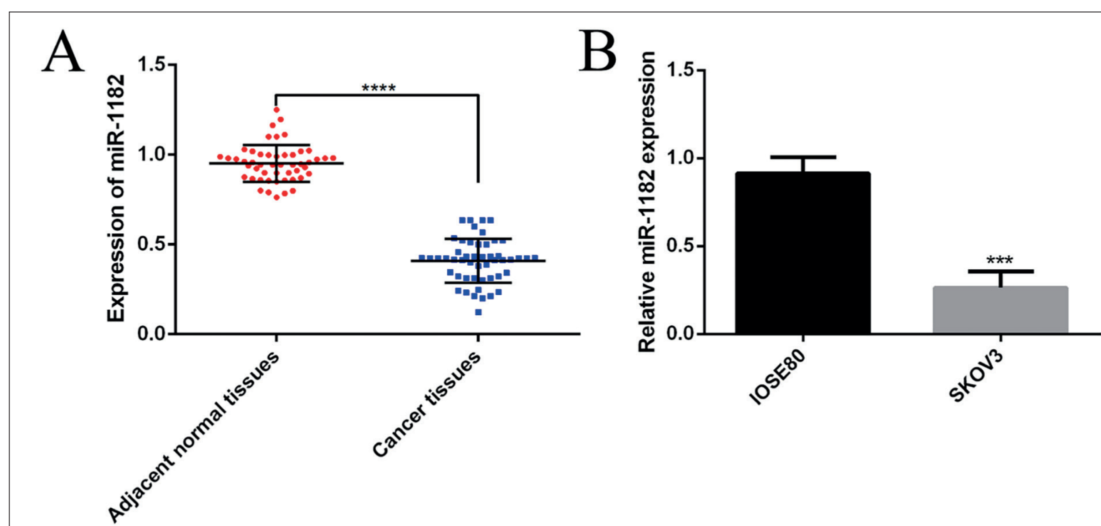

Figure 1. The expressions of miR-1182 in ovarian cancer tissue samples and ovarian cancer cell. ***A,*** Difference in the expression of miR-1182 between OC tissues and corresponding adjacent normal ovarian tissues. (****$p < 0.0001$ compared with adjacent normal gastric tissue). ***B,*** The expression of miR-1182 in OC cell line (SKOV3) and normal ovarian cells (IOSE80). (***$p < 0.001$ compared with IOSE80).

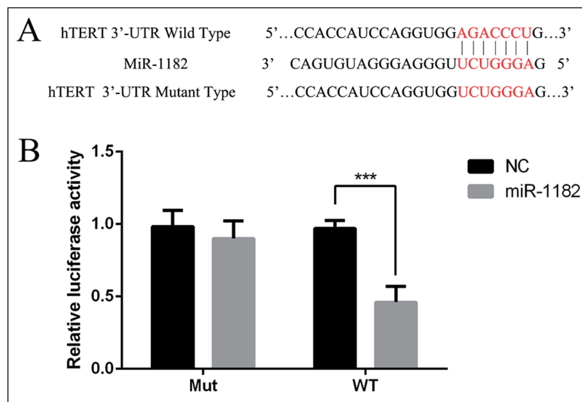

Figure 2. hTERT is a direct and functional target of miR-1182. SKOV3 cells were transfected with miR-1182 mimics and inhibitor. ***A,*** Diagram of putative miR-1182 binding sites of hTERT. ***B,*** Relative activities of luciferase reporters (***$p < 0.001$).

by mimics. In contrast, down-regulated miR-1182 expedited the growth of OC cells (Figure 3).

MiR-1182 Decreased the Expression Level of hTERT

SKOV3 cells were divided into three groups (miR-NC group, miR-1182 mimics group, and mimics + hTERT group) receiving similar experiments. It was found that the expression level of hTERT was decreased by up-regulation of miR-1182 in SKOV3 cells in both Real-time PCR analysis and Western blotting (Figure 4A, B and C). These data indicated that hTERT could be negatively regulated by miR-1182.

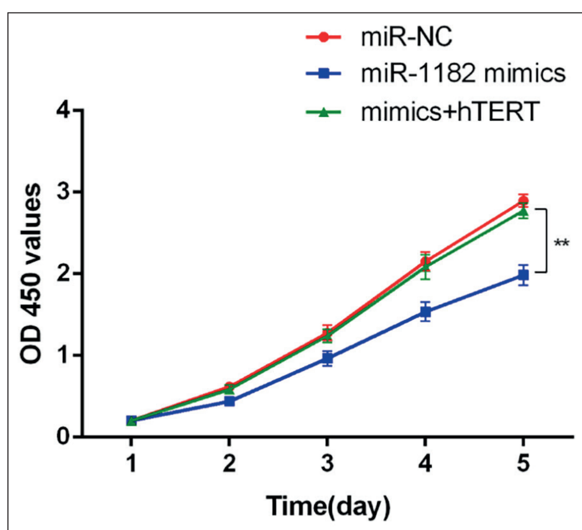

Figure 3. MiR-1182 inhibits the proliferation of OC cell. (**$p < 0.01$).

