Synergistic effect of oxaliplatin and nanocurcumin in dendrosomal carrier to inhibits ovarian cancer cells invasion and metastasis through the long non-coding RNA MEG3

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Abstract

Background: Ovarian cancer (OC) is a common female cancer with a high mortality rate. Maternally Expressed Gene 3 (MEG3) is a long non-coding RNA (lncRNA) located on chromosome 14q32.3 and acts as an anti-tumor factor in various cancers. In this study we aimed to determine the role of siRNA-mediated MEG3 knockdown under dendrosomal nanocurcumin (DNC) and Oxaliplatin (OXA) treatment on ovarian cancer cell lines, also the expression levels of cancer-associated gene of Bcl-2, BAX, MMP-2 and MMP-9.

Methods: We performed the MTT assay, flow cytometry for cell cycle analysis and Annexin V-FLUOS approach to evaluate the apoptosis, transwell migration and invasion assay in ovarian cancer cell lines. The expression analysis of lncRNA MEG3 and Bcl-2, BAX, MMP-2 and MMP-9 genes was also done using real-time PCR.

Results: As result, we found MEG3 expression was significantly increased in two cell lines while it was in a time-dependent manner with OXA (24h and 48h) (P <0.01) and DNC (24h and 48h) (P <0.001) in OVCAR3 cell line. Also, siRNA-mediated MEG3 could significantly suppress many aspects of DNC and OXA anticancer effects in ovarian cancer cell lines. Real time analysis data demonstrated the increased expression of MMP-2 only in DNC (P <0.01) and combination treatment (P <0.001) and increased MMP-9 expression level only in OXA treatment after MEG3 downregulation (P <0.01).

Conclusion: The findings of the current study illustrate MEG3 knockdown potentially can affect anticancer effects of DNC treatment in migration and invasion of ovarian cancer cell lines by change the expression levels of metastasis-associated genes MMP-2 and MMP-9. Then, it seems DNC and OXA combination treatment act as novel and efficient therapy in ovarian cancer, also MEG3 as a potential biomarker and therapeutic target for drug resistance ovarian cells.

Introduction

Ovarian cancer (OC) is the most common cancer and the fifth leading cause of cancer-associated mortality among women worldwide (1, 2). Chemoresistance is still one of the major clinical challenge in the treatment of patients with ovarian cancer. The efforts to overcome these problems has been led to recently use of novel chemoprevention agents with more efficacy and lesser side effect in cancer therapy. Oxaliplatin (OXA), as an anticancer chemotherapeutic drug, is a third-generation platinum compound used clinically alone or in combination with other anti-cancer drugs against to ovarian cancer (3). Recently, for improve the efficacy of OXA, it has been combined with natural phytochemical agents such as curcumin (Cur) (4). Curcumin is a natural polyphenolic compound extracted from the rhizome turmeric with potent anti-tumor effect (5, 6). Dendrosomal curcumin (DNC) has been recently used as a novel nanoparticle formulation of curcumin, due to therapeutic limitation of curcumin such as water insolubility, low adsorption, and rapid metabolism (7). Dendrosome is an efficient neutral nano-carrier synthesized firstly by Babaei et al. in laboratory at 2012 for safely delivering genes into different cell
lines (7, 8). A growing number of evidences demonstrated anticancer effect of DNC and OXA combination treatments on multiple biological processes of tumor proliferation, apoptosis and cell cycle arrest in ovarian cancer (8). Recent studies have also revealed the crucial role of specific long non-coding RNAs (lncRNAs) in affecting sensitivity to the therapeutic drugs or even drug-resistant in ovarian cancer cells. Long non-coding RNAs (lncRNAs) with longer than 200 nucleotides have be found to be associated with the development of many types of cancers (9-11). Among them, maternally expressed gene 3 (MEG3) is known for its contribution in response to chemotherapeutic agents such as curcumin and OXA (12). MEG3 is a 1.6 kb long non-coding RNA (lncRNA) located on chromosome 14q32.3 and consists of ten exons (13). Previous studies have illustrated that MEG3 may affect proliferation and apoptosis of tumor cells (14-18). However, little is known about the role of MEG3 in the development of chemo resistant in ovarian cancer. Based on results of our previous study, the combination of DNC and OXA had a synergistic inhibitory effect on the expression levels of a panel of long non-coding RNAs and affecting cell proliferation and apoptosis in OVCAR3 cell lines (11). Earlier studies in our research group found the anticancer properties of DNC are mediated by regulating the expression of lncRNA MEG3 and apoptotic-associated markers of Bcl-2 and BAX in ovarian cancer (19). Several studies have also reported that curcumin exert its anti-cancer activity in cancer cells via interacting with a variety of cancer-associated molecular targets such as BAX, BCL-2, MMPs (MMP-9, MMP-2), cyclin D1, TP53 via modulating signaling pathways involved in cancer development (20, 21). Matrix metalloproteinase (MMPs) such as MMP-2 and MMP-9 facilitate tumor progression and EMT-associated metastasis directly by degrading the basement membrane components, allowing cancer cells to invade into the surrounding (22-25). Better understanding the mechanisms of chemotherapeutic drug resistance and chemo sensitivity to DNC and OXA in ovarian cancer cell lines, facilitate the development of more effective anti-tumor drugs, and thereby improve the survival rate of the patients. Therefore, the aim of this study is to further explore of mechanisms underlying the role of MEG3 expression levels on anti-cancer effects of DNC, OXA and combination treatment, via mediating MMP-2 and MMP-9 involved in tumor cell invasion and metastasis in OVCAR3 and SKOV3 ovarian cancer cell lines.

