

Regulation of miRNAs on c-met protein expression in ovarian cancer and its implication

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Abstract. – **OBJECTIVE:** HGF/c-met signal pathway exerts important roles in tumor pathogenesis. The study of c-met related regulatory mechanism provides the basis for finding anti-tumor molecular drugs. MiRNAs can effectively regulate gene expression and work as gene therapy. The identification of miRNAs for c-met regulation and study of related mechanism are of critical importance.

MATERIALS AND METHODS: Bioinformatics approach was used to search for possible miRNAs with regulatory functions on c-met gene. Using pcDNA3.1-EGFP as the scaffold, miRNAs over-expression and inhibitor plasmids were constructed for electroporation-transfection in ovarian cell line ES-2, and pcDNA3.1-EGFP empty plasmid was used as the control group. qRT-PCR and Western blot were applied to measure c-met mRNA and protein expression, followed by transwell chamber *in vitro* assay for the evaluation of invasion potency.

RESULTS: Bioinformatics prediction showed favorable regulatory function on c-met gene by miR-204. The differential expressions of EGFP were observed between pcDNA3.1-EGFP-204-up and inhibitor plasmid pcDNA3.1-EGFP-204-down. After transfection for 24 h and 48 h, c-met expression in miR-204 over-expression group gradually decreased ($p < 0.05$ compared to control group), accompanied with reducing cell migration or invasion potency in a time dependent manner ($p < 0.05$). In contrast, no significant difference in the level of c-met was found in the inhibitor group and control group ($p > 0.05$).

CONCLUSIONS: The up-regulation of miR-204 suppressed the expression of c-met in ovarian cancer cells and inhibited cell infiltration. The

suppression of miR-204 expression, however, presented no significant impact on cell infiltration potency.

Key Words:

MiR-204, Ovarian cancer, Cell migration, Cell infiltration.

Introduction

Ovarian cancer is the most common and deadly malignant tumor in females, the incidence of which has been gradually increasing in recent years. About 70% of ovarian cancer cases belong to moderate to terminal stage once being diagnosed, presenting severe threats to public health¹⁻³. The study showed that ovarian cancer pathogenesis was affected by various factors, including environment and genetics. With advancement of medical science and laboratory methods, early diagnosis of ovarian cancer has become feasible at present, and it largely decreases overall mortality. However, major treatment approaches against ovarian cancer still depend on chemotherapy or radiotherapy^{4,5}. The development of biotechnology and genetic engineering has provided novel insights for tumor treatment. The study of differential gene expressions during tumor pathogenesis can benefit individualized treatment and drug delivery, thus making it critical to find effective target gene in cancer therapy^{6,7}.

Normal HGF/c-Met signal pathway predominantly participated in the regulation of embryonic development and angiogenesis, and its abnormal activation was closely correlated with tumor pathogenesis and metastasis. Currently, anti-tumor drugs targeting this signal pathway have obtained major effects^{8,9}. This pathway mainly included the activation of intracellular tyrosine kinase phosphorylation via binding between HGF and c-met, further regulating multiple downstream signal pathways containing PI3K, ERK1/2 and FAK, thus modulating various biological functions of tumor cells¹⁰. A previous research showed that up-regulation of c-met could decrease cell-to-cell adhesion and facilitate cell metastasis¹¹, suggesting the possibility of using the inhibitors of c-met gene and related factors/genes as novel treatment targets. MiRNA has potent regulatory effects on target gene expression, and becomes the research focus in current biological treatment. It is widely distributed in cells and tissues, and exerts the regulatory function via complete complementary binding with target genes to directly degrade target gene mRNA or block gene translation at post-transcriptional stage or translational stage. In this regard, miRNA exerted its functions as oncogene and tumor suppressor gene to regulate biological behaviors including cell proliferation or apoptosis, and exhibited close correlation with tumor onset and progression^{12,13}. Current technology of miRNA study has gained major advances as the findings of its important regulatory roles in multiple human diseases, and some miRNAs have become markers for early tumor occurrence, or targets for clinical drugs¹⁴. In this work, we aimed to illustrate the effect of miRNAs on ovarian cancer and related regulatory mechanism.

