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miR-145 regulates proliferation and chemotherapy sensitivity of ovarian carcinoma

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Summary

Previous work showed that miR-145 is downregulated in human serous epithelia ovarian carcinoma (SEOC) tissue and SKOV3 cells. However, the molecular mechanisms of miR-145 used to regulate ovarian proliferation and chemotherapy sensitivity remain to be determined. The present research demonstrate that miR-145 inhibit SKOV3 cells proliferation and promote chemotherapy sensitivity to paclitaxel according to MTT assay. MiR-145 inhibits Mucin1 (MUC1) post-transcriptional expression by binding to its 3'-untranslated region (UTR). The epithelial mesenchymal transition (EMT) marker E-cadherin (E-cad), which is downstream molecule of MUC1, is promoted by miR-145 overexpression. Furthermore, the E-cad protein level is inversely correlated with the expression of MUC1 in SKOV3 cells. It showed that promotion of E-cad signaling induced by miR-145 was released by MUC1 inhibition. Taken together, miR-145 serves as a tumor suppressor which can upregulate E-cad expression by targeting MUC1, leading to the inhibition of tumor proliferation and chemotherapy sensitivity. The miR-145 could be a rationale for therapeutic applications in ovarian carcinoma in the future.

Key words: Ovarian carcinoma; miR-145; MUC1; Proliferation; Chemotherapy sensitivity.

Introduction

In China, 52,100 new cases of ovarian cancer will be diagnosed and approximately 22,500 of them will be estimated deaths in 2015 [1]. The majority patients were diagnosed at advanced Stages (III or IV) with peritoneal seeding. Although they accepted systematic therapy such as surgery and chemotherapy, the five-year overall survival (Stage III: 40%–60%; Stage IV: 17%) has unchanged over the past 30 years because of chemoresistance and lacking of screening tests [2].

MiR-145, a member of 143/145 cluster, was down-regulated in many cancers, including glioma [3], breast cancer [4], renal cell carcinoma [5], and prostate cancer [6]. The present research shows that miR-145 dramatically low-expressed in SKOV3 cells and SEOC tissue, these results are similar to those from previous studies [7]. Recently miR-145 was validated to play pivotal roles in regulating various cellular functions such as cell apoptosis, cell proliferation, invasion, and metastasis in different malignant tumors, including breast cancer, glioma, colon carcinoma, cervix cancer, and so on [8]. However, the patho-biological significance of aberrant miR-145 expression in serious epithelial ovarian carcinoma has not been obviously elucidated and the role of miR-145 in serous epithelia ovarian carcinoma (SEOC) remains to be demonstrated.

In the current study, the authors investigated the aberrant expression and functional role of miR-145 in SEOC. They demonstrated that overexpression of miR-145 in SEOC significantly suppressed proliferation and improve chemotherapy sensitivity in vitro. Furthermore, Mucin1 (MUC1), an oncogene that was highly expressed in SEOC, was identified as a direct target of miR-145. Therefore, they both correlated with the poor prognosis of SEOC patients.

Materials and Methods

All cell lines were purchased from Cell bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (NTCC). Ovarian cancer cell lines SKOV3, LvX-MOCK-CMV-RFP SKOV3, LvX-miR-145-CMV-RFP SKOV3 were grown in RPMI 1640, supplemented with 10% FBS. HEK-293T cells were cultured in DMEM supplemented with 10% FBS. All media contained 2 mM glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Cells were incubated at 37°C and supplemented with 5% CO2 in the humidified chamber.

SKOV3 cells were transfected with miR-145 mimic using RNAfectin reagent (lipofectmin 2000) following the manufacturer's protocol.

miRNAs was reverse transcribed in a 20 μl reaction using the one step primescript miRNA cDNA synthesis kit. Forward primer sequences has-miR-145: GTCCAGTTTTCCCAGGAATC. Quantitative real-time PCR (QPCR) was performed on a real-time PCR system using Power- SYBR Green PCR Master Mix in a 20-μl reaction and U6 as an endogenous control, miRlet-7 as positive control, result was determined using the $2^{-\Delta\Delta CT}$. The QPCR experiments were run thrice within each experiment run and relative expression values were normalized to standard deviations from the mean.

