Identification of Differentially Expressed microRNAs in Metastatic Ovarian Cancer

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Yang Gu
Shengjing Hospital of China Medical University

Shulan Zhang
Shengjing Hospital of China Medical University

✉ zsl0909@126.com Corresponding Author
ORCID: https://orcid.org/0000-0002-4182-2233

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Abstract

Background: The molecular mechanisms of ovarian cancer (OC) remain unclear. We sought to comprehensively identify miRNAs that are aberrantly expressed in OC.

Methods: Differentially expressed miRNAs were screened from six pairs of primary and metastatic OC tissues; their possible functions were then analyzed and target genes were predicted by bioinformatics. Then gene expression profiling results were established by reverse transcription quantitative polymerase chain reaction and western blot. Target binding between miR-7-5p and TGFβ2 was validated by dual-luciferase reporter assay.

Results: Fifteen miRNAs and 10 target mRNAs were differentially expressed in primary and metastatic OC tissues. ITGB3, TGFβ2 and TNC correlated to miRNA function in metastatic OC. Compared with primary OC, RNA levels of hsa-miR-141-3p, hsa-miR-7-5p and hsa-miR-187-5p in metastatic OC tissues were potently decreased (p < 0.05). However, a statistically prominent difference in hsa-miR-584-5p level between the two groups (p > 0.05) was not observed. Comparing to primary OC, TGFβ2 and TNC were markedly increased (p < 0.05). Luciferase activity was remarkably decreased after co-transfection of a wild-type TGFβ2 3’-UTR plasmid and miR-7-5p compared with a control plasmid, but no remarkable change after co-transfection of mutant TGFβ2 3’-UTR and miR-7-5p was demonstrated.

Conclusions: Fifteen miRNAs and 10 mRNAs were differentially expressed in metastatic OC tissues compared with primary OC tissues, which suggested that they may participate in invasive and metastatic processes. Hsa-miR-141-3p, hsa-miR-187-5p and hsa-miR-7-5p expression was prominently lower in metastatic OC than in primary OC, while TGFβ2 and TNC expression was markedly higher in metastatic OC tissues. Hsa-miR-7-5p may bind to the TGFβ2 3’-UTR to inhibit its expression.

Background

Ovarian cancer (OC) is the deadliest gynecological cancer due to the absence of symptoms at the early stage [1, 2]. More concretely, due to the non-specificity of the symptoms of OC, a diagnosis is always made at advanced stages of cancer that have poor 5-year overall survival rate which is about 30–40% [3]. Moreover, the widespread and distant metastasis is observed in 59% OC patients and
closely correlated with poor prognosis of ovarian cancer [4]. Therefore, it is pivotal to study invasive and metastatic mechanisms of OC. The incomplete elucidation of the molecular pathways underlying OC development poses a great challenge to improving clinical outcomes and having a greater depth of understanding underlying the mechanisms of cancer metastasis that would contribute toward combating this disease.

MicroRNAs (miRNA), small RNA molecules (21–23 nucleotides [nt]), function as potent modulators of gene expression through mRNA translation blockade or RNA interference [5], and have become a hotspot of tumor research over the recent years. The dysregulation of miRNAs may act as novel oncogenes or anti-oncogenes in ovarian cancer by regulating target genes [6]. For instance, miR-7 was found to depress EGFR/ERK pathway to decrease OC cell invasion [7]. Stated thus, a hypothesis can be drawn that miRNAs participate in OC metastasis via their target genes. Therefore, differentially expressed miRNAs and mRNAs were screened from primary and metastatic OC tissues using chip technique. We found 15 miRNAs and 10 mRNAs that were differentially expressed and that participate in the invasive and metastatic processes of OC. The chip results were validated by reverse transcription quantitative polymerase chain reaction (RT-qPCR), western blot and dual-luciferase reporter assay.

Methods
Study participants
All 31 pairs of metastatic and primary OC tissues were collected from patients who had received surgery at Shengjing Hospital of China Medical University from 2014–2016. Of these, six pairs were used for chip experiments and 25 pairs were employed for RT-qPCR validation. Tissue samples resected during operations were immediately placed into 1.5-mL centrifuge tubes with RNase-removal high-pressure treatment, rapidly placed in liquid nitrogen for quick freezing, and then transferred to a -80°C cryogenic freezer for preservation. All patients were diagnosed with ovarian serous cystadenocarcinoma by postoperative paraffin pathology, and their clinical staging was made according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for ovarian cancer (2009). None of the patients had received radiotherapy, chemotherapy or other
special treatments prior to surgery. Patients who provided tissue specimens for chip experiments were aged 48–64 years (mean: 57.5 years), and those providing samples for RT-qPCR validation were aged 38–80 years (median: 57 years). Data and tissues were harvested upon receiving of the informed consent of patients and approval by the ethics committee of Shengjing Hospital of China Medical University.

RNA extraction from tissues
A Trizol Kit (Invitrogen Inc., Carlsbad, CA, USA) was applied for isolation of total RNA from metastatic and primary OC tissues. RNA purity was estimated using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and assessed by the ratio of optical density (OD) 260 nm/280 nm. A ratio between 1.8 and 2.0 signified the sample to be highly pure.

MiRNA expression profiling
Six pairs of metastatic and primary OC tissues were obtained from Shengjing Hospital of China Medical University. Total RNA was extracted from tissues using a Trizol Kit. cDNA was synthesized, purified and hybridized based on the instructions of an Arraystar miRNA First-Strand cDNA Synthesis Kit (Cat# AS-MR-004), which was fully compatible with an miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array (Arraystar, Rockville, MD, USA). After standardization of the original data from fluorescence quantitative PCR, fold changes in miRNAs and mRNAs were calculated based on a comparison between metastatic and primary OC tissues. A fold change > 1.5 and \( p < 0.05 \) were considered as a noteworthy up-regulation while fold change < -1.5 and \( p < 0.05 \) were considered as a striking down-regulation.