Materials and Methods

Major Equipment and Materials

Human ovarian cancer cell line ES-2 was purchased from Beinuo Biotech (Shanghai, China) and was cryopreserved in our department. Dulbecco's Modified Eagle Medium (DMEM)/F12 medium for cell culture and fetal bovine serum (FBS) were produced by Sigma-Aldrich (St. Louis, MO, USA). Trypsin and transwell chamber along with Matrigel were produced by BD Biosciences (Franklin Lakes, NJ, USA). Phosphate-buffered saline (PBS) and dual antibiotics were purchased from Tiangen Bio (Beijing, China). Liposome transfection reagent, SYBR fluore-

scent dye, cDNA synthesis kit were produced by Promega (Madison, WI, USA). Human c-met and β -actin antibody from mouse polyclone and goat polyclonal secondary antibody were purchased from Xinlei Bio (Shanghai, China). Western blot reagents were produced by Beyotime (Beijing, China). pcDNA3.1-EGFP plasmid was purchased from You Bio (Changsha, Hunan, China). Restriction endonuclease was produced by TaKaRa (Otsu, Shiga, Japan). Electroporation transfection reagent and equipment were produced by Loza (Encino, CA, USA). Fluorescent quantitative PCR cyclor was produced by ABI (Vernon, CA, USA). Microplate reader was a product of Thermo-Fisher Scientific (Waltham, MA, USA).

Gene Sequence Analysis and Plasmid Construction

Based on human c-met gene sequence accessed from NCBI (NM_000245.3), TargetScan Human 7.1 was used to predict and to analyze possible regulatory miRNA. Results showed that miR-204 had the highest score of 84.2. Based on miR-204 sequence in miBase, NEB cutter (<http://nc2.neb.com/NEBcutter2/>) was used to analyze enzymatic digestion site on gene sequence. NotI and XhoI restricted digestion sites were introduced on upstream and downstream. Sense and anti-sense sequences of pre-miR-204 were synthesized by Invitrogen (Carlsbad, CA, USA) with length of 263bp. Using pcDNA3.1-EGFP as the plasmid scaffold, dual enzymatic digestion was used to construct miR-204 over-expression and inhibitor plasmids. PCR method was used to investigate construction of plasmid, using forward primer (5'-CTAGA GAACC CACTG CTTAC-3') and reverse primer (5'-TAGAA GGCAC AGTCG AGG-3') under 95°C 5 min, followed by 30 cycles each containing 95°C 30 s, 55°C 35 s and 72°C 30 s. Those plasmids after positive screening were sequenced for selecting those targets for further study.

Cell Culture and Transfection

Human ovarian cancer cells ES-2 were resuscitated by routine method, and were cultured in DMEM medium containing 10% FBS and 1% dual antibiotics in a 37°C chamber with 5% CO₂ for 24 h. PBS buffer was used for rinsing cells repeatedly. Cells were digested with trypsin for 2-3 min until becoming shrinkage to rounding. Serum-containing medium was introduced to stop the digestion. Cell suspension was saved and centrifuged at 400 g for 3 min. The supernatant was discarded, with the addition of fresh medium for repeating rinsing to

prepare single cell suspension. Cells were counted and transferred to 60 mm dish at 1×10^6 density for 24 h incubation. Cell transfection was performed when reaching 90% confluence with covering the dish bottom. Transfection plasmid concentration was set as 5 μg using 250 V electrical filed for 5 ms. After transfection, cell suspension was transferred to 6-well plate for culture using pcDNA3.1-EGFP empty plasmid as the control group. Transfection efficiency was deduced by the expression of green fluorescence. Single cell colony was collected for further culture.