Total protein was extracted from frozen tissues. Protein con-

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tents were measured with a DC protein assay kit. Ten micrograms of lysate protein for Western blotting of MUC1 were separated by SDS-PAGE using polyacrylamide gels and electroblotted onto a PVDF membrane. The antibodies used were anti-human MUC1 mouse monoclonal antibody properly diluted with PBS-T containing 2% bovine serum albumin and 0.01% sodium azide. The expression levels of MUC1 was calculated by densitometry and expressed as the MUC1/tublin ratio.

SKOV3 cells were transfected by miR-145 mimics. After 48 hours when they were grown to logarithmic phase, cells were digested by trypsin, then radicaled into cell suspension with 10% FBS cultured in RPMI1640 with 2×10^3 cells per hole which were seeded in 96-well plates, respectively. Cells proliferation was detected after 24, 48, and 72 hours of culture, respectively, in each hole by adding 50 μl MTT of 0.5 mg/ml concentration, after incubated for four hours in CO2 incubator, suspension in the holes were sucted then added 150 μl of two dimethyl sulfoxide (DMSO), gently shocked for 10 minutes. The optical density (OD value) of each hole was measured by 490 nm. Each assay was performed in triplicate. OD value for the vertical draw cell growth curve.

Cell viability of paclitaxel-treated SKOV3 cells was determined by MTT assay method. Cells were seeded on 96-well plates with density of 2×10^3 cells/well in 200 μl medium for 24 hours and then treated with paclitaxel at different concentrations (25, 50, 100, μg/ml) for 24, 48, and 72 hours. Each 20 μl MTT solution (5.0 mg/ml) was added to each well and incubated at 37°C for four hours. Then 150 μl DMSO was added in each well to dissolve the MTT formazan crystals and the optical density value (OD value) was measured at 490 nm with a microplate reader. Inhibitory rate (%) = [1 - (paclitaxel-treated OD/untreated OD)] × 100%. The 50% inhibitory concentrations (IC_{50}) for 24, 48, and 72 hours were calculated by regression analysis, respectively.

Different groups of SKOV3 cells in 96-well plates were washed with phosphate-buffered saline (PBS) thrice and fixed with 4% paraformaldehyde in PBS buffer for 30 minutes at room temperature. Cells were stained with DAPI for ten minutes in the dark and then washed thrice. The unstained and stained cells were observed under a fluorescence microscope. Five coverslips were used as replicates in each group and the apoptotic nuclei of each cells was visualized.

The 3′UTR of MUC1 containing two putative miR-145 binding sites (580 bp) was amplified and cloned into pmirGLO vector using the SacI and XbaI sites to generate the wild type construct. For mutant plasmid (MT), overlap extension PCR assay was used. Cells were cultured in 96-well plates and transfected with 100 ng of WT or MT MTDH 3′UTR constructs by lipofectamine 2000 assay. Twenty-four hours after transfection, luciferase activity was measured using the Dual-Glo Luciferase Assay System. Renilla luciferase activity was normalized to corresponding firefly luciferase activity and plotted as a percentage.

Analysis were carried out by BioMad CFX system, GraphPad Prism software and statistical software SPSS 20.0. The risk ratio and its 95% confidence interval were recorded for each marker. *P*-values < 0.05 were considered statistically significant in all of the analysis.

