MicroRNA-302b Suppresses Human Epithelial Ovarian Cancer Cell Growth by Targeting RUNX1

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Key Words
miR-302b • RUNX1 • Epithelial ovarian carcinoma • STAT3

Abstract

Background: The microRNA (miR)-302 family functions as a tumor suppressor in human cancer. However, its role in epithelial ovarian carcinoma (EOC) remains unknown. Here, we investigated the role of miR-302b and its target gene RUNX1 in EOC. Methods: The expression levels of miR-302b and RUNX1 were assessed by quantitative real-time PCR and western blotting. The effects of ectopic expression of miR-302b were evaluated by the MTT assay, colony forming assay and flow cytometry. RUNX1 was identified as a target of miR-302b and their interaction was confirmed by luciferase activity assays, RUNX1 silencing and overexpression of a RUNX1 mutant construct lacking the 3′UTR. The effect of miR-302b on the suppression of tumor growth was investigated in vivo in a xenograft mouse model. Results: Micro-302b levels were markedly decreased in EOC specimens. Ectopic expression of miR-302b in EOC cells inhibited cell proliferation and colony formation, induced G0/G1 arrest, and promoted apoptosis. RUNX1 was identified as a direct target of miR-302b, and knockdown of RUNX1 inhibited cell growth in a manner similar to miR-302b overexpression, whereas introduction of a 3′UTR mutant of RUNX1 reversed the suppressive effect of miR-302b. Furthermore, miR-302b overexpression led to the inactivation of the STAT3 signaling pathway in EOC cells and inhibited tumor growth in a xenograft mouse model. Conclusions: MiR-302b functions as a tumor suppressor in EOC by targeting RUNX1 and modulating the activity of the STAT3 signaling pathway.
Introduction

Runt-related transcription factor 1 (RUNX1) belongs to a small family of heterodimeric transcription factors involved in cell lineage determination that play a role in various types of cancer by activating or repressing the transcription of key regulators of growth, survival and differentiation [1]. RUNX1 is one of the most frequently mutated genes in human leukemias [2]. RUNX proteins have been implicated in the transcriptional regulation of cell cycle related proteins such as Cyclin D1 and p21, and RUNX expression levels vary during the cell cycle. RUNX1 levels increase by two- to four-fold during the S and G2/M phases compared to the G1 phase in hematopoietic cells, and experiments have suggested that RUNX1 is a positive regulator of the G1 to S transition [3, 4]. RUNX1 can act as a tumor suppressor or an oncogene in different cells and tissues through the regulation of cancer-related genes such as p21 and signal transducer and activator of transcription 3 (STAT3) [5]. RUNX1 represses p21 and promotes the activation of STAT3, a transcription factor with oncogenic properties, by facilitating its phosphorylation through the Jak/Stat pathway [6, 7]. Phosphorylation of STAT3 on its conserved tyrosine residue promotes its homodimerization and translocation to the nucleus to regulate the transcription of genes involved in survival, proliferation and invasion, and constitutive activation of STAT3 is associated with malignant transformation and poor clinical outcomes in several cancers [8-10]. In keratinocytes and skin cancer cells, RUNX1 maintains STAT3 in an active state by repressing cytokine signaling suppressors (SOCS) [6]. Recently, RUNX1 was shown to be upregulated in human epithelial ovarian carcinoma (EOC) tissues and associated with EOC cell proliferation, migration and invasion [11].

MicroRNAs (miRNAs) are small (22-nucleotide) noncoding single stranded RNAs that regulate gene expression by binding to the 3′ untranslated region (3′UTR) of their target mRNAs, modulating mRNA stability and/or translation [12]. An increasing body of evidence suggests that miRNAs play a role in the pathogenesis of cancer through the modulation of genes and signaling pathways involved in tumorigenesis [13]. In EOC, miRNA profiling studies have identified miRNAs associated with tumor progression and chemotherapy resistance [14-16]. MiR-302b is a member of the miR-302 cluster, which consists of four homologous miRNAs that are transcribed as a noncoding RNA cluster and are specifically expressed in human and mouse embryonic stem cells [17]. MiR-302b acts as a tumor suppressor in esophageal squamous cell carcinoma and it is downregulated in hepatocellular carcinoma, where it inhibits cell proliferation by targeting EGFR [18, 19]. Although genome-wide miRNA expression profiling studies showed that miR-302b is downregulated in ovarian carcinoma [14, 20], the exact role of miR-302b in EOC remains unknown.