MiR-1182 Inhibited Invasion and Metastasis of OC Cells

Metastasis and invasion are the two most key factors in cancer cell proliferation. Transwell experiment results demonstrated that the invasion and metastasis abilities of SKOV3 cells were restricted by up-regulation of miR-1182 with mimics, while inhibiting miR-1182 resulted in the gaining of these abilities of OC cells ($p < 0.05$) (Figure 4D, E and F).

Discussion

Ovarian cancer is the most common type of malignant tumor of the female reproductive system worldwide. Due to its hidden onset, difficult early diagnosis and no reliable early treatment, its mortality rate ranks first in the gynecologic malignant tumor. Therefore, it has important clinical significance to actively explore the mechanism of invasion and metastasis of ovarian cancer, applying the target drug treatment and searching the effective drug treatment targets. To date, there have been many studies on the miRNA expression profile in ovarian cancer, but only a few miRNA target genes have been confirmed to be related to the occurrence of ovarian cancer. Yang et al[13] found that miR-214 can affect the tumor prognosis and drug resistance in ovarian cancer through regulating the target gene PTEN. Corney et al[14] found that miR-34b and miR-34c are expressed lowly in ovarian cancer, and the tumor cell proliferation and clone formation can be inhibited through p53 signaling pathway when they are highly expressed. Moreover, Guan et al[15] found that miR-125b expression is down-regulated in ovarian cancer, and the overexpression of miR-125b in ovarian cancer cells can result in cell cycle arrest and inhibition of proliferation and clone formation via inhibiting the BCL3 gene expression. Therefore, further studies are still needed to clarify the specific mechanism and target-signaling pathway of miRNAs in the occurrence and development of ovarian cancer. Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, is one of the core components of telomerase, as well as the most important rate-limiting factor for telomerase to exert its function[16-18]. As a special form of protective structure at the end of chromosome, telomere shortens itself to avoid the loss of chromosomal gene information in DNA replication, thus maintaining the stability of genome[19]. In most somatic