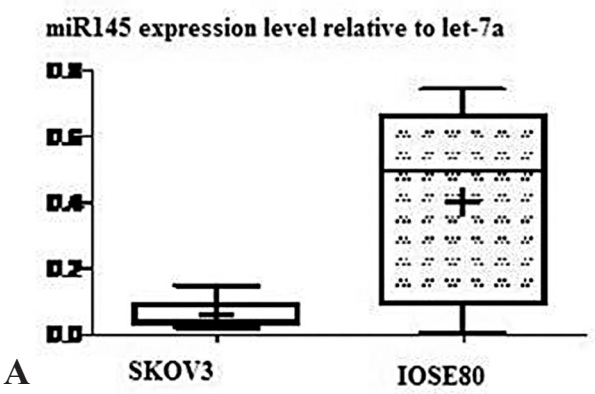
Results

It was reported that miR-145 was downregulated in freshly frozen ovarian carcinoma samples based on microarray analysis. To further elucidate the ectopic expression of miR-145 in ovarian carcinoma, the authors detected the expression level of miR-145 in human SEOC cell line SKOV3 and the normal human ovarian epithelial cell line IOSE80. Moreover, they detected the expression level of miR-145 in 50 freshly frozen human SEOC samples and the 30 normal human ovarian epithelial biopsy. MiR-145 was demonstrated to be significantly reduced in SKOV3 cell line (Figure 1A, $p < 0.05$). Also, results of qRT-PCR showed that in 50 freshly-frozen SEOC biopsy, expression of miR-145 was downregulated and relative fold was 0.15, so the authors determined that miR-145 expression was obviously decreased in SEOC tissues than in normal ovarian tissue samples (Figure 1B, $p < 0.05$).

To investigate whether miR-145 affects the proliferative ability of SEOC cells, the authors transiently transfected SKOV3 cells with human miR-145 mimics or negative control, then performed MTT assay. Twenty-four hours after transfection, cells were resuspended into cell suspension in each experimental group, counted, and plated. A total of five 96-well plates were seeded, and tests were continuously conducted 24, 48, and 72 hours after transfection. Compared with the negative control, mock and blank groups, miR-145 revealed a significant inhibitory effect on SKOV3 cell proliferation. Compared to the other three groups, SKOV3 cell proliferation in the miR-145 group was slow, compared with the blank group at 24, 48, and 72 hours after inoculation, the activity of miR - 145-5 p mimics group were 54.3%, 46.4%, 43.4% (Figure 2).

To understand how miR-145 influenced on SKOV3 cell chemotherapy sensitivity, the authors used miR-145 and negative control treating SKOV3 cells and cells' viability was determined by MTT assay and tested OD490 value at different time points. Results demonstrated that 72 hours after transfection, compared to negative control, mock and blank groups, OD490 value was significantly decreased in miR-145 mimic group ($p < 0.05$) (Figure 3A), Compared with the blank group at 72 hours after inoculation, the relative activity of miR-145 mimic group were 50.5%, 46.2%, and 43.7%. Then the authors used different concentrations of miR-145 treating SKOV3 cells and their viability was significantly decreased as treated by miR-145 with 100 μmol/L (Figure 3B). These results showed that overexpression of miR-145 can promote SKOV3 cells' sensitivity to paclitaxel.

Apoptotic morphological changes of SKOV3 cells after 48 hours miR-145 transfection were observed under a fluorescence microscopy (Figure 4). With DAPI nucleus staining, some cells showed typical apoptotic signs, including chromatin condensation, karyopyknosis, and nuclear fragmentation, which are characteristic features of apoptotic cells. However compared to control group, miR-145 group did not obviously display increased number of detached cells in round and shrunken shapes. After treated by paclitaxel, in accordance with the MTT assay, a concentration-dependent manner of toxal treatment was found since more apparent morphological alterations and more apoptotic cells presented with increased paclitaxel concentration.