In the present study, we showed that miR-302b is downregulated in EOC tissues compared to normal ovarian tissues. Ectopic expression of miR-302b inhibited cell proliferation and colony formation, induced G0/G1 cell cycle arrest and apoptosis in ovarian cancer cells, and inhibited tumor formation in a xenograft mouse model. We identified RUNX1 as a direct target of miR-302b, and showed that its tumor suppressor effect is mediated by the downregulation of RUNX1 and the inhibition of STAT3 signaling.

Materials and Methods

Clinical specimens and cell lines

This study was approved by the Ethics Committee of Harbin Medical University. Animal experiments were performed with the approval of the Animal Ethical Committee of Harbin Medical University and in conformity with national guidelines for the care and use of laboratory animals. Human EOC specimens and normal ovarian tissues were collected from Affiliated Tumor Hospital of Harbin Medical University after obtaining written informed consent from all patients. Tissues were snap-frozen in liquid nitrogen and stored at -80 °C following surgery for qRT-PCR. The human EOC cell lines SKOV3 and OVCAR3 were purchased from
American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained in McCoy’s 5A medium and RPMI-1640, respectively, supplemented with 10% fetal bovine serum (FBS).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. For miR-302b analysis, RT primer and real-time primer sequences used were as follows: RT primer, 5′-GTCGTATCCAGTCCAGGGACCGAGGACTGGATAGACCTACTA-3′; miR-302b forward, 5′-CGTAATGTGTTACCATGTTT-3′ and miR-302b reverse, 5′-TCCAGGACCGAGGA-3′. For the detection of RUNX1 expression, the following primers were used: RUNX1 forward, 5′-CGAAGACATCGGCAGAAA-3′ and reverse, 5′-TAAAGGGACTGAGGTGTTCA-3′. U6 and β-actin were used to normalize miRNA and mRNA respectively. All RT-PCR experiments were performed on a BioRad iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The expression levels relative to U6 and β-actin were calculated using the formula 2^−ΔΔCT.

Western blotting

For western blot analysis, protein lysates were prepared by resuspending cell pellets in Laemmli sample buffer containing 5% β-mercaptoethanol. Lysates were separated in 6-12% Tris-glycine gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking in 5% nonfat dry milk in TBST (20 mmol/L TRIS-HCl, 0.5 M NaCl and 0.1% Tween 20), membranes were incubated in primary antibodies at 4°C overnight, followed by three 15 min washes in TBST and incubation in horseradish peroxidase-conjugated secondary antibodies and signal detection using the enhanced chemiluminescence system (Thermo Fisher Scientific). Primary antibodies used were as follows: anti-RUNX1 (1:200), anti-Bcl-2 (1:500), anti-Bax (1:200), anti-β-actin (1:5,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-STAT3 (Tyr705) (1:1000), anti-Cyclin D1 (1:1000), and anti-Cleaved caspase-3 (Asp175) (1:1000) (Cell Signaling Technology, Danvers, MA, USA).

Generation of stable miR-302b-expressing EOC cells

A miR-302b-expression vector was generated by PCR-amplifying a DNA fragment encoding the miR-302b-precursor using the following primers: forward, 5′-CGGGATCCGCTCTTCAACTTTAACATGGAAGTGCTTTCTGTGACTTTAAA-3′ and reverse, 5′-CCCAAGCTTACTCCTACTAAAACATGGAAGCACTTACTTTTAAAGTC-3′, and cloning it into the BamHI and HindIII sites of the pSilencer vector (Life Technologies). The miR-302b carrying and control vectors were transfected into SKOV3 or OVCAR3 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), followed by selection with neomycin (Gibco, Grand Island, NY, USA) to generate stable cell lines.

