Abstract. This study investigated the inhibitory effects of miR-124 and miR-152 on the growth of human ovarian cancer (OC) SKOV3 cell line subcutaneous xenografts in nude mice. Twenty-eight healthy nude mice were selected and divided into the experimental group 1 (n=4), experimental group 2 (n=4), negative control group 1 (n=4), negative control group 2 (n=4), blank control group 1 (n=4), blank control group 2 (n=4) and observation group (n=4) according to the principle of similarity in body weight. The transfected SKOV3 cells were inoculated subcutaneously into the nape of the nude mice. After tumorigenesis, miR-124 mimics, miR-152 mimics, and their negative controls were transiently transfected into human OC SKOV3 cells via lipofection method. The expression levels of miR-124 and miR-152 were detected via reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and those of Ki-67 and caspase-3 were detected by western blotting. After transfection, the expression levels of miR-124 and miR-152 in the SKOV3 cells were significantly upregulated. The nude mice were sacrificed 36 days later, and tumor nodes of nude mice transfected with miR-124 and miR-152 grew slowly. Compared with that in the experimental groups, tumor size in the blank control and negative control groups was gradually increased with the increment of days (P<0.05). The volume of subcutaneous xenografts in nude mice of miR-124 and miR-152 experimental groups was obviously smaller than that in the blank control and negative control groups (P<0.05). Besides, the inhibition of tumor size in the observation group was more significant than that in the experimental groups (P<0.05). Thus, miR-124 and miR-152 inhibit the growth of human epithelial OC xenografts in nude mice, and they are expected to become new targets for gene-based therapy of OC.

Introduction

Epithelial ovarian cancer (EOC) is a malignant tumor with the highest incidence rate in ovarian cancer (OC). According to statistics, EOC patients account for more than 80% of OC patients, and approximately 120,000 EOC patients die each year around the world, showing an increasing trend year by year (1). The occurrence and pathogenesis of EOC have remained unclear through the study on EOC for years (2). EOC has high malignancy, rapid development, and no good biological or molecular markers in the early diagnosis. Many patients are in the middle and late stages when attending the hospital (3). Besides, its prognosis is not ideal, and statistics reveal that the 5-year survival rate of patients with advanced EOC is not >30%. Therefore, a practical and effective treatment method is needed in clinical practice (4).

A micro ribonucleic acid (miRNA) is a small non-coding RNA molecule with a length of approximately 21-25 nucleotides (5). miRNAs are involved in such important cell physiological processes as cell regulation, energy metabolism, and apoptosis, which can degrade mRNA or inhibit protein translation by binding to the 3’ untranslated region (UTR) of downstream target genes. At present, many studies on miRNAs have revealed that miRNAs become more closely related to mental illness, cardiovascular disease and tumors (6,7). In particular, in the research of tumors, miRNAs participate in the occurrence, development, diagnosis, treatment and prognosis of tumors, and different miRNAs are differentially expressed in different tumors (8). Moreover, literature has manifested that expression of both miR-124 and miR-152 are low in EOC, which can significantly inhibit the proliferation of SKOV3 cells (9,10).

Therefore, a nude mouse model of subcutaneous xenografts was established in this experiment to observe the inhibitory effects of miR-124 mimics and miR-152 mimics on tumors, thus inserting new ideas and laying a foundation for clinical treatment of EOC.
Materials and methods

Main reagents and materials. Human OC SKOV3 cells (cat. no. FDCC-HLC207) were purchased from Shanghai Ruilu Biotechnology Co., Ltd. (Shanghai, China). A total of 10% bovine fetal serum (FBS), Roswell Park Memorial Institute (RPMI)-1640 medium, Lipofectamine\textsuperscript{\tiny\textregistered} 2000, and TRIzol reagents (cat. nos. 10099141, 61870044, 11668019 and 15596026, respectively) were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) kit was purchased from Sigma-Aldrich (QR0100; St. Louis, Missouri, USA) and complementary deoxyribonucleic acid (cDNA) synthesis kit was purchased from Roche Diagnostics (cat. no. 11483188001; Basel, Switzerland). Primary antibodies (rabbit anti-mouse caspase-3, Ki-67 and β-actin polyclonal antibodies) and a secondary antibody [horseradish peroxidase (HRP) goat anti-rabbit antibody] (cat. nos. AC033, AF1738, A0208 and AF0003, respectively) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). A 7900 fluorescence real-time qPCR instrument was purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). Uv spectrophotometer Alpha-1860SPlus was purchased from Shanghai Lab-Spectrum Instruments Co. Ltd. (Shanghai, China).