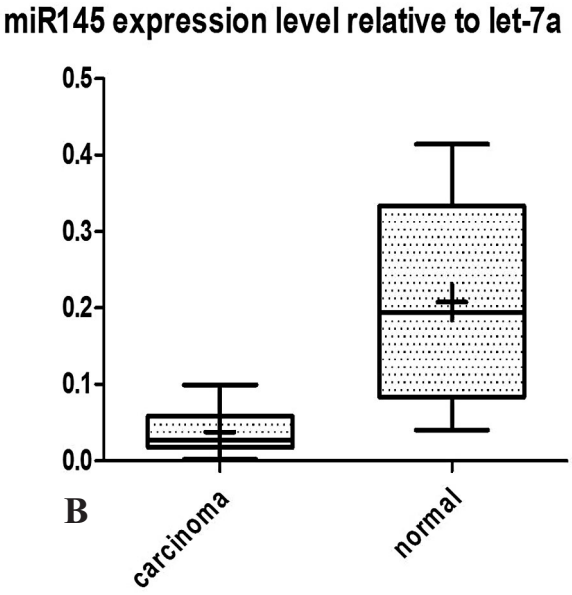


Figure 1. — Downregulation of miR-145 in ovarian cancer tissue and cell lines. (A) Relative miR-145 expression levels of ovarian cancer cell line, SKOV3 cells, and normal ovarian epithelial cell line, IOSE80 are examined with qRT-PCR assay. (B) qRT-PCR validation of miR-145 expression in normal cervical tissue and SEOC tissue.

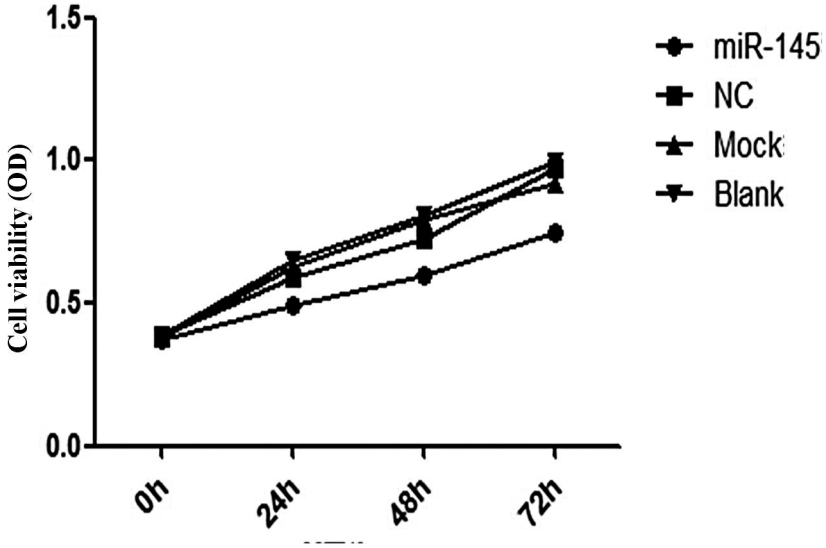


Figure 2. — Effect of miR-145 on cell proliferation of ovarian carcinoma SKOV3 cells determined by MTT assay.

According to three public databases (TargetScan, MicroCosm, and miRanda) the authors identify potential targets of miR-145, and selected MUC1 3'UTR sequence for analysis (Figure 5A). They first cloned the wild-type or mutant miR-145 target sequences of the MUC1 3' UTR into luciferase reporter vectors. Co-transfection with human miR-145 mimics (0.452 ± 0.066) obviously reduced the luciferase activity of the wild-type (WT) reporter gene compared with negative control (0.912 ± 0.087), but co-transfected with the mutant-type (MT) reporter gene, the luciferase activity of miR-145 mimic group (0.918 ± 0.100) showed no difference with negative control (0.942 ± 0.086) (Figure 5B, $p < 0.05$). Taken together, these findings demonstrated that miR-145 could bind to MUC1 through 3'UTR.