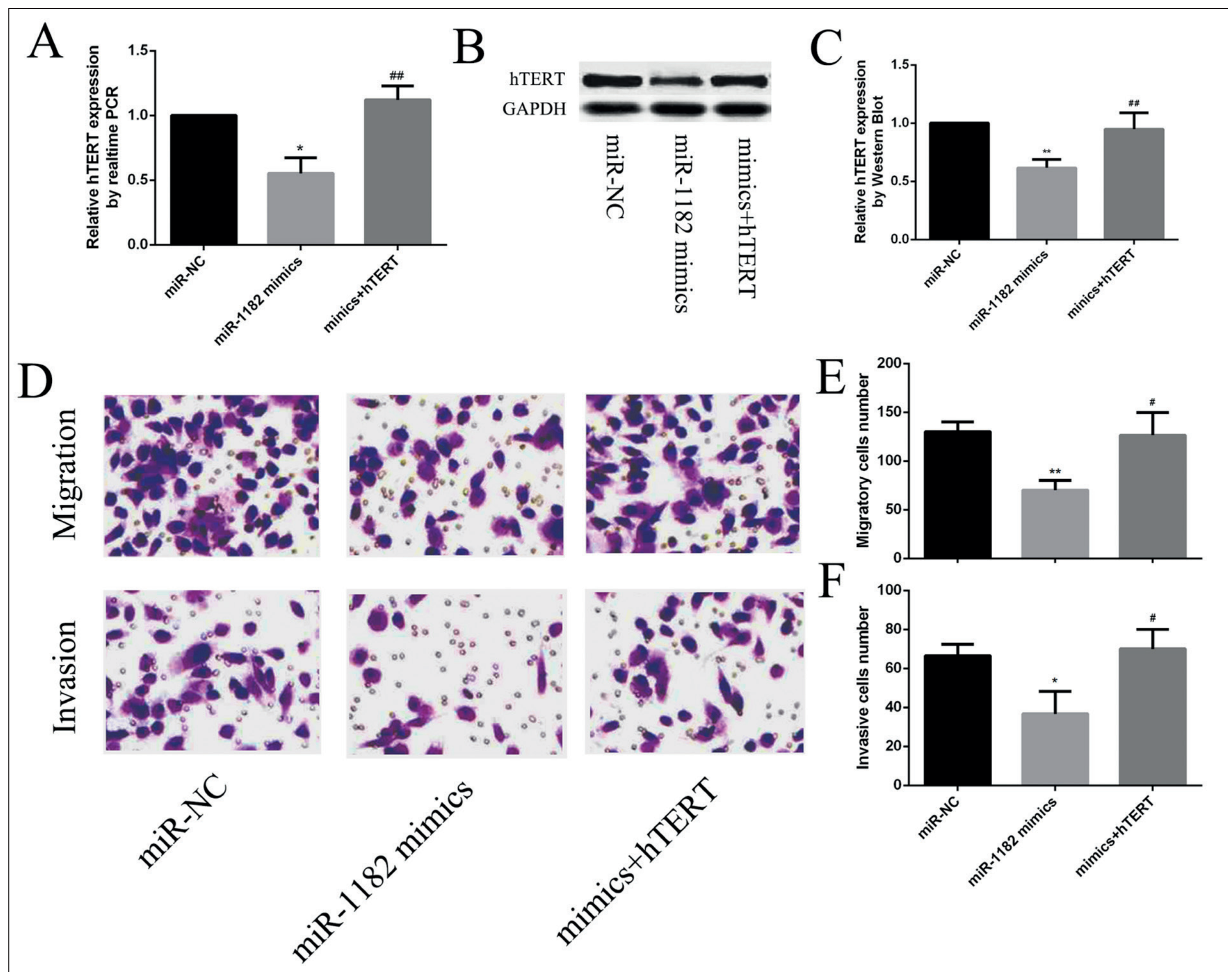

Figure 4. MiR-1182 decreases the expression level of hTERT and inhibits the invasion and metastasis of OC cell. ***A,*** Expression level of hTERT by Real-time PCR analysis. ***B,*** Protein expression of hTERT by Western blot. ***C,*** Expression level of hTERT by Western blot experiment. (*$p < 0.05$, **$p < 0.01$ *vs.* NC group; ##$p < 0.01$ *vs.* Mimics group). D, The invasion and metastasis of OC cells were analyzed using transwell assay and detected by microscope (× 200). ***E,*** and ***F,*** statistical analysis of D. Data were presented as means ± standard deviations. (*$p < 0.05$, **$p < 0.01$ *vs.* NC group; ##$p < 0.01$ *vs.* Mimics group).

cells, cells will face senescence and death after telomere shortens to a certain extent[20]. Therefore, the extension of telomeres is necessary for tumor cells to gain the unlimited proliferation capacity[21]. The most critical one in the three subunits of telomerase is hTERT, whose expression level directly determines the activity of telomerase[22]. The expression level of hTERT in most tumors is up-regulated, and it is positively correlated with the malignant degree of tumor. Some researches[23-25] have shown that hTERT is highly expressed in a variety of tumors, thereby up-regulating the telomerase activity, and transforming normal cells into tumor cells. This shows that hTERT is of great significance in the occurrence and development of tumors, and the study on hTERT will contribute to the diagnosis, treatment and prognosis of cancer[26]. However, there are relatively few studies on the correlation between hTERT and progression of ovarian cancer.

We analyzed the expression of miR-1182 in OC tissues founding that the expression level of miR-1182 was significantly decreased in OC tissues. To study the molecular mechanism of abnormally low expression of miR-1182 in OC, it was found, first through bioinformatics, that hTERT was a regulatory target of miR-1182. Subsequent analyses showed that the expression level of hTERT was significantly increased in OC cell, and miR-1182 might be a tumor suppressor gene. Existing studies have shown that hTERT is closely related to the tumor progression, and it has been reported that hTERT is abnormally expressed in various cancers, such as colon cancer[27], cervical cancer[28],

and gastric cancer[29], which is closely related to the proliferation, metastasis, and invasion of tumors. Moreover, after the up-regulated expression of miR-1182 in OC cells, the proliferation, metastasis, and invasion capacities of OC cells were significantly decreased, suggesting that the miR-1182/hTERT regulatory axis may contribute the occurrence and progression of OC, providing a new detection direction for the diagnosis and treatment of OC.

Although the application of miRNA in the treatment of disease is still a new area, miRNA plays an important role in the diagnosis of ovarian cancer and the development of therapeutic regimens. Ovarian cancer has often been in the advanced stage when diagnosed, leading to a high mortality rate; a very important reason for this is the lack of early diagnosis methods. Studies[30,31] have found that the expressions of many miRNAs related to ovarian cancer are different compared with those in normal ovarian tissues, and the mature or ovarian cancer-associated miRNA precursors can be detected in peripheral blood. Therefore, the research on diagnosis of ovarian cancer via the differential expression of miRNA in peripheral blood is of great significance. These miRNAs associated with the biological characteristics of ovarian cancer, in addition to being a screening method for ovarian cancer, can also serve as targets for the tumor treatment, providing new methods for tumor treatment.

Conclusions

hTERT, as a target for the treatment of ovarian cancer, may become a feasible and new method of tumor treatment. In conclusion, the study on miRNA regulatory mechanism provides a new perspective for the research and treatment of ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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