Functional analysis
GO (gene ontology) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were employed to analyze differentially expressed mRNAs in OC based on a David database (https://david.ncifcrf.gov/). GO analysis provided annotations and function classifications for differentially expressed mRNAs by analyzing the classifications of Biological Processes, Cellular Components and Molecular Function. Pathway analysis was based on KEGG results combined with Fisher's exact test and a t test. A \( p < 0.05 \) was regarded statistically distinct.

The miRNA–mRNA conjoint analysis
Using an miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array chip, we carried out the screening of six pairs of metastatic and primary OC tissues in 184 cancer-related miRNAs and 178 target mRNAs. The miRNA–mRNAs with a potential targeting relationship were screened out from miRNA microarray analysis and then confirmed by the online tools targetscan (http://www.targetscan.org/vert_71/) and miranda (http://34.236.212.39/microrna/home.do).

**RT-qPCR**
A total of 25 pairs of metastatic and primary OC tissues frozen at -80 °C were collected. Extraction of total RNA from tissues and cells was conducted using TRIzol reagent (Invitrogen). A mixture (14.5 µL) of 2.0 µg RNA, 1 µL Oligo, 1.6 µL deoxy-ribonucleoside triphosphate Mix, and H2O were incubated at 65 °C in water for 5 min and then put on ice for 2 min. After centrifugation, the mixture was mixed with reverse transcription reaction liquid, and placed at 37 °C for 1 min. The mRNA was reversely transcribed into cDNA, and the sample then temporarily placed on ice. All cDNA template samples were prepared in a PCR amplification system that included 5 µL of 2 × Taq MasterMix (Arraystar), 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), and the volume made up to 8 µL with H2O. PCR reaction conditions were as follows: pre-denaturation at 95 °C for 10 s and 40 cycles of denaturation at 94 °C for 5 s, annealing at 60 °C for 60 s and extension at 95 °C for 15 s to detect the fluorescence value of SYBR Green. PCR primer sequences (Table 1) were designed by Sangon Biotech (Shanghai, China). The relative expression level of TGFβ2 and TNC or hsa-miR-141-3p, hsa-miR-187-5p, hsa-miR-7-5p and hsa-miR-584-5p was standardized to β-actin or U6 expression and was calculated using the $2^{-\Delta\Delta Ct}$ method.
Table 1
Primer sequences for RT-qPCR

| Genes          | Primer sequences           |
|----------------|----------------------------|
| hsa-miR-141-3p | Forward: 5'-GGGGTAAACACTGTCTGGTAA-3'  |
|                | Reverse: 5'-GTGCCGTCTGGTAAAGTC-3'    |
| hsa-miR-187-5p | GSP: 5'-GGGGAGGCTACAACACAGGA-3'      |
|                | Reverse: 5'-GTGCCGTCTGGTAAAGTC-3'    |
| hsa-miR-7-5p   | GSP: 5'-GGGGGTGGAAGACTAGTGATTT-3'    |
|                | Reverse: 5'-GTGCCGTCTGGTAAAGTC-3'    |
| hsa-miR-584-5p | GSP: 5'-TGCCAATATGTTTGCGCTGG-3'      |
|                | Reverse: 5'-GTGCCGTCTGGTAAAGTC-3'    |
| TGFβ2          | Forward: 5'-CTGCTAATGTTATTGCCCTCCTAC-3' |
|                | Reverse: 5'-CCTGTAACAACGCATCTCATATT-3' |
| TNC            | Forward: 5'-GTGACAGAAGTGACGGAAGAC-3'  |
|                | Reverse: 5'-GATGGCAAATACACGGATAAAGT-3' |
| U6             | Forward: 5'-GCTTCGGCAGCACATATACATAT-3' |
|                | Reverse: 5'-CGCTTCACGAATTTGCCTCAT-3'  |
| β-actin        | Forward: 5'-GTGGGCGGAGACTTTTGATTG-3'  |
|                | Reverse: 5'-CCTGTAACAACGCATCTCATATT-3' |

Note: hsa; Homo sapiens; miR; microRNA; TGFβ2, transforming growth factor beta 2; TNC, tenascin-C; RT–qPCR, reverse transcription quantitative polymerase chain reaction; GSP, genome-specific primer.

Western blot

Tissues were immersed in radioimmunoprecipitation assay lysis buffer (P0013B; Beyotime, Shanghai, China) containing phenylmethyl sulfonylfluoride, and put on ice for 30 min, followed by 20-min centrifugation at 12,000 g. The supernatant was collected and transferred to an EP tube to estimate the total protein concentration according to the instructions of a bicinchoninic acid kit (KC-430, Shanghai Kangchen Biological Engineering Co., Ltd., Shanghai, China). The samples were separated at 100V for 1.5 h, and electroblotted onto a polyvinylidene fluoride membrane using a sandwich method at 80 V for 1.5 h. After 1-h blocking in 5% bovine serum albumin-Tris-buffered saline (TBST), the membrane was incubated with diluted (1:1000) rabbit anti-human TGFβ2 primary antibody overnight at 4 °C. Afterwards, the membrane was then re-probed with goat anti-rabbit secondary antibody (1:5000; Shanghai Kangchen Biological Engineering Co., Ltd., Shanghai, China) complexed to horseradish peroxidase at RT for 1 h. After rinsing with TBST, membranes were soaked in chemiluminescence BeyoECL plus (P0018, Beyotime) for 1 min, developed, photographed and recorded. Glyceraldehyde phosphate dehydrogenase (GAPDH, 1:10000; Shanghai Kangchen Biological Engineering Co. Ltd, Shanghai, China) served as an endogenous control for TGFβ2. A Gel-Pro Analyzer 4.0 program (Media Cybernetics, Silver Spring, MD, USA) was used to analyze gray values. The gray value ratio of each target protein band to the endogenous control band was calculated as the relative expression of each protein. Experiments were independently conducted three times.
Dual-luciferase reporter assay
Binding sites for hsa-miR-7-5p and TGFβ2-3'UTR were predicted using Genebank and Targetscan combined with chip analysis results. A Dual-Luciferase® Reporter Assay System (E2910; Promega, Fitchburg, WI, USA) was adopted. TGFβ2-3'-UTR WT, TGFβ2-3'-UTR MT Luciferase plasmid and miR-7-5p mimics/negative control (NC) were constructed by Shanghai GeneChem Co., Ltd (Shanghai, China). After plasmid transfection, a. 293T cells in the logarithmic growth phase were prepared into a suspension that was then cultured in a 24-well plate so as to achieve 85% confluence at 24 h. b. Six groups were used in the experiment (NC + TGFβ2-3'-UTR-WT, miR-7-5p + TGFβ2-3'-UTR-WT, NC + TGFβ2-3'-UTR-MT, and miR-7-5p + TGFβ2-3'-UTR-MT). After 48-h transfection, the cells were lysed to obtain the supernatant that was centrifuged for 3–5 min. The luciferase activity of TGFβ2 3'-UTR was detected by using the Dual-Luciferase® Reporter Assay System (E2910, Promega). The fluorescence intensity was measured by Multiskan Sky (Thermo Fisher Scientific). The experiment was repeated three times.