To test whether miR-145 regulates the expression of MUC1 and E-cad at protein level in ovarian carcinoma, the authors transfected SKOV3 cells with miR-145 mimics and negative control sequence, then performed q-RT PCR and Western blot analysis. Quantitative RT-PCR and Western blotting showed that overexpressing of miR-145 remarkably reduced the protein expression of MUC1, but not mRNA expression level (Figures 6A and 6B, $p < 0.05$). The authors found that E-cad protein expression was signifi-

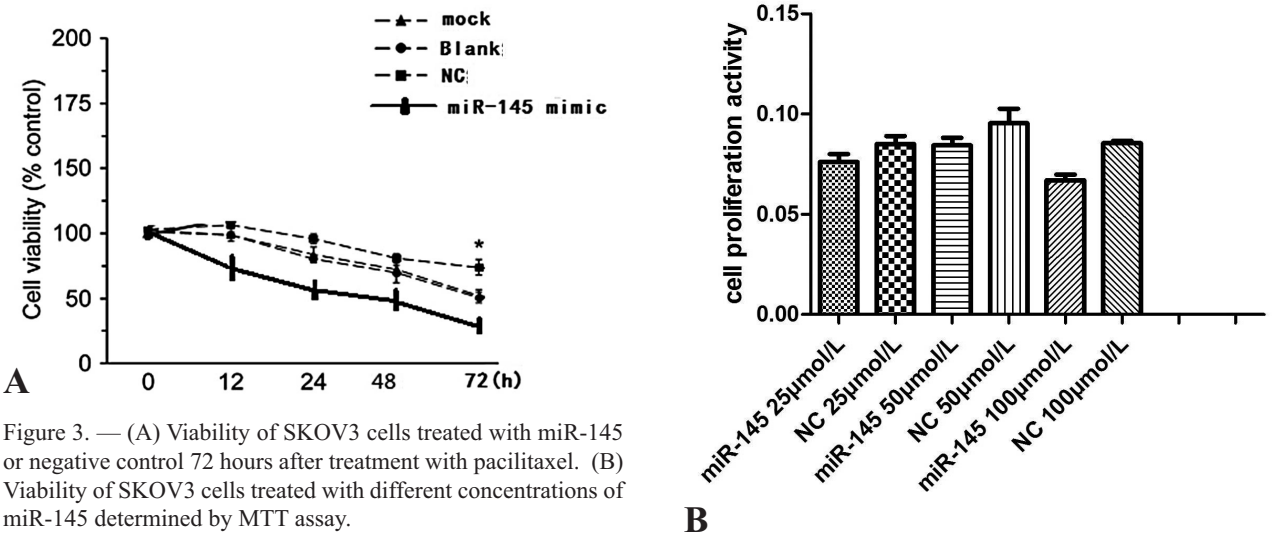


Figure 3. — (A) Viability of SKOV3 cells treated with miR-145 or negative control 72 hours after treatment with pacilitaxel. (B) Viability of SKOV3 cells treated with different concentrations of miR-145 determined by MTT assay.

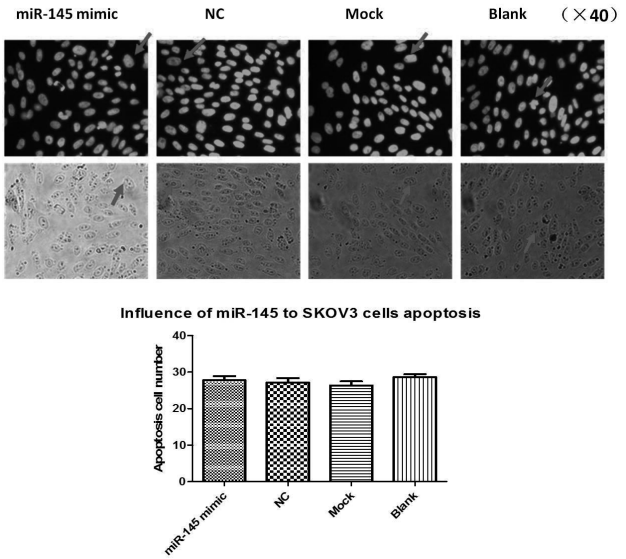


Figure 4. — Morphological observation on SKOV3 cells by DAPI staining (×40).