Animal sources. A total of 28 female, specific-pathogen-free (SPF)-grade BALB/c nude mice, aged 5 weeks and weighing 18-20 g, were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China). The mice were fed by designated persons in the SPF-grade animal laboratories under uniform temperature (20±2°C) and humidity (80±5%), with chow and water ad libitum. The mice were kept in separate cages with 12 h day and 12 h night. The study was approved by the Ethics Committee of Yudu Central Hospital of Weifang (Weifang, China).

Methods

Cell culture and transfection. SKOV3 cells were cultured in RPMI-1640 medium (containing 10% FBS), and further cultured in an incubator with 5% CO\textsubscript{2} at a constant temperature (37°C). Then the cells were observed, and 0.25% trypsin was used for digestion and passage after they were adherent to the wall. The SKOV3 cells were inoculated on a 6-well plate to observe cell growth. When the degree of cell fusion reached approximately 80%, Lipofectamine\textsuperscript{\tiny\textregistered} 2000 kit was applied for plasmid transfection. In this study, mice were divided into 7 groups: the observation group, the experimental group 1 (miR-124 mimics), the experimental group 2 (miR-152 mimics), the control group 1 (NCs of miR-124 mimics), the control group 2 (NCs of miR-152 mimics), the blank group 1 (untreated cells), and the blank group 2 (untreated cells). miR-124 mimics or NCs of miR-124 mimics (20 µl) were added to 100 µl serum-free medium, and then 3 µl Lipofectamine\textsuperscript{\tiny\textregistered} 2000 was added. After 5 min of standing, the reagents were evenly mixed, followed by standing for 20 min. Then the prepared mixed solution was added to the 6-well plate containing SKOV3 cells, and the volume was adjusted to 1 ml using RPMI-1640 medium (without penicillin-streptomycin double antibody), and the medium was changed 6 h later. After transfection, the cells were collected for standby application after further culture for 48 h. The above experiments were repeated for the transcription of miR-152.

Detection of the expression levels of miR-124 and miR-152 in cells and cancer tissues via RT-qPCR. Cells or tissues were lysed by using TRIzol reagent, and the total RNA was extracted in strict accordance with the TRIzol reagent manufacturer’s protocol. Cell or tissue gridding was conducted on ice, followed by the addition of TRIzol reagent for extraction. After that, RNA concentration, purity and integrity were measured via an ultraviolet spectrophotometer and 1% denatured agarose gel electrophoresis. The optical density (OD) value of the total RNA solution: A\textsubscript{260}/A\textsubscript{280} should be within the range of 1.8-2.1, and if it did not meet the standard, the extraction would be conducted again. Then, it was reverse transcribed to cDNA with reverse transcription kit (Beyotime, Shanghai, China). The expression levels of miR-124 and miR-152 in cells and cancer tissues after transfection were detected via RT-qPCR. miR-124 and miR-152 primer sequences are shown in Table 1. PCR reaction system: 2’ SYBR Green Taq ReadyMix for RT-qPCR (10 µl), upstream and downstream primers each 0.5 µl, cDNA 1 µl, MgCl\textsubscript{2} 0.6 µl and finally added to 20 µl with DEPC. PCR amplification conditions: at 95°C for 10 min, 95°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec, and a total of 40 cycles. U6 was used as an internal reference gene, and the experiments were repeated 3 times. The results were expressed by using 2^-ΔΔCq (11).

Animal model establishment. A total of 28 fed nude mice (BALB/c) were divided into 7 groups [the observation group, miR-124 groups (experimental group 1, control group 1 and blank group 1), and miR-152 groups (experimental group 2, control group 2 and blank group 2)] with 4 mice each according to the principle of similarity in body weight. After cells were transfected for 24 h (the density of cells was adjusted to 5x10\textsuperscript{6} cells/well by resuspension in serum-free medium), they were subcutaneously injected in the right axillary fossa of nude mice. After 1 week, tumorigenesis was observed, and the tumor

Table I. Gene sequences.

| Gene name | Forward primers | Reverse primers |
|-----------|-----------------|-----------------|
| miRNA-124 | 5'-GATACTCATAAGGCACCGGC-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| miRNA-152 | 5'-CCAGTCAGTGACATGACAGA-3' | 5'-GTGCAGGGTCCGAGGTATTC-3' |
| U6        | 5'-GATACTCATAAGGCACCGGC-3' | 5'-GTGCAGGGTCCGAGGT-3' |

LIU et al: EFFECTS OF miR-124 AND miR-152 ON EPITHELIAL OVARIAN CANCER
Expression of miR-124 mimics and miR-152 mimics in SKOV3 cells after transfection. The expression of miRNA-124 and miRNA-152 mimics in SKOV3 cells after transfection was detected by RT-qPCR. The results revealed that both miRNA-124 mimics and miRNA-152 mimics in the experimental groups were obviously highly expressed in SKOV3 cells, and were remarkably higher than those in the control and blank groups, displaying statistically significant differences (P<0.01) (Table II).