qRT-PCR for miR-204 Expression in Cells Before and After Transfection

Following the manual instruction of RNA extraction and cDNA synthesis kit, total RNA was extracted from cells at 24 h or 48 h after transfection, followed by cDNA synthesis. qRT-PCR was used to detect miR-204 expression, using forward primer (5'-GGCTA CAGTC TTTCT TCAT-3') and reverse primer (5'-GCCAG TGA-TG ACAAT TG-3'). GAPDH was used as the internal reference (forward primer: 5'-CGGAG TCAAC GGATT TGGTC GTA-3'; reverse primer: 5'-AGCCT TCTCC ATGGT GGTGA AG-3'). In a 20 μL qRT-PCR system, 10 μL SYBR pre-mixture, 0.6 μL primer mixture, 0.8 μg cDNA and nuclease-free water were added and mixed. PCR was performed under the following conditions: 95°C for 2 min, followed by 40 cycles each containing 95°C 10 s, 55°C 15 s and 72°C 10 s, ended with 95°C 1 min, 60°C 30 s and 95°C 30 s. Each group was tested in triplicates for average values to reflect miR-204 expression.

Western Blot for c-met Expression in Cells Before and After Transfection

Total cellular proteins were collected at 24 h or 48 h after transfection. In brief, culture medium was removed, and cells were washed by PBS for 3-5 times. Cells were then mixed with lysis buffer containing proteinase inhibitor and phosphatase inhibitor for 25 min iced incubation. Lysate was saved and centrifuged at 12000 g under 4°C for 15 min. The supernatant was saved and quantified for protein contents using BCA method. Protein samples were separated by 10% separating gel, and were transferred to PVDF membrane using semi-dry apparatus (35 V, 30 min). The membrane was blocked using 5% bovine serum albumin (BSA) for 2 h room temperature incubation, followed by 4°C incubation in 1:200 diluted primary antibody. The membrane was washed by PBS

and Twee 20 (PBST), and was cultured at room temperature for 2 h using 1:500 diluted secondary antibody. Using β -actin as the internal reference, expression of c-met protein was measured.

Scratch Assay for the Effect of Transfection on Cell Migration

After transfection for 24 h, when cells paved all over dish bottom, sterile 200 μL pipette tip was used to draw vertical lines on the dish bottom. Detached cells were removed by PBS rinsing for 2-3 times. Culture medium was then added for 24 h incubation. Three scratch sites were randomly selected under 200 \times microscope for imaging to evaluate migration distance among all groups.

Transwell Assay for Cell Invasion Potency

Matrigel was diluted in serum-free culture medium at 1:5 ratio, and was added into the chamber (30 μL each), which was air-dried under 4°C. 24 h after transfection, cells were digested by trypsin and prepared for single cell suspension using medium containing 0.1% FBS. Cells were counted and seeded into the chamber with matrix gel pavement at $1 \times 10^5/\text{ml}$. The chamber was incubated for 24 h, and was washed three times in PBS. Cells were fixed in paraformaldehyde and stained. Three fields were randomly selected for imaging under an inverted microscope for counting.

Statistical Analysis

Data were statistically analyzed by SPSS 11.3 (SPSS Inc., Chicago, IL, USA) and express as means \pm standard deviation (SD). Student *t*-test was used to test significant difference between groups. Statistical significance was defined when $p < 0.05$, whilst extreme significance was defined when $p < 0.01$.

Results

Plasmid Construction and Confirmation

By dual enzymatic digestion and directed ligation, three plasmids were randomly selected for PCR assay. As shown in Figure 1, all plasmids included positive cloning, indicating successful construction of the plasmids. No mutation or missing of base pair was found after sequencing, suggesting that they can be used for further study.

Observation of cell Transfection

Cells were transfected by plasmids pcDNA3.1-EGFP, pcDNA3.1-EGFP-204-up and pcD-

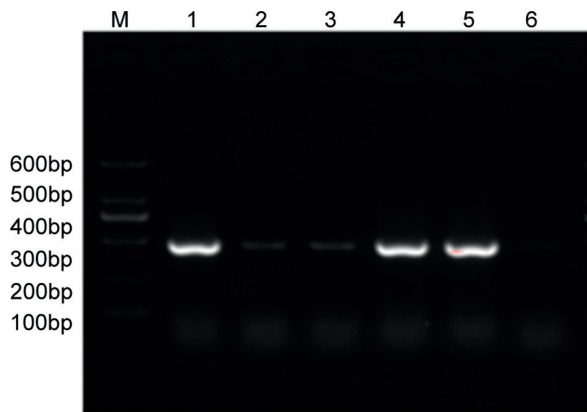


Figure 1. PCR for plasmid construction. M, DNA marker; Lane 1-3, pcDNA3.1-EGFP-204-up; Lane 4-6, pcDNA3.1-EGFP-204-down.