cantly increased by miR-145 compared to negative control in SKOV3 cells. However, overexpression of MUC1 by transfected MUC1 plasmids into SKOV3 cells downregulated protein expression of E-cad compared to negative control plasmids. While transfected SKOV3 cells with both miR-145 mimics and MUC1 plasmid, protein expression of E-cad was lower than that with miR-145 mimics only (Figure 6C). The results of Western blotting revealed that E-cad expression levels were decreased in SKOV3 cells with increased MUC1 expression. Taken together it showed that promotion of E-cad signaling induced by miR-145 was released by MUC1 inhibition.

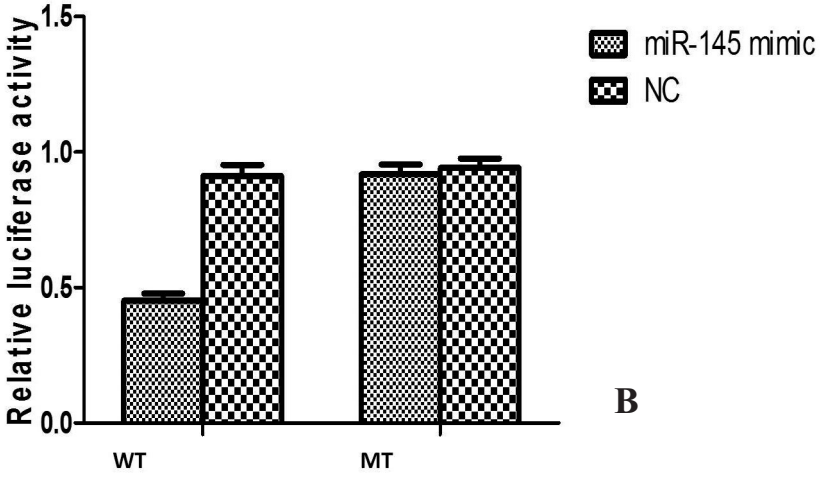
Discussion

DNA methylation in the upstream sequence of miR-145 contributes to the downregulation of miR-145 in prostate cancer [9]. Post-transcriptional regulation is also critical for miR-145 expression. Breast cancer 1 (BRCA1) recognizes the root of the stem-loop structure of pri-miR-145, directly associates with Drosha and DDX5 of the Drosha complex, and interacts with Smad3, p53, and DHX9 RNA helicase, promoting miR-145 processing [10]. Conversely, BCDIN3D is a methyltransferase that modifies the 50-monophosphate end of miRNAs, including pre-miR-145, which affects their recognition by Dicer. BCDIN3D depletion reduced the level of premiR-145 and increased the level of mature miR-145 in breast cancer cells[11].

To determine the potential roles of miR-145 in SEOC, SKOV3 cells were separately transfected with miR-145 mimics and negative control sequence and were challenged in cell viability assay and DAPI staining, then the proliferation, apoptosis, and chemotherapy sensitivity were assessed. The present findings revealed that upregulated miR-145 significantly inhibits the proliferation and promote chemotherapy sensitivity of SKOV3 cells, but had no significant effect on apoptosis.