Cell treatment
HEK 293T cells were cultured in Dulbecco minimum essential medium (DMEM; 12800017; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; FBS500-S; Ausgenex, Brisbane, Australia) in an incubator (BB15; Thermo Fisher Scientific) with saturated humidity and 5% CO₂ at 37 °C. The medium was changed every 24 h and subcultures were undertaken every 72 h. After removal of medium, cells were trypsinized for 3 min and then the reaction was ended by adding DMEM containing 10% FBS. Afterward, cells were made into a single cell suspension using pipette tips.

Statistical analysis
All data were summarized as mean ± standard deviation and analyzed by t test using SPSS 23.0 statistical software (IBM Corp., Armonk, New York, USA). A value of p < 0.05 was considered appreciable.

Results
Correlation of several aberrant miRNAs and mRNAs with metastatic OC
Differentially expressed miRNAs and mRNAs in metastatic OC were screened using miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array analysis. Fifteen noticeably and differentially
expressed miRNAs were screened out from metastatic and primary OC tissues (Fig. 1A): (in sequence of absolute fold change) miR-200a-3p, miR-141-3p, miR-200b-3p, miR-10a-5p, miR-15a-5p, miR-187-5p, miR-16-5p, miR-9-3p, miR-195-5p, miR-7-5p, miR-584-5p, miR-27a-3p, miR-23a-3p, miR-27b-3p, and SNORD95-F (p < 0.05). In contrast to primary OC tissues, the expression levels of five miRNAs (miR-10a-5p, miR-15a-5p, miR-16-5p, miR-195-5p and miR-27a-3p) in metastatic OC tissues were observably higher (p < 0.05, fold change > 1.5; Table 2), while those of seven miRNAs (miR-141-3p, miR-7-5p, miR-187-5p, miR-200a-3p, miR-200b-3p, miR-584-5p and miR-9-3p) were markedly lower (p < 0.05, fold change > 1.5; Table 3). A total of 10 target mRNAs were differentially expressed in a potent manner and screened from metastatic and primary OC tissues (p < 0.05); compared with primary OC tissues, the expression levels of BCL2L1, TGFβ2, TNC, HOXB5, HOXB7, HOXB8, ITGB3 and MTSS1 in metastatic OC tissues were substantially up-regulated (p < 0.05, fold change > 2; Table 4 and Fig. 1B), while those of PAK1 and FOXA2 were down-regulated (p < 0.05, fold change > 2; Table 5).

### Table 2

| Genes          | Metastatic OC (2^−ΔCt) | Primary OC (2^−ΔCt) | p value     | Fold change |
|----------------|------------------------|---------------------|-------------|-------------|
| hsa-miR-10a-5p | 9.10E-03               | 3.20E-03            | 0.014557    | 2.85        |
| hsa-miR-15a-5p | 2.90E-01               | 1.30E-01            | 0.008198    | 2.24        |
| hsa-miR-16-5p  | 7.30E-01               | 4.00E-01            | 0.042838    | 1.84        |
| hsa-miR-195-5p | 1.00E + 00             | 6.10E-01            | 0.047072    | 1.66        |
| hsa-miR-27a-3p | 7.60E-01               | 4.90E-01            | 0.003931    | 1.54        |

Notes: hsa, Homo sapiens; miR, microRNA; OC, ovarian cancer

### Table 3

| Genes          | Metastatic OC (2^−ΔCt) | Primary OC (2^−ΔCt) | p value     | Fold Change |
|----------------|------------------------|---------------------|-------------|-------------|
| hsa-miR-141-3p | 3.10E-02               | 1.70E-01            | 0.013728    | -5.64       |
| hsa-miR-187-5p | 9.50E-02               | 1.80E-01            | 0.031732    | -1.9        |
| hsa-miR-200a-3p| 5.60E-03               | 3.40E-02            | 0.023611    | -5.99       |
| hsa-miR-200b-3p| 1.20E-02               | 5.20E-02            | 0.022363    | -4.53       |
| hsa-miR-584-5p | 5.40E-02               | 8.50E-02            | 0.032832    | -1.58       |
| hsa-miR-7-5p   | 2.20E-01               | 3.60E-01            | 0.020499    | -1.65       |
| hsa-miR-9-3p   | 1.90E-03               | 3.40E-03            | 0.040069    | -1.76       |

Notes: hsa, Homo sapiens; miR, microRNA; OC, ovarian cancer
Table 4
Significantly up-regulated mRNAs in metastatic OC compared with primary OC

| Genes | Metastatic OC (2^−ΔCt) | Primary OC (2^−ΔCt) | P value | Fold regulation |
|-------|------------------------|----------------------|---------|-----------------|
| BCL2L1 | 4.70E-02               | 2.30E-02             | 0.047085 | 2.07            |
| TGFβ2  | 2.00E-03               | 3.50E-04             | 0.017231 | 5.68            |
| TNC    | 5.20E-02               | 2.50E-03             | 0.006661 | 20.7            |
| HOXB5  | 6.10E-04               | 1.00E-04             | 0.035099 | 6.11            |
| HOXB7  | 1.20E-02               | 2.50E-03             | 0.033375 | 4.9             |
| HOXB8  | 4.10E-02               | 5.70E-03             | 0.046631 | 7.19            |
| ITGB3  | 5.60E-03               | 1.40E-03             | 0.021938 | 3.9             |
| MTSS1  | 5.10E-03               | 1.00E-03             | 0.034451 | 5.08            |