NA3.1-EGFP-204-down. GFP expression was observed under an inverted fluorescent microscope. As shown in Figure 2, the expression of GFP in all three groups was shown at 24 h after transfection.

qRT-PCR for miR-204 Expression in Cells After Transfection

Total RNA was extracted at 24 h and 48 h after transfection. The level of miR-204 was detected by using qRT-PCR. As shown in Figure 3, after transfection for 24 h or 48 h, miR-204 expression in pcDNA3.1-EGFP-204-up group was gradually elevated, whilst the level was decreased in pcDNA3.1-EGFP-204-down group ($p < 0.05$ at 24 h post-transfection, and $p < 0.01$ at 48 h post-transfection compared to pcDNA3.1-EGFP control group), suggesting that those recombinant vectors could effectively mediate miR-204 expression.

Western Blot for c-met Protein Expression in Cells After Transfection

After 24 h and 48 h transfection, intracellular c-met protein expression was measured by Western blot. As shown in Figure 4A and Figure 4B of gray value analysis, c-met expression in pcDNA3.1-EGFP-up group gradually increased at 24 h post-transfection ($p < 0.05$ compared to other two groups). The expression of c-met in pcDNA3.1-EGFP-204-down group was marginally increased but showed no significant difference compared to pcDNA3.1-EGFP control group ($p > 0.05$).

Transwell Chamber in vitro Assay for Cell Invasion Potency

Cells after transfection were continuously cultured for 24 h. After being stained with crystal violet (Figure 5), cells were counted and invasion percentage was calculated (Table I). Compared to pcDNA3.1-EGFP group, cells in pcDNA3.1-EGFP-204-up group presented significantly lower invasion rate (45.98%, $p < 0.05$ compared to control group). Cells in pcDNA3.1-EGFP-204-down group exhibited invasion rate of 94.17%, indicating no significant difference comparing to control group ($p = 0.652$).

Discussion

The occurrence of ovarian cancer represents an extremely complex process with family inheritance property, and is one of the three most common malignant tumors in female reproductive system, besides cervical cancer and uterus cancer. It leads to relatively high mortality and is associated