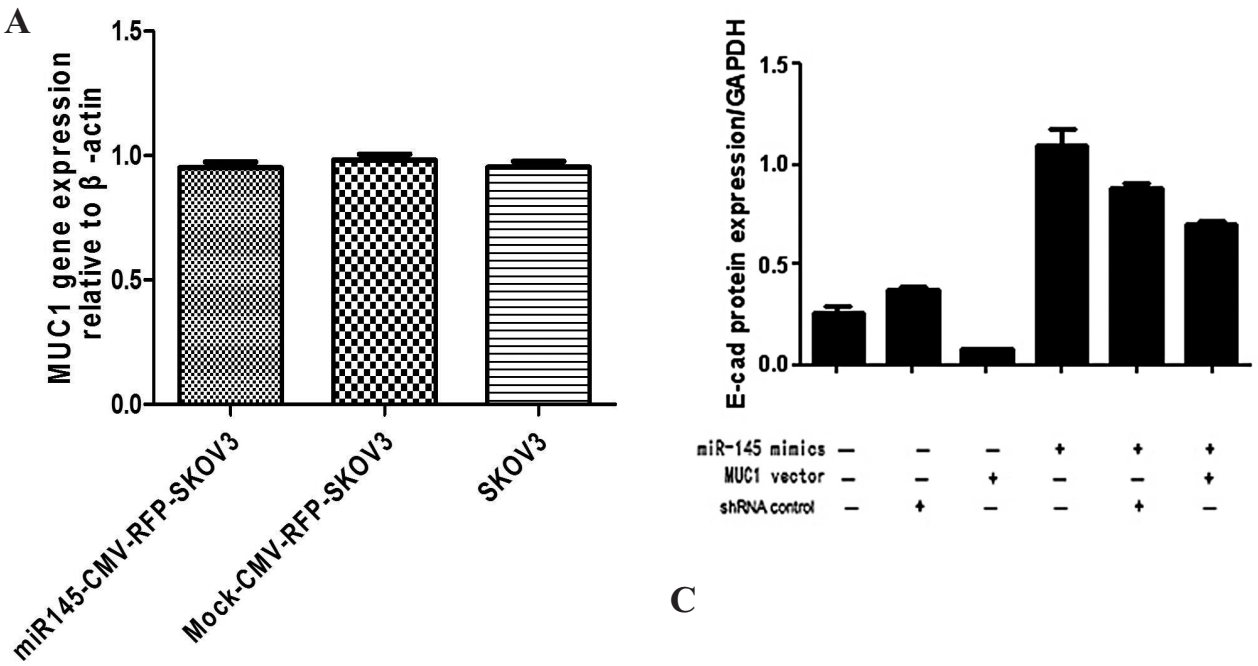
Transcriptional regulation of miR-145 seems to be complex. Since p53 mutations occurred in almost all SEOC (96%), downregulation of miR-145 in SEOC might be related to p53 mutation. The transcriptional factor E2f1 can be activated as a tumor suppressor and function to inhibit cell proliferation, migration, and invasion and induce apoptosis in cancer cells [12, 13]. E2f1's tumor suppressing effect is always exerted through p53-dependent mechanism,

A



B

Figure 5. — (A) Predicted complementary sequence of miR-145 to predicted target gene MUC1. (B) Luciferase reporter assay reveals miR-145 suppressed MUC1 3′UTR luciferase activity. Histobars illustrate the relative luciferase activity in SKOV3 cells with and without miR-145 overexpression when transfected with WT or MT luciferase plasmids.



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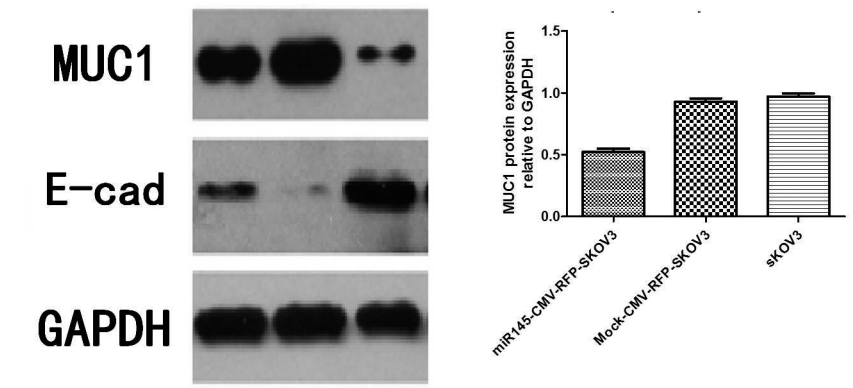