Notes: BCL2L1, B-Cell CLL/lymphoma 2-like 1; TGFβ2, transforming growth factor beta 2; TNC, tenasin-C; HOX, homeobox; ITGB3, integrin beta-3; MTSS1, metastasis suppressor 1; OC, ovarian cancer

Table 5
Significantly down-regulated mRNAs in metastatic OC compared with primary OC

| Genes | Metastatic OC (2^−ΔCt) | Primary OC (2^−ΔCt) | P value | Fold change |
|-------|------------------------|----------------------|---------|-------------|
| FOXA2 | 1.70E-05               | 1.10E-04             | 0.034065 | -6.48       |
| PAK1  | 2.60E-03               | 5.40E-03             | 0.006704 | -2.04       |

Notes: FOXA2, forkhead box A2; PAK1, p21-activated kinase

ITGB3, TGFβ2 and TNC are associated with miRNA function in metastatic OC

To explore which mRNAs affected miRNA function in metastatic OC, GO analysis of the function of differently expressed mRNAs was undertaken. KEGG pathway analysis was applied to the pathways associated with OC. The results of GO analysis are presented in Fig. 2. For Biological Processes, differentially expressed genes mostly participated in anatomical structure morphogenesis, embryo development, cell differentiation and cellular developmental processes (p < 0.001, Enrichment score > 8; Table 6). For cellular components, differentially expressed genes mostly participated in ruffles, adherens junction, anchoring junction and cell junctions (p < 0.001, Enrichment score > 3.3; Table 7).

For molecular functions, differentially expressed genes mostly took part in RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription (p < 0.001, Enrichment score > 3; Table 8). The results of KEGG enrichment analysis are presented in Fig.s 3–4. Pronounced differences between 10 pathways were found, of which ITGB3, TGFβ2 and TNC were linked to the pathway-MicroRNAs in Cancer.
Table 6
Nodes in Biological Processes found by Gene Ontology

| GO.ID     | Term                                      | Count | P value    | Enrichment score |
|-----------|-------------------------------------------|-------|------------|------------------|
| GO:0009653 | anatomical structure morphogenesis        | 12    | 7.67E-11   | 10.11505         |
| GO:0009790 | embryo development                         | 9     | 1.88E-09   | 8.72576          |
| GO:0030154 | cell differentiation                       | 12    | 4.76E-09   | 8.322078         |
| GO:0048869 | cellular developmental process             | 12    | 9.76E-09   | 8.010594         |
| GO:0048729 | tissue morphogenesis                       | 7     | 2.67E-08   | 7.573257         |
| GO:0048731 | system development                         | 12    | 3.86E-08   | 7.412918         |
| GO:0035295 | tube development                           | 7     | 5.16E-08   | 7.287302         |
| GO:0043009 | chordate embryonic development             | 7     | 6.66E-08   | 7.176411         |
| GO:0009792 | embryo development ending in birth or egg hatching | 7     | 7.21E-08   | 7.142            |
| GO:0060429 | epithelium development                     | 8     | 1.02E-07   | 6.989655         |

Table 7
Nodes in Cellular Components found by Gene Ontology

| GO.ID      | Term                  | Count | P value    | Enrichment score |
|------------|-----------------------|-------|------------|------------------|
| GO:0001726 | ruffle                | 3     | 0.000125   | 3.901568         |
| GO:0005912 | adherens junction     | 4     | 0.000167   | 3.777312         |
| GO:0070161 | anchoring junction     | 4     | 0.000195   | 3.709575         |
| GO:0030054 | cell junction          | 5     | 0.000474   | 3.324369         |
| GO:0031091 | platelet alpha granule | 2     | 0.000817   | 3.087852         |
| GO:0031252 | cell leading edge      | 3     | 0.001105   | 2.956574         |
| GO:0030175 | filopodium             | 2     | 0.00113    | 2.946761         |
| GO:0032587 | ruffle membrane        | 2     | 0.00113    | 2.946761         |
| GO:0005902 | microvillus            | 2     | 0.001389   | 2.857181         |
| GO:0005925 | focal adhesion         | 3     | 0.001841   | 2.73493          |
Table 8
Nodes in Molecular Functions found by Gene Ontology

| GO.ID     | Term                                                                 | Count | P value      | Enrichment score |
|-----------|----------------------------------------------------------------------|-------|--------------|------------------|
| GO:0001205| RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription | 2     | 0.000122     | 3.911881         |
| GO:0003705| RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity | 2     | 0.001717     | 2.765282         |
| GO:0043565| sequence-specific DNA binding                                         | 4     | 0.001875     | 2.727084         |
| GO:0003700| sequence-specific DNA binding transcription factor activity           | 4     | 0.006255     | 2.203762         |
| GO:0001071| nucleic acid binding transcription factor activity                    | 4     | 0.006277     | 2.202281         |
| GO:0042802| identical protein binding                                            | 4     | 0.006603     | 2.180266         |
| GO:0050839| cell adhesion molecule binding                                       | 2     | 0.006752     | 2.170542         |
| GO:0005172| vascular endothelial growth factor receptor binding                  | 1     | 0.007614     | 2.118391         |
| GO:0051400| BH domain binding                                                    | 1     | 0.007614     | 2.118391         |
| GO:0019904| protein domain specific binding                                       | 3     | 0.008101     | 2.091458         |

Screening of miRNA-mRNA with potential target relationship
Through miRNA microarray analysis, miRNA-mRNA with a potential target relationship was screened and their binding sites analyzed on a bioinformatics prediction website (Fig. 5).

Hsa-miR-7-5p and TGFβ2 selected as target miRNAs
The expression of four miRNAs and TGFβ2 and TNC mRNA expression were determined by RT-qPCR.