Vector construction and luciferase reporter assay

The 3′UTR region of RUNX1 containing the wild-type or mutant potential target site for miR-302b was synthesized and inserted into the pMIR-reporter vector (Ambion, Carlsbad, CA, USA) at Spe I and Hind III sites. The RUNX1 3′UTR oligos were synthesized as follows: RUNX1-wt 3′UTR, sense, 5′-CTAGTATTATTGTCTTTACACACATGAGAGACTTTTCTGGTGAAGAA-3′ and antisense, 5′-AGCTTTCTTACAAATCGTACCAGTGTGTTAAACAATAAT-3′; RUNX1-mut 3′UTR, sense, 5′-CTAGTATTATTGTCTTTACACACATGAGAGACTTTTCTGGTGAAGAA-3′ and antisense, 5′-AGCTTTCTTACAAATCGTACCAGTGTGTTAAACAATAAT-3′. For luciferase assay, SKOV3 cells were co-transfected with RUNX1-wt 3′UTR or RUNX1-mut 3′UTR and miR-302b or anti-miR-302b (Ambion) or control using Lipofectamine 2000 (Invitrogen). Cell lysates were prepared 24 h after transfection, and luciferase activity was measured using a Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA).

RUNX1-expression vector without miR-302b binding sites

A full-length RUNX1 cDNA lacking the 3′UTR was generated by PCR amplification using the following primers: sense, 5′-GGGACATCCGCCACATGCTACGATCCCTCACA-3′ and antisense, 5′-GACGCCCTCAGTCCAGTACCAGGCTTCCCACA-3′. The construct was inserted into the pcDNA3.1(+) (Invitrogen) vector between the EcoR I and Xho I sites to generate a RUNX1 expression vector without miR-302b binding sites. RUNX1 siRNA and negative control oligonucleotides were purchased from GenePharma (Shanghai, China).
Cell Proliferation Assay
SKOV3 or OVCAR3 cells (2 × 10^4 cells/ml) were seeded in 96-well plates 24 h post-transfection. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 20 µl, 5 mg/ml) (Sigma-Aldrich, St Louis, MO, USA) was added to each well and cells were incubated for another 4 h. The reaction was stopped by addition of 150 µl DMSO. The absorbance of the solution was read at 570 nm using an iQuant Universal Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

 Colony formation
SKOV3 or OVCAR3 cells were treated as indicated and seeded at a density of 200 cells per 35-mm culture dish. After 14 days, the dishes were washed twice with PBS, fixed with cold methanol, stained with Giemsa solution (Sigma-Aldrich) for 15 min, washed with water and air-dried. The number of colonies was determined by imaging with a Multimage Cabinet (Alpha Innotech Corporation).

Cell cycle analysis
SKOV3 or OVCAR3 cells were harvested 48 h post-transfection, washed with prechilled PBS, fixed with 70% ethanol and stored at 4°C overnight. Cells were rehydrated with PBS for 10 min and stained with a propidium iodide (PI) solution containing 50 µg/ml PI, 2 µg/ml DNase-free RNase A and 0.2% NP-40 in PBS for 15 min at 37°C. Cell cycle analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Apoptosis assay
For detection of apoptotic cells, SKOV3 or OVCAR3 cells were harvested 48 h post-transfection, washed twice with prechilled PBS and resuspended in 100 µl binding buffer Annexin V and PI double staining was performed using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences) and flow cytometric analysis was performed on a FACSCalibur flow cytometer (BD Biosciences).

Tumor xenograft model and tumorigenicity assay
SKOV3 cells (5 × 10^6 cells/ml) stably transfected with miR-302b or control vector were subcutaneously injected into 4-week-old male nude mice. Tumor volume and weight was monitored over a period of 32 days. Tumor samples were analyzed by immunohistochemistry using anti-Ki-67 antibody (1:100) and anti-PCNA antibody (1:100, Dako, Carpinteria, CA, USA). MiR-302b and RUNX1 levels in xenograft tumor tissues were analyzed by qRT-PCR and western blot, respectively.

Statistical analysis
All results were expressed as mean ± SD. Data analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was analyzed using Student's t test or one-way ANOVA. Differences with $P<0.05$ were considered statistically significant.