Detection of the relative expression level of miRNA in animal model tissues in each group via RT-qPCR. According to the detection of the relative expression levels of miRNA-124 and miRNA-154 in the nude mouse tumor model in each group, the relative expression level of miR-124 in the experimental group 1 (5.84±1.67) was significantly higher than that in the control group 1 (1.33±0.47) and blank group 1 (1.28±0.32) among the miRNA-124 groups, with statistical differences (P<0.01 in all comparisons). Besides, among the miRNA-152 groups, the relative expression level of miR-152 in the experimental group 2 (6.34±2.17) was markedly higher than that in the control group 2 (1.64±0.87) and the blank group 2 (1.58±0.64), displaying statistical differences (P<0.01 in all comparisons). However, the relative expression levels of miRNA-124 and miRNA-152 in the observation group were 6.21±1.84 and 6.98±1.77, respectively, manifesting no differences in comparisons with those in the experimental group 1 and 2 (P>0.05) (Fig. 2).
Protein expression level in each group. The relative expression levels of Ki-67 and caspase-3 proteins were detected by means of western blotting. The results illustrated that among the Ki-67 groups, the expression level of Ki-67 in the experimental group 1 (0.492±0.084) was obviously lower than those in the control group 1 (0.841±0.148) and blank group 1 (0.813±0.137), manifesting statistical differences (P<0.01 in all comparisons). The expression level of Ki-67 in the experimental group 1 (0.492±0.084) was obviously lower than those in the control group 1 (0.841±0.148) and blank group 1 (0.813±0.137), manifesting statistical differences (P<0.01 in all comparisons). The expression level of Ki-67 in the experimental group 1 (0.492±0.084) was obviously lower than those in the control group 1 (0.841±0.148) and blank group 1 (0.813±0.137), manifesting statistical differences (P<0.01 in all comparisons).
experimental group 2 (0.501±0.084) was decreased markedly compared with that in the control group 2 (0.862±0.152) and blank group 2 (0.823±0.147), and the differences were statistically significant (P<0.01 in all comparisons). In addition, among the caspase-3 groups, the expression level of caspase-3 in the experimental group 1 (0.724±0.117) was significantly higher than that in the control group 1 (0.392±0.067) and blank group 1 (0.411±0.077), and the differences were statistically significant (P<0.01 in all comparisons). The expression level of caspase-3 in the experimental group 2 (0.748±0.122) was elevated notably compared with that in the control group 2 (0.387±0.058) and blank group 2 (0.406±0.062), and the differences are statistically significant (P<0.01 in all comparisons). Nevertheless, the expression levels of Ki-67 and caspase-3 in the observation group were 0.506±0.088 and 0.794±0.110, respectively, and there were no differences in comparison with those in the experimental group 1 and 2 (P>0.05) (Figs. 3-5).

Figure 3. (A) Ki-67 protein expression levels in miR-124 groups. Western blotting is applied to detect the protein expression level in each group, which reveals that the expression level of Ki-67 in the experimental group 1 (0.492±0.084) is markedly lower than that in the control group 1 (0.841±0.148) and blank group 1 (0.813±0.137), showing statistically significant differences (P<0.01 in all comparisons). (B) Ki-67 protein expression levels in miR-152 groups. Western blotting is applied to detect the protein expression level in each group, which reveals that the expression level of Ki-67 in the experimental group 2 (0.501±0.084) is markedly low compared with that in the control group 2 (0.862±0.15) and blank group 2 (0.823±0.147), displaying statistically significant differences (P<0.01 in all comparisons). (A and B) *P>0.05, not statistically significant.

Figure 4. (A) Caspase-3 protein expression levels in miR-124 groups. Detection of the protein expression level in each group through western blotting indicates that the expression of caspase-3 in the experimental group 1 (0.724±0.117) is remarkably higher than that in the control group 1 (0.392±0.067) and blank group 1 (0.411±0.077), and the differences are statistically significant (P<0.01 in all comparisons). (B) Caspase-3 protein expression levels in miR-152 groups. Results of western blotting in detecting the protein expression level in each group indicate that the expression of caspase-3 in the experimental group 2 (0.758±0.122) is increased notably compared with that in the control group 2 (0.387±0.058) and blank group 2 (0.406±0.062), and the differences are statistically significant (P<0.01 in all comparisons). (A and B) *P>0.05, not statistically significant.