As documented in Fig. 6, hsa-miR-7-5p, hsa-miR-141-3p and miR-187-5p expression was markedly decreased in metastatic compared with primary OC tissue (p < 0.05). As displayed in Fig. 7, TGFβ2 and TNC mRNA expression was enhanced in metastatic OC compared with primary OC tissue (p < 0.05). Results coincided with the results of microarray analysis. TGFβ2 was chosen as our target gene.

The protein level of TGFβ2 is elevated in metastatic OC tissue
TGFβ2 protein level was evaluated by western blot. TGFβ2 protein level was elevated in metastatic OC tissue versus that in primary OC tissue (p < 0.05; Fig. 8). The enhanced TGFβ2 may contribute to metastasis in OC progression.

Negative regulation of TGFβ2 by hsa-miR-7-5p
The relationship of hsa-miR-7-5p with TGFβ2 was evaluated by dual luciferase reporter assay. As demonstrated in Fig. 9, luciferase activity was severely decreased after co-transfection of wild-type TGFβ2 3'-UTR plasmid and hsa-miR-7-5p; however, a marked change in mutant TGFβ2 3'UTR plasmid was not observed. Hsa-miR-7-5p directly bound TGFβ2 3'-UTR.

Discussion

MicroRNAs play a pivotal part in multiple cellular functions such as proliferation, apoptosis, differentiation and development. However, aberrant miRNAs occurs in carcinogenesis and metastasis during cancer progression [8]. Mounting evidence highlights a crucial role of miRNAs in OC progression. For instance, alterations in miR-101, miR-206, miR-200a and miR-203 are associated with OC cell proliferation and invasion [9-11]. In recent years, increasing numbers of studies elucidated that miRNAs participated in the invasive and metastatic processes of tumors. One miRNA can regulate nearly 100 different mRNAs; in addition, a single mRNA can bind several different miRNAs, thus forming a miRNA-target gene network regulatory relationship [12, 13]. Different types of cancer samples show varying gene expression patterns; even the same miRNAs often regulate different target genes in different types of cancer. In studies on OC, the application of a microRNA chip technique is helpful in identifying differentially expressed miRNAs affecting proliferation, apoptosis, invasion and metastasis in OC in a more rapid and comprehensive manner. Using a microRNA chip technique, Fu et al. [14] screened and identified eight differentially expressed miRNAs, including miR-93 from cisplatin-resistant OC cell strains and OC parental cell strains. With the application of a miRCURY LNA™ microRNA Array chip technique, Liu et al. [15] screened and identified a total of 31 differentially expressed miRNAs from another two pairs of cisplatin-resistant OC cell lines, of which the expression of 21 miRNAs was up-regulated and that of 10 miRNAs was down-regulated. Cheng et al.[16] found by using an Affymetrix miRNA 3.0 Array chip that 37 miRNAs were differentially expressed in adult and juvenile granulosa cell tumors (p < 0.05, fold change ≥ 2), and that these could be used as markers for the diagnosis and recurrence of ovarian granulosa cell tumors.

In studies on the mechanism of metastasis in OC, it is critical to screen differentially expressed
miRNAs influencing the invasion and metastasis of OC. Conventional miRNA detection platforms only support the detection of miRNA expression but fail to allow the parallel detection of miRNA and target genes; as a result, it is impossible to comprehensively understand microRNA-target gene regulatory networks. In our study, we analyzed the miRNA expression profiles of primary OC tissues and their respective metastases from six patients using a miRStar™ Human Cancer Focus miRNA and Target mRNA PCR Array chip. This chip contained 184 cancer-related miRNAs and 178 target mRNAs. All 184 miRNAs were carefully screened and identified from the latest literature, including miRNAs relating to such common cancers as gastric, liver, lung, breast, colorectal, and prostate. Numerous studies provided support for 178 target mRNAs, and their expression correlated to the functional activity of miRNAs in tumors. With an innovative experimental design, the miRStar™ Human Cancer Focus miRNA and Target mRNA PCR Array chip allows the synchronous detection of miRNA and target mRNA. It can be used not only to rapidly detect expression changes of cancer-related miRNAs in test samples, but can also be used to evaluate the activity of these miRNAs against target mRNAs, thus facilitating the screening of core functional miRNAs in diseases and the rapid comprehension of their regulatory mechanisms and functions.

It was found that 15 miRNAs and 10 mRNAs were dysregulated in both primary and metastatic OC tissues. Meanwhile, miRNAs and mRNAs with a possible targeting relationship were obtained by microRNA-mRNA conjoint analysis. By using our chip and combining the experimental results obtained from microRNA and mRNA chips, and the famous target gene prediction websites, targetscan and Miranda, possible target genes were predicted as follows: TGFβ2, MTSS1, HOXB5 and TNC (for miR-200a-3p); TGFβ2, MTSS1 and HOXB5 (for miR-141-3p); TGFβ2, CD34 and HOXB5 (for miR-7-5p); and HOXB5 (for miR-187-5p). Furthermore, GO and Pathway analysis was performed on mRNAs with a substantial difference in microRNA-mRNA conjoint analysis. GO analysis consisted of three parts: Biological Process, Cellular Component and Molecular Function. For Biological Process, we identified a total of 668 molecular function nodes showing a prominent difference (p < 0.05); differential genes were mainly involved in anatomical structure morphogenesis, embryo development, cell differentiation and cellular developmental processes. For Cellular Component, we identified a total of
52 molecular function nodes showing a remarkable difference (p < 0.05); differential genes primarily participated in ruffles, adherens junctions, anchoring junctions and cell junctions. For Molecular Function, we identified a total of 39 molecular function nodes showing a noteworthy difference (p < 0.05). Differential genes dominantly joined in RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity involved in the positive regulation of transcription. In this study, we listed Enrichment Score Top 10 nodes in Biological Process, Cellular Component and Molecular Function. Using Pathway analysis, we found 21 strikingly different pathways (p < 0.05) and listed the top 10 appreciably different pathways, of which the pathway of MicroRNAs in Cancer was associated with TGFβ2, TNC and ITGB3. Based on these study results, we found several differentially expressed miRNAs and mRNAs, as well as miRNAs and mRNAs with a possible targeted regulatory relationship in metastatic and primary OC tissues that may play a critical part in invasion and metastasis links of OC.