Results
Decreased miR-302b and increased RUNX1 expression in human EOC specimens
The expression of miR-302b and RUNX1 was examined in 38 EOC and 16 normal ovarian tissues by qRT-PCR. The results showed significantly lower levels of miR-302b and significantly higher levels of RUNX1 mRNA in EOC tissues than in normal ovarian tissues ($P<0.001$ both) (Fig. 1A and B). Western blot analysis of RUNX1 protein expression showed a significant upregulation of RUNX1 in EOC compared to normal ovarian tissues ($P<0.01$). A representative blot and quantitative analysis of protein levels by densitometry are shown in Fig. 1C.

MiR-302b inhibits growth and promotes apoptosis in EOC cells
To determine the effect of miR-302b on EOC cell viability, SKOV3 or OVCAR3 ovarian cancer cells were transfected with miR-302b expressing vector, anti-miR-302b or their respective controls. The miR-302b vector caused a more than 20-fold upregulation of the
expression of miR-302b compared to the miR-control in both cell lines, whereas anti-miR-302b significantly downregulated the levels of miR-302b \((P<0.001)\) (Fig. 2A). Assessment of cell viability over a period of 72 h using the MTT assay showed significant inhibition of cell growth in miR-302b transfected cells in both cell lines \((P<0.01)\), whereas the anti-miR-302b construct significantly stimulated cell growth compared to the respective controls \((P<0.01)\) (Fig. 2B). Similar results were obtained in the colony formation assay, in which ectopic expression of miR-302b significantly inhibited colony forming ability compared to the controls in SKOV3 and OVCAR3 cells, whereas anti-miR-302b had the opposite effect \((P<0.01)\) (Fig. 2C). Analysis of cell cycle distribution showed that miR-302b significantly increased the number of cells at G0/G1 phase, with a concomitant decrease in the proportion of cells in S phase, and anti-miR-302b had the opposite effect (Fig. 2D). To determine the mechanism of cell death induced by miR-302b, SKOV3 or OVCAR3 cells transfected as described above were analyzed by Annexin V-FITC/PI flow cytometry. The results indicated that overexpression of miR-302b significantly increased the rate of apoptosis, whereas downregulation of its expression by anti-miR-302b had the opposite effect (Fig. 2E). Taken together, these results indicated that miR-302b inhibits the growth and colony forming ability of EOC cells by inducing cell cycle arrest at G0/G1 phase and promoting apoptosis.

**MiR-302b directly targets RUNX1**

Conserved binding sites for miR-302b in the 3’UTR of RUNX1 were identified in several species by TargetScan, PicTar, and DIANA-microT and are shown in Fig. 3A. A mutant construct was generated by modifying eight residues in the 3’UTR of RUNX1. To verify RUNX1 as a direct target of miR-302b, constructs containing wild-type (wt) and mutant (mut) RUNX1 3’UTR were cloned into a luciferase reporter vector and co-transfected with miR-302b or anti-miR-302b and their respective negative controls into SKOV3 cells. Luciferase activity was significantly decreased by miR-302b mimics only in the presence of RUNX1-wt 3’UTR, but not in cells carrying RUNX1-mut 3’UTR, and this effect was reversed by anti-miR-302b, confirming that miR-302b directly targets the 3’UTR of RUNX1 (Fig. 3B). To determine whether miR-302b affects the expression of RUNX1, miR-302b, anti-miR-302b and their respective negative controls were transfected into SKOV3 cells and the mRNA expression of RUNX1 was assessed by qRT-PCR, which showed a significant downregulation of RUNX1 expression by miR-302b, whereas anti-miR-302b had the opposite effect (Fig. 3C). Assessment of protein levels by western blotting showed that miR-302b downregulated RUNX1 protein levels by approximately two-fold, whereas anti-miR-302b caused an approximately two-fold increase in RUNX1 protein levels compared to the negative control \((P<0.01)\) (Fig. 3D).
RUNX1 mediates miR-302b induced growth inhibition and apoptosis promotion in SKOV3 cells