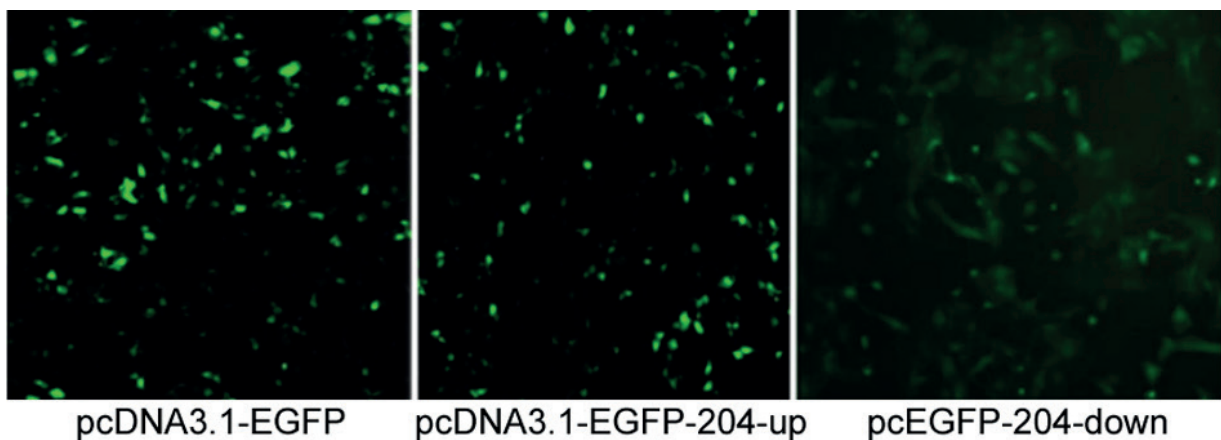


Figure 2. GFP expression at 24 h after transfection using pcDNA3.1-EGFP, pcDNA3.1-EGFP-204-up and pcDNA3.1-EGFP-204-down (10 \times).

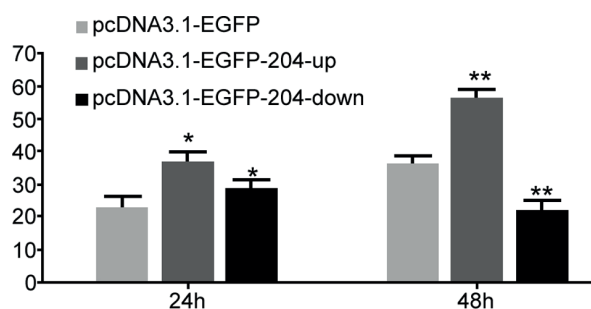


Figure 3. qRT-PCR for miR-204 expression at 24 h and 48 h after transfection. *, $p < 0.05$ compared to control group; **, $p < 0.01$ compared to control group.

with multiple pathogenic factors. A direct factor is the change of cancer cell migration or infiltration potency, which severely threatens prognosis of patients with ovarian cancer¹⁵⁻¹⁷. Previous investigations showed that the pathogenesis process of tumor is frequently accompanied with over-expression of stem cell-related genes and factors, such as over-expression of c-met and COX-2/1 in breast cancer, lung cancer and ovarian cancer tissues^{18,19}. Through tyrosine kinase activity, c-met expression can affect cell-to-cell adhesion, and its over-expression can facilitate cell migration and invasion. Via the bind with HGF for protein phosphorylation, it can activate various signal pathways of tumors, such as Raf/MAPK, PI3K and JAK/STAT, further modulating tumor cell proliferation, differentiation, migration and infiltration potency. Therefore, HGF/c-met signal pathway plays a critical role in occurrence and progression of multiple tumors. The study of this pathway thus has become the hot-spot for tumor targeting therapy. The study confirmed that in normal cells, low expression or absence of c-met was observed for homeostasis. Under the circumstance of tumor transformation, c-met immediately performed abnormally high expression. Therefore, the blockade of HGF/c-met pathway and decrease of c-met expression demonstrated an effective measure to inhibit further progression of tumors. The study of c-met in tumor cell expression and related regulatory mechanism provided it

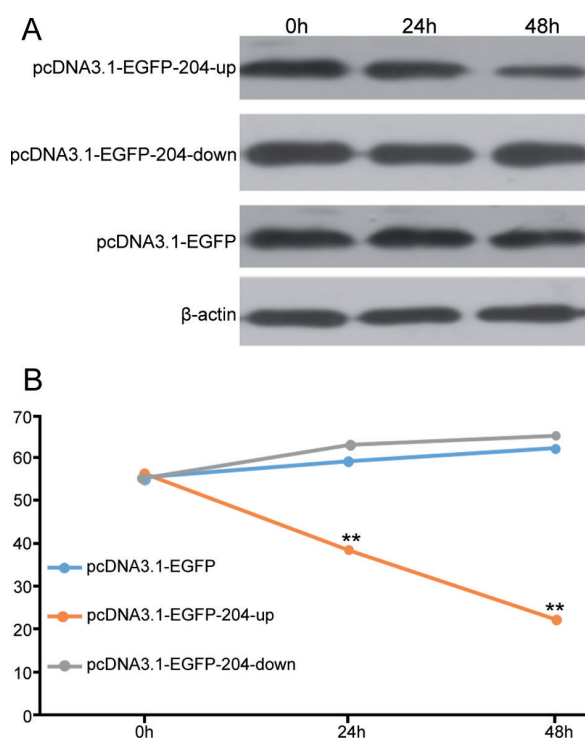


Figure 4. Western blot for c-met expression in all groups after transfection. *, $p < 0.05$ compared to control group; **, $p < 0.01$ compared to control group.