As is well known, miRNAs can post-transcriptionally mediate a number of genes through a combination of specific sequences in target mRNA molecules [17]. MicroRNAs were differently expressed in OC [18]. Furthermore, miR-187 has been found to exert a dual role in OC by regulating the disabled homolog-2 gene [19]. Gao et al. also found that miR-141 acted as a potential diagnostic and prognostic biomarker for OC [20], which was concurred with our results. Zhu et al. demonstrated that miR-7-5p suppresses cell migration and invasion by targeting SOX18 in pancreatic ductal adenocarcinoma [21]. Another study also claimed that miR-7-5p under-expression was associated with recurrence in glioblastoma patients, and that its overexpression decreased glioblastoma cell stemness [22]. Furthermore, it was also elucidated that ectopic expression of miR-7 functioned as an anti-oncogene in OC by repressing cell invasion and proliferation [7]. MiR-7-5p has been found to target and regulate the genes as SATB1 and PARP1 in some cancer diseases as we have mentioned before [23, 24]. Furthermore, in this study, we found that TGFβ2 was targeted by hsa-miR-7-5p. The results of miRNA-mRNA conjoint analysis with our chip showed that hsa-miR-7-5p and TGFβ2 were differentially expressed in metastatic and primary OC tissues at a striking level, and may have a targeted regulatory relationship. To confirm this, we further validated
the expression of four miRNAs (including hsa-miR-7-5p), TGFβ2 and TNC in 25 pairs of metastatic and primary OC tissues, a larger sample size. We found that TGFβ2 was negatively targeted by miR-7-5p using a dual luciferase reporter assay. MiR-7-5p may have several target genes similar to miR-137; such target genes are critical oncogenic factors, including TGFβ2, that could further regulate brain tumorigenesis [25]. In addition, we found that OC metastases expressed higher levels of TGFβ2 and TNC compared with primary OC tissues. This suggested that TGFβ2 was closely associated with OC metastasis. In studying potential mechanisms, we identified TGFβ2 as an mRNA target using bioinformatics analysis, as well as functional and binding assays. Taken together, our findings suggested that hsa-miR-7-5p and TGFβ2 were inversely correlated with regard to mRNA and protein expression; they demonstrated a targeted regulatory relationship that could influence the invasive and metastatic processes of OC. The TGFβ pathway takes part in many cellular processes, including cell proliferation, differentiation, extracellular matrix accumulation, tissue repair, immune and inflammatory responses. TGFβ2 is an isoform of TGFβ in mammals [26] and is abnormally expressed in various cancers such as human melanoma and hepatocellular carcinoma [27, 28]. In addition, many researchers have also explored the role of TGFβ in OC. Cao and his colleagues reported that TGFβ-induced transglutaminase gave rise to EMT and a cancer stem cell phenotype that consequently enhanced ovarian tumor metastasis [29]. TGFβ induced EMT and a more invasive phenotype in epithelial OC cells in collaboration with the EGF pathway, indicating TGFβ may be a promising target candidate for the treatment of metastatic OC in future [30]. TNC is an extracellular matrix glycoprotein that shows forced expression in cell proliferation and migration, and in EMT during organogenesis [31]. Moreover, a recent study highlighted that serum TNC levels were much higher in patients with epithelial OC than in normal controls. Such high serum TNC levels were related to poorer overall survival, which was consistent with our findings [32].

Conclusion
In conclusion, our study suggested that several differentially expressed miRNAs and mRNAs exist as putative target genes of these miRNAs in primary and metastatic OC tissues, which may mediate invasion and metastasis by OC. As shown by PCR validation with a larger sample size, three miRNAs
(hsa-miR-141-3p, hsa-miR-187-5p, and hsa-miR-7-5p) in metastatic OC tissues were obviously down-regulated versus primary OC tissues, while TGFβ2 and TNC were markedly up-regulated. Hsa-miR-7-5p and TGFβ2 were negatively correlated in terms of mRNA and protein expression. Hsa-miR-7-5p directly bound the TGFβ2 3′-UTR to inhibit its expression and thus affect the invasive and metastatic processes of OC. Hsa-miR-7-5p may prove to be a potential strategy for targeted metastatic OC therapies. However, several limitations existed in the present study. Firstly, the present study had a small sample size. Thus, it is necessary to conduct further studies with a larger number of samples to validate our results. Furthermore, future studies will be required to investigate the underlying molecular mechanisms between hsa-miR-7-5p and metastatic OC.

**Abbreviations**

OC, Ovarian cancer; RT-qPCR, reverse transcription quantitative polymerase chain reaction; OD, optical density; KEGG, Kyoto Encyclopedia of Genes and Genomes; TBST, Tris-buffered saline; GAPDH, Glyceraldehyde phosphate dehydrogenase; NC, negative control;

**Declarations**

**Authors’ contributions**

Yang Gu wrote the main manuscript text, Shulan Zhang collected the data, and prepared all tables. All authors reviewed the manuscript.

**Author details**

Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, Shenyang 110004, P. R. China

**Competing interests**

The authors have declared that no competing interests exist.

**Availability of data and materials**

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable
Ethics approval and consent to participate

All 31 pairs of metastatic and primary OC tissues were collected from patients who had received surgery at Shengjing Hospital of China Medical University from 2014-2016. Of these, six pairs were used for chip experiments and 25 pairs were employed for RT-qPCR validation. Tissue samples resected during operations were immediately placed into 1.5-mL centrifuge tubes with RNase-removal high-pressure treatment, rapidly placed in liquid nitrogen for quick freezing, and then transferred to a -80°C cryogenic freezer for preservation. All patients were diagnosed with ovarian serous cystadenocarcinoma by postoperative paraffin pathology, and their clinical staging was made according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for ovarian cancer (2009). None of the patients had received radiotherapy, chemotherapy or other special treatments prior to surgery. Patients who provided tissue specimens for chip experiments were aged 48–64 years (mean: 57.5 years), and those providing samples for RT-qPCR validation were aged 38–80 years (median: 57 years). Data and tissues were harvested upon receiving of the informed consent of patients and approval by the ethics committee of Shengjing Hospital of China Medical University.