To further examine the regulation of RUNX1 expression by miR-302b and its effect on cell growth and apoptosis, a RUNX1 expression vector lacking the miR-302b binding site was generated and transfected into SKOV3 cells ectopically expressing miR-302b. In addition, RUNX1 expression was knocked down using a specific siRNA. Western blot analysis
showed that co-expression of miR-302b and empty vector significantly downregulated the expression of RUNX1 protein, whereas in the presence of the mutant RUNX1 construct, the levels of RUNX1 protein increased to a level above that of the control \((P<0.01)\) (Fig. 4A). SiRNA-mediated knockdown of RUNX1 significantly downregulated the expression of the RUNX1 protein, similar to the effect of miR-302b overexpression \((P<0.01)\) (Fig. 4A). We then examined the effects of these constructs on cell viability, colony forming ability, cell cycle progression and apoptosis induction. The results showed that while miR-302b overexpressing SKOV3 cells showed decreased cell viability, cell cycle arrest at the G0/G1 phase, decreased colony forming ability and a significantly increased rate of apoptosis, co-transfection with the mutant RUNX1 expression vector lacking the miR-302b 3'UTR binding site abolished these effects \((P<0.01\) all) (Fig. 4B-E). SiRNA mediated silencing of RUNX1 expression decreased cell viability, colony forming ability, caused cell cycle arrest at G0/G1 and increased the rate of apoptosis, similar to the effects of miR-302b overexpression (Fig. 4B-E). Taken together, these results confirm that RUNX1 is a direct target of miR-302b and mediates its effects on cell growth and apoptosis in ovarian cancer.

**MiR-302b inhibits the STAT3 pathway**

STAT3 is frequently activated in ovarian cancer and promotes tumor growth and survival by upregulating the expression of oncogenes such as Cyclin D1 and antiapoptotic proteins such as Bcl-2. In addition, STAT3 activation is induced by the suppression of the expression of SOCS3 and 4 by RUNX1, supporting the role of RUNX1/STAT3 signaling in promoting cell proliferation and tumorigenesis [6]. Therefore, we examined the potential effect of miR-302b
and its target RUNX1 on the activity of STAT3 and its downstream effectors in SKOV3 cells. Western blot analysis showed that miR-302b downregulated phospho-STAT3, Cyclin D1 and Bcl-2 and upregulated the pro-apoptotic protein Bax and Cleaved caspase 3, whereas anti-

**Fig. 4.** RUNX1 mediates miR-302b induced growth inhibition and apoptosis promotion. A. The protein levels of RUNX1 were measured in SKOV3 cells co-transfected with miR-302b and a RUNX1 mutant construct lacking the 3’UTR, and in RUNX1 siRNA transfected cells. B. Cell viability was measured by MTT assay. C. Colony formation assay. D. Cell cycle progression. E. Cell apoptosis by Annexin V/FITC flow cytometry. **P<0.01.
miR-302b had the opposite effects (Fig. 5). Knockdown of RUNX1 suppressed the activation of STAT3 and its downstream effectors, downregulated Cyclin D1 and promoted apoptosis, similar to the effect of miR-302b overexpression. Taken together, these results suggested
that miR-302b acts as a tumor suppressor in ovarian cancer by modulating the activity of the STAT3 pathway through its target RUNX1.

**MiR-302b suppresses tumor growth in vivo**

To examine the effect of miR-302b in vivo, a tumor xenograft model was established using miR-302b overexpressing cells and tumor growth was monitored over a period of 32 days. Tumor growth was significantly inhibited in mice inoculated with miR-302b overexpressing cells compared to vector control inoculated mice (Fig. 6A). Tumor weight at 32 days was approximately 50% lower in mice inoculated with miR-302b-expressing cells than in those inoculated with control vector-transfected cells (P<0.01) (Fig. 6B). Immunohistochemical analysis showed lower levels of Ki-67 antigen-positive cells and PCNA staining in tissues of mice inoculated with miR-302b expressing cells than in vector control expressing tumors, indicating a lower rate of cell proliferation in miR-302b overexpressing tumors (Fig. 6C). Analysis of miR-302b expression in tumor tissues by qRT-PCR showed significantly higher levels of miR-302b mRNA in tumors generated from miR-302b overexpressing cells (Fig. 6D). Conversely, RUNX1 expression was significantly lower in miR-302b overexpressing tumors than in the controls, as determined by western blotting (Fig. 6E).