Figure 5. The expression levels of Ki-67 and caspase-3 proteins in the observation and two experimental groups. Comparisons illustrate that the expression levels of Ki-67 and caspase-3 proteins in the observation group are 0.506±0.088 and 0.794±0.110, respectively, which are not statistically different from those in the experimental group 1 (0.492±0.084, 0.501±0.084) and experimental group 2 (0.724±0.117, 0.748±0.122) (P>0.05).
Discussion

OC is one of the malignant cancers with a fairly high mortality rate in women, and it ranks sixth among cancer in women worldwide. EOC, occupying the highest proportion of cancers with the highest malignancy, is hard to be detected, and patients are in the advanced stage when diagnosed, so they lost the best time for treatment. Nowadays, early diagnosis and treatment of EOC are the major problems faced with the society (12). Cancerous adhesions triggered by late lesions of EOC cannot be completely removed during surgery, and distal metastasis may occur, which cannot be solved via surgery. These problems, in turn, are also the main causes of poor prognosis of EOC patients (13). At present, the best treatment option is surgery combined with chemotherapy as well as cisplatin and paclitaxel combined chemotherapy, but the drug resistance resulted from the long-term application makes clinical treatment efficacy worse and worse, so there is a need to find a more effective treatment method for EOC, thus alleviating the burden on patients (14).

In recent years, miRNA has become a hot topic. As a non-coding RNA molecule, miRNAs can inhibit the degradation of target genes by binding to them, causing differential expression of post-transcriptional regulatory genes. miRNAs are involved in most of the complex biological processes in the organism, especially the occurrence and development of tumors, which have attracted increasing attention from scholars (15). A study has illustrated that (16) miR-124 is expressed in a variety of cancers such as liver cancer, gastric cancer, pancreatic cancer, and OC. A study by Silber et al (17) demonstrated that the high expression of miR-124 can reduce the proliferative capacity of neuroblasts and decrease the growth rate of tumorigenic cells in in vivo experiments. However, miR-152 has been confirmed to play an important role in the occurrence and development of many tumors, as well as cell proliferation and apoptosis (18).

In this study, the inhibitory effects of miR-124 and miR-152 in EOC were investigated. An EOC animal model was successfully established, and it was revealed through the regular injection of miR-124 mimics and miR-152 mimics that overexpressed miR-124 mimics and miR-152 mimics markedly inhibited the growth of tumors. Moreover, the combined injection of miR-124 and miR-152 indicated that the tumor size detected by the combined injection was significantly larger than that detected via the injection of miRNA-124 mimics or miRNA-152 mimics alone. Furthermore, subsequent RT-qPCR detection results revealed that the expression of miR-124 and miR-152 in both experimental groups were obviously increased compared with those in other groups, manifesting significant differences, and there were no differences in comparison with the observation group. A study by Zhang et al (19) demonstrated that miR-124 expression is low in OC, and this low expression is more obvious in highly metastatic OC. In addition, Shu et al (20) found from in vitro experiments that highly expressed miRNA can inhibit the proliferation, migration and invasion, and induce apoptosis of OC cells. According to the study of Zhou et al (21), the overexpression of miR-152 can significantly suppress the proliferation of OC cells. The study results are similar to those of our experiment. Ki-67 protein is closely related to cell proliferation, differentiation, invasion and apoptosis and participates in the pathological processes of all malignant tumors (22). However, caspase-3 protein, as a member of the caspase family, is involved in apoptosis and is the initiator of apoptosis (23). The expression levels of Ki-67 and caspase-3 proteins in tissues were detected via western blotting, which manifested that the expression of Ki-67 protein in the two experimental groups was significantly lower than that in other groups, with significant differences, but there was no difference compared with that in the control groups. However, caspase-3 protein was highly expressed in the two experimental groups compared with that in the other groups, but this expression was not different from that in the observation group. The underlying cause may be the binding of miRNA-124 and miRNA-152 to downstream target genes (24,25) [GLI family zinc finger 3 (Gli3) and phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3)].

However, there are still some defects in this experiment. First of all, this experiment was an animal model experiment and not conducted in clinical practice. Whether the observed results are biased is not clear due to the few experimental samples. Besides, the modeling time is short, and patients were in the middle and advanced stage when they were clinically diagnosed, so we expect to carry out research through clinical trials in the future, so as to improve the treatment and prognosis of EOC.

In conclusion, miR-124 and miR-152 exert inhibitory effects on the growth of EOC xenografts in nude mice and are expected to serve as new targets for EOC treatment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

WL and LZ wrote the manuscript and were responsible for cell culture and transfection. JW performed PCR. XW constructed the animal model. HS contributed to performing western blotting. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Yidu Central Hospital of Weifang (Weifang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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