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References

1. Moufarrij S, Dandapani M, Arthofer E, Gomez S, Srivastava A, Lopez-Acevedo M, Villagra A, Chiappinelli KB. Epigenetic therapy for ovarian cancer: promise and progress. Clin Epigenetics. 2019;11:7.

2. Franier B, Thompson M. Early stage detection and screening of ovarian cancer: A research opportunity and significant challenge for biosensor technology. Biosens Bioelectron. 2019;135:71-81.

3. Emmings E, Mullany S, Chang Z, Landen CN, Jr., Linder S, Bazzaro M. Targeting Mitochondria for Treatment of Chemoresistant Ovarian Cancer. Int J Mol Sci. 2019;20:
4. Hoter A, Naim HY. Heat Shock Proteins and Ovarian Cancer: Important Roles and Therapeutic Opportunities. Cancers (Basel). 2019;11:

5. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol. 2019;20:21-37.

6. Chen SN, Chang R, Lin LT, Chern CU, Tsai HW, Wen ZH, Li YH, Li CJ, Tsui KH. MicroRNA in Ovarian Cancer: Biology, Pathogenesis, and Therapeutic Opportunities. Int J Environ Res Public Health. 2019;16:

7. Cui X, Sun Y, Shen M, Song K, Yin X, Di W, Duan Y. Enhanced Chemotherapeutic Efficacy of Paclitaxel Nanoparticles Co-delivered with MicroRNA-7 by Inhibiting Paclitaxel-Induced EGFR/ERK pathway Activation for Ovarian Cancer Therapy. ACS Appl Mater Interfaces. 2018;10:7821-31.

8. Lin CW, Chang YL, Chang YC, Lin JC, Chen CC, Pan SH, Wu CT, Chen HY, Yang SC, Hong TM, Yang PC. MicroRNA-135b promotes lung cancer metastasis by regulating multiple targets in the Hippo pathway and LZTS1. Nat Commun. 2013;4:1877.

9. Semaan A, Qazi AM, Seward S, Chamala S, Bryant CS, Kumar S, Morris R, Steffes CP, Bouwman DL, Munkarah AR, Weaver DW, Gruber SA, Batchu RB. MicroRNA-101 inhibits growth of epithelial ovarian cancer by relieving chromatin-mediated transcriptional repression of p21(waf(1)/cip(1)). Pharm Res. 2011;28:3079-90.

10. Li S, Li Y, Wen Z, Kong F, Guan X, Liu W. microRNA-206 overexpression inhibits cellular proliferation and invasion of estrogen receptor alpha-positive ovarian cancer cells. Mol Med Rep. 2014;9:1703-8.

11. Wang S, Zhao X, Wang J, Wen Y, Zhang L, Wang D, Chen H, Chen Q, Xiang W. Upregulation of microRNA-203 is associated with advanced tumor progression and poor prognosis in epithelial ovarian cancer. Med Oncol. 2013;30:681.

12. Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP. Expanding the
microRNA targeting code: functional sites with centered pairing. Mol Cell. 2010;38:789-802.

13. de Giorgio A, Krell J, Harding V, Stebbing J, Castellano L. Emerging roles of competing endogenous RNAs in cancer: insights from the regulation of PTEN. Mol Cell Biol. 2013;33:3976-82.

14. Fu X, Tian J, Zhang L, Chen Y, Hao Q. Involvement of microRNA-93, a new regulator of PTEN/Akt signaling pathway, in regulation of chemotherapeutic drug cisplatin chemosensitivity in ovarian cancer cells. FEBS Lett. 2012;586:1279-86.

15. Liu MX, Siu MK, Liu SS, Yam JW, Ngan HY, Chan DW. Epigenetic silencing of microRNA-199b-5p is associated with acquired chemoresistance via activation of JAG1-Notch1 signaling in ovarian cancer. Oncotarget. 2014;5:944-58.

16. Cheng WT, Rosario R, Muthukaruppan A, Wilson MK, Payne K, Fong PC, Shelling AN, Blenkiron C. MicroRNA profiling of ovarian granulosa cell tumours reveals novel diagnostic and prognostic markers. 2017;9:72.

17. Elton TS, Yalowich JC. Experimental procedures to identify and validate specific mRNA targets of miRNAs. EXCLI J. 2015;14:758-90.

18. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol. 2008;110:13-21.

19. Chao A, Lin CY, Lee YS, Tsai CL, Wei PC, Hsueh S, Wu TI, Tsai CN, Wang CJ, Chao AS, Wang TH, Lai CH. Regulation of ovarian cancer progression by microRNA-187 through targeting Disabled homolog-2. Oncogene. 2012;31:764-75.

20. Gao YC, Wu J. MicroRNA-200c and microRNA-141 as potential diagnostic and prognostic biomarkers for ovarian cancer. Tumour Biol. 2015;36:4843-50.

21. Zhu W, Wang Y, Zhang D, Yu X, Leng X. MiR-7-5p functions as a tumor suppressor by targeting SOX18 in pancreatic ductal adenocarcinoma. Biochem Biophys Res
22. Jia B, Liu W, Gu J, Wang J, Lv W, Zhang W, Hao Q, Pang Z, Mu N, Zhang W, Guo Q. MiR-7-5p suppresses stemness and enhances temozolomide sensitivity of drug-resistant glioblastoma cells by targeting Yin Yang 1. Exp Cell Res. 2019;375:73-81.

23. Yin CY, Kong W, Jiang J, Xu H, Zhao W. miR-7-5p inhibits cell migration and invasion in glioblastoma through targeting SATB1. Oncol Lett. 2019;17:1819-25.