**Discussion**

In the present study, we investigated the role of miR-302b in EOC, which led to the identification of RUNX1 as its direct target and the elucidation of a potential mechanism underlying the tumor suppressor function of miR-302b. Over 60 miRNAs have been predicted to target RUNX1, and several of these have been validated including miRNAs 17, 20a, 18a, 27a, 30c and 106a [20]. The present study is the first to show the modulation of RUNX1 expression by miR-302b in EOC. We showed that miR-302b is downregulated in EOC tissues in association with RUNX1 upregulation, and ectopic expression of miR-302b inhibited cell proliferation and colony formation and induced cell cycle arrest and apoptosis via the downregulation of its target RUNX1. Although RUNX1 can act as a tumor suppressor or an oncogene, its critical role in cancer cell growth and survival has been demonstrated in several solid tumors [5]. In skin squamous cell carcinoma, RUNX1 is overexpressed and its action is thought to be mediated by the repression of p21 and activation of STAT3. RUNX1 is associated with tumorigenesis in colon cancer, whereas in breast cancer, its role as a tumor suppressor or oncogene remains unclear [21, 22]. RUNX1 is highly overexpressed in invasive endometrial carcinoma, and the colocalization, upregulation and codistribution of RUNX1 with matrix metalloproteases in both endometrioid endometrial and ovarian endometrioid carcinoma (OEC) suggests that it plays a role in the progression and invasion of OEC [23, 24]. In an analysis of primary cultures derived from patients with EOC, RUNX1 overexpression was associated with advanced (metastatic) EOC [25], and the oncogenic role of RUNX1 in EOC was confirmed in SKOV3 cells, in which RUNX1 promoted cell proliferation, cell cycle progression and migration/invasion [6]. RUNX1 is overexpressed in various epithelial tumors including EOC and its association with tumor initiation was shown to be mediated by a RUNX1/STAT3 signaling axis [6].

Constitutive activation of STAT3, which has been detected in several cancers including ovarian cancer, can lead to cellular transformation and tumorigenesis [26]. Furthermore, STAT3 activation is correlated with disease stage, lymph node metastasis, drug resistance and poor survival in ovarian cancer [27-29]. The expression of the phosphorylated form of STAT3, p-STAT3, is higher in ovarian cancer cell lines and in EOC tissues compared to benign ovarian tumors or normal ovarian tissues [27, 30]. The nuclear localization of p-STAT3 has been detected in a significant proportion of ovarian cancer patients in association with poor prognosis [28, 31]. Treatment with the STAT3 inhibitor corosolic acid inhibited EOC cell proliferation and increased the sensitivity of EOC cells to anticancer drugs in a recent study, confirming the link between STAT3 activity and tumorigenesis in EOC [32]. In the present
study, we showed that overexpression of miR-302b decreased the levels of p-STAT3 as well as those of its downstream effectors in SKOV3 cells, indicating that the tumor suppressor effects of miR-302b may be mediated by the inactivation of STAT3 in EOC.

In conclusion, in the present study we showed that miR-302b is downregulated in EOC and its ectopic expression inhibits cell proliferation and colony formation and induces cell cycle arrest and apoptosis in ovarian cancer cells. We identified RUNX1 as a direct target of miR-302b, and showed that silencing of RUNX1 mimicked the effects of miR-302b overexpression in EOC, indicating that the tumor suppressor effects of miR-302b are mediated by the downregulation of RUNX1. MiR-302b inhibited the STAT3 signaling pathway, and RUNX1 silencing had a similar effect, demonstrating that miR-302b regulates STAT3 signaling through the modulation of its target gene RUNX1. Our results provide insight into the role of miRNAs in EOC tumorigenesis and reveal a novel mechanism involving the transcription factor RUNX1 and STAT3, suggesting potential prognostic biomarkers and therapeutic targets for the treatment of ovarian cancer.

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