B

Figure 6. — (A) Expression of MUC1 gene in SKOV3 cells with and without miR-145 overexpression. (B) Protein expression of MUC1 and EMT marker E-cad in SKOV3 cells with and without miR-145 overexpression. (C) Different expression of EMT marker E-cad with miR-145 or MUC1 overexpression in SKOV3 cells.

leading to upregulation of p53 expression. As a crucial transcription factor, p53 controls apoptosis through activating the transcription of hundreds of genes, including the members of Bcl-2 family and caspase family [14]. Expectably, it is reported overexpression of not only transcriptional factor genes (E2F1 and p53) but also downstream genes (GADD45, BAX, BCL-2, BIM, CASP 7, CASP 8, CASP 9, and CASP 3) in miR-145-treated malignant cells, suggesting a p53 pathway-mediated apoptosis induced by miR-145. It is showed that gene upregulation of CASP 9, CASP 7, and CASP 3 as well as protein upregulation of active-caspase 3 [15] and cleavage-PARP inhibited proliferation and promoted apoptosis of non-small cell lung cancer (NSCLC) cells, indicating intrinsic caspases pathway as a main pathway involved in apoptosis [16]. Sachdeva *et al.* found that p53 could increase miR-145 expression by directly binding to the p53 response elements-2 (p53RE-2) in the miR-145 promoter, which was possibly the mechanism of p53-mediated repression of c-Myc [17]. Moreover, the positive feedback regulation between miR-145 and p53, partially deal to impairing of the murine double minute 2 (MDM2)-p53 feedback loop [18, 19].

Some findings elucidate the role of miR-145 as an important regulator of chemoresistance in ovarian cancer; it was confirmed that both Cdk6 and Sp1 are targets of miR-145. miR-145 led to a reduction in Cdk6 and Sp1 along with downregulation of P-gp and pRb. These changes resulted in increased accumulation of antineoplastic drugs and G1 cell cycle arrest, which rendered the cells more sensitive to paclitaxel in vitro and in vivo. Intriguingly, demethylation with 5-aza-dC led to reactivation of miR-145 expression in drug-resistant ovarian cancer cell lines, which also resulted in increased sensitivity to paclitaxel [20].

Many studies demonstrated that miR-145 influence tumor development by mediating different downstream targeting mRNAs, including c-Myc in breast cancer [21] and human lung cancer [22], FSCN1 in prostate cancer [23], ANGPT2 and NEDD9 in renal cell carcinoma [24], and Sox2 in human choriocarcinoma cells [25].

The present research validated that miR-145 mimics inhibited the protein expression level of MUC1 in SKOV3 cells since dual luciferase reporter gene assay prove miR-145 is able to directly bind with the 3'UTR of MUC1 mRNA. Furthermore, E-cadherin could be upregulated by overexpression of miR-145, while this management could be repressed by MUC1. Thus, this study provides a new link between miR-145, MUC1, and E-cadherin in the regulation of tumor proliferation and chemotherapy sensitivity in SEOC.

MUC1 was overexpressed in breast, colon, pancreas, and bladder tumors and was often associated with EMT of different cancer cells [26, 27]. By promoting focal adhesion assembly, MUC1 promotes also tumor cell growth and facilitates proliferation [28]. It is indicated that MUC1 gene silencing induces growth inhibition in SMMC-7721 cells through Bax-mediated mitochondrial and caspase-8-mediated death receptor apoptotic pathways [29]. Yin *et al.* demonstrated that inhibiting the expression of MUC1 significantly attenuated the effects of pro-adhesion, Akt-activation, and pro-survival [30]. Upregulation of MUC1 contributes to DEX-induced pro-adhesion and protects ovarian cancer cells from chemotherapy [30].

MiR-145-based therapy for ovarian cancer is at an early stage and further research of miR-145 may lead to novel therapeutic strategies.

Acknowledgements

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