24. Lai J, Yang H, Zhu Y, Ruan M, Huang Y, Zhang Q. MiR-7-5p-mediated downregulation of PARP1 impacts DNA homologous recombination repair and resistance to doxorubicin in small cell lung cancer. BMC Cancer. 2019;19:602.

25. Zhao J, Tao Y, Zhou Y, Qin N, Chen C, Tian D, Xu L. MicroRNA-7: a promising new target in cancer therapy. Cancer Cell Int. 2015;15:103.

26. Xie B, Zhang C, Kang K, Jiang S. miR-599 Inhibits Vascular Smooth Muscle Cells Proliferation and Migration by Targeting TGFB2. PLoS One. 2015;10:e0141512.

27. Li JR, Wang JQ, Gong Q, Fang RH, Guo YL. MicroRNA-328 inhibits proliferation of human melanoma cells by targeting TGFbeta2. Asian Pac J Cancer Prev. 2015;16:1575-9.

28. Dropmann A, Dediulia T, Breitkopf-Heinlein K, Korhonen H, Janicot M, Weber SN, Thomas M, Piiper A, Bertran E, Fabregat I, Abshagen K, Hess J, Angel P, Coulouarn C, Dooley S, Meindl-Beinker NM. TGF-beta1 and TGF-beta2 abundance in liver diseases of mice and men. Oncotarget. 2016;7:19499-518.

29. Cao L, Shao M, Schilder J, Guise T, Mohammad KS, Matei D. Tissue transglutaminase links TGF-beta, epithelial to mesenchymal transition and a stem cell phenotype in ovarian cancer. Oncogene. 2012;31:2521-34.

30. Xu Z, Jiang Y, Steed H, Davidge S, Fu Y. TGFbeta and EGF synergistically induce a more invasive phenotype of epithelial ovarian cancer cells. Biochem Biophys Res
31. Yoshida T, Akatsuka T, Imanaka-Yoshida K. Tenascin-C and integrins in cancer. Cell Adh Migr. 2015;9:96-104.

32. Didem T, Faruk T, Senem K, Derya D, Murat S, Murat G, Oznur K. Clinical significance of serum tenascin-c levels in epithelial ovarian cancer. Tumour Biol. 2014;35:6777-82.

Figures
A number of miRNAs and mRNAs showed abnormal expression in OC. Panel A, MicroRNAs (miRNAs) were up- or down-regulated in metastatic ovarian cancer (OC) tissues compared with primary OC tissues. Panel B, mRNAs were up- or down-regulated in metastatic OC tissues compared with primary OC tissues. The color red represents up-regulated expression and the color green represents down-regulated expression. hsa, Homo sapiens; miR; microRNA; BCL2L1, B-Cell CLL/lymphoma 2-like 1; TGFβ2, transforming growth factor beta 2; TNC, tenascin-C; FOXA2, forkhead box A2; HOXB5, homeobox B5; HOXB7, homeobox B7; HOXB8, homeobox B8; ITGB3, integrin beta-3; MTSS1, metastasis suppressor 1; PAK1, p21-activated kinase.
Differentially expressed genes in ovarian cancer are associated with 10 functional gene ontology (GO) terms of predicted microRNA (miRNA) target genes by GO analysis.
Figure 3

Top ten pathways regulated by differentially expressed genes in ovarian cancer. HLTV-1, human T cell leukemia virus type 1; ECM, extracellular matrix; DE gene, differentially expressed gene.
ITGB3, TGFβ2 and TNC genes participate in regulation of miRNA in cancer. TGFβ2, transforming growth factor beta 2; TNC, tenascin-C; ITGB3, integrin beta-3; miRNAs, microRNAs; KEGG, Kyoto Encyclopedia of Genes and Genomes; CRC, colorectal cancer; FGFR3, fibroblast growth factor receptor 3; CIS, cisplatin; EMT, epithelial-mesenchymal transition.
Some miRNA–mRNAs have a potential target relationship. Panel A, Micro (mi)RNA microarray analysis revealed the target genes of miR-141-3p, miR-7-5p, miR-187-5p, miR-200a-3p, and miR-200b-3p. Panels B and C, Bioinformatics prediction indicated that there were binding sites between the target genes and miR-141-3p, miR-7-5p, miR-187-5p, miR-200a-3p, and miR-200b-3p. TGFβ2, transforming growth factor beta 2; TNC, tenascin-C; HOXB5, homeobox B5; MTSS1, metastasis suppressor 1.

The expression of hsa-miR-7-5p, miR-187-5p, and miR-141-3p is decreased in metastatic OC tissue as measured by RT-qPCR. *p < 0.05 vs. primary OC tissues; hsa, Homo sapiens; miR, microRNA; RT-qPCR, reverse transcription quantitative polymerase chain reaction.
The mRNA expression of TGFβ2 and TNC are elevated in metastatic OC tissue as measured by RT-qPCR. Panels A and B, The mRNA expression of TGFβ2 and TNC in metastatic OC tissue increased when compared with primary OC tissue as shown in bar graphs. Panels C and D, The mRNA expression of TGFβ2 and TNC in metastatic OC tissue increased when compared with primary OC tissue as shown in scatter plots; *p < 0.05 vs. primary OC tissue; TGFβ2, transforming growth factor beta 2; TNC, tenascin-C; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

Elevated TGFβ2 protein level in metastatic OC tissue detected by western blot analysis. Panel A, The protein level of TGFβ2 in metastatic OC tissue was increased when compared with primary OC tissue as shown by gray protein bands. Panel B, The protein level of TGFβ2 in metastatic OC tissue was increased when compared with primary OC tissue as shown by bar graphs. *p < 0.05 vs. metastasis OC tissue; TGFβ2, transforming growth factor beta 2; GAPDH, glyceraldehyde phosphate dehydrogenase.
A. Predicted binding site of miR7-5p on TGFβ2 3′-UTR. B. Hsa-miR-7-5p inversely modulated the expression of TGFβ2 as detected by dual luciferase reporter assay; ***p < 0.001 vs. 3′-UTR-NC + miRNA group; miRNA, microRNA; NC, negative control; MT, mutant; UTR, untranslated region; hsa, Homo sapiens.