as an effective biological treatment target in clinics. MiRNAs exerted regulatory function in proliferation, apoptosis, and migration of various cancer cells. For instance, miR-34 regulated cancer cell behaviors via mediating p53 signal pathway, whilst miR-21 and miR-27a affected liver cancer pathogenesis and progression via expression of tumor suppressor gene sprouty 2 in liver tissues²⁰⁻²³. This work utilized bioinformatics approach to analyze c-met stem cell like gene, and searched for potential miRNAs targeting c-met. MiR-204 was revealed to possess multiple functional targets to c-met gene, with the highest score among miRNAs. Previous reports showed that miR-204 was involved in progression of multiple tumors including cervical cancer, kidney cancer and ovarian cancer. In this study, the effect of miR-204 was evaluated by overexpression or reduction of

Table I. Transwell chamber assay for cell invasion rate.

	Invasion cell number per field	Invasion rate (%)	p
pcDNA3.1-EGFP	53.344		
pcDNA3.1-EGFP-204-up	24.532	45.98	0.001
pcDNA3.1-EGFP-204-down	50.236	94.17	0.652

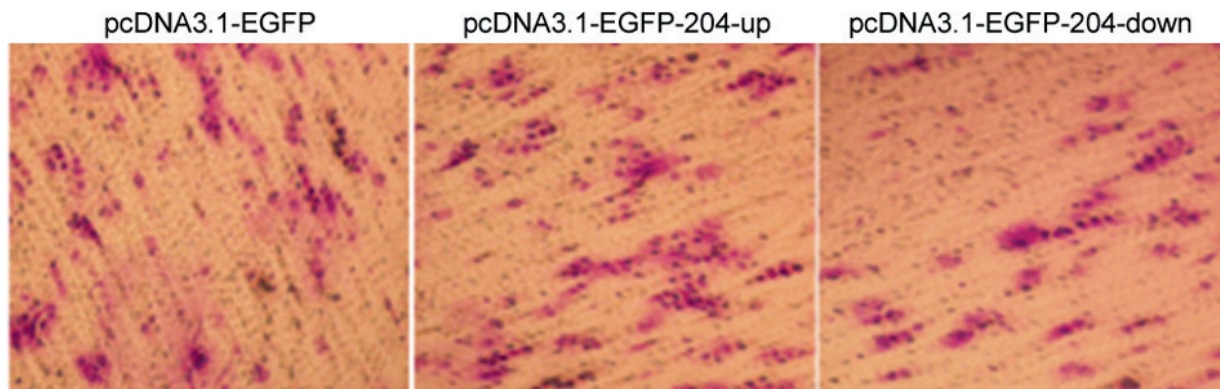


Figure 5. Transwell assay at 24 h after transfection.

miR-204. We constructed miR-204 over-expression or inhibition vectors using pcDNA3.1-EGFP as the scaffold. PCR and sequencing were used to identify ovarian cancer cell line ES-2 with correct transfection, followed by qRT-PCR and Western blot to measure mRNA and protein expression of c-met at 24 h or 48 h post-transfection. Transwell chamber assay was used to determine *in vitro* invasion potency of cells. The effect of miR-204 on c-met expression at the cellular level was also investigated. Results showed that our constructed miR-204 over-expression and inhibitor plasmids all effectively facilitated or inhibited miR-204 expression, respectively. Further assays showed that over-expression of miR-204 significantly suppressed expression of c-met, whilst down-regulation had no significant effect on c-met expression. This is probably due to multiple factors regulating c-met, or because of homeostatic mechanism of c-met level.

Conclusions

MiR-204 effectively suppressed the expression of c-met in ovarian cancer cell ES-2 can, further impeded cell migration or infiltration potency.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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