Methods And Materials

Cell lines and culture conditions

OVCAR3 and SKOV3 ovarian cancer cell lines used in this study were purchased from Pasteur Institute of Iran (Tehran, Iran). The cells cultured in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) and 100 units/ml penicillin–streptomycin (Gibco, Scotland) in 5% CO2 at 37 °C in a humidified incubator.

Agents

OXA and Cur powders was purchased from Merck (Darmstadt, Germany) with purity of 95%. OXA was initially dissolved in dimethyl formamide (DMF) followed by dilution with milli Q (mQ) water (at a ratio 1:5) to produce a 1mM stock solution.
Dendrosome (polymeric micelle OA400 carrier) preparation

Dendrosomal nanoparticles (Den 400) and DNC (a combination of curcumin powder and liquid dendrosome), were prepared based on previous protocol (7, 11, 26). Briefly, the OA400 carrier (Dendrosome nanoparticle specified Den 400) was synthesized by esterification of oleoyl chloride (0.01 mol) and polyethylene glycol 400 (0.01 mol) in the presence of triethyl amine (0.012 mol) and chloroform as the solvent at 25 °C for 4 hr. After filtration of trimethylamine hydrochloride salt from organic phase, chloroform was eliminated from OA400 through evaporation in a vacuum oven at 40 °C for 4 h. For DNC preparation, different weight/weight ratios of dendrosome/curcumin (about 50:1 to 10:1) were investigated by spectrophotometry (Infinite®200 PRO, Tecan, Mannedorf, Switzerland) in order to select an appropriate ratio of 1:25 as the optimum ratio. The curcumin was dissolved in dendrosome nanoparticles using protocols Maling Gou et al (27) Prepared DNC at a concentration of 2700 mM was kept at 4 °C and kept away from light until use. For in vitro experiments, DNC was diluted in culture medium before use in any assay. Fourier transform infrared (FTIR) Spectrum analysis was performed to confirm the dendrosomal chemical structure. Dynamic light scattering (DLS) analysis confirmed the mean diameter of ≤200 nm for DNC and transmission electron microscopy (TEM) exhibited DNC nanoparticles were sphere shaped. Also, the efficient encapsulation of curcumin in the dendrosomal nano-carrier was very high (87%). Additionally, acceptable value of the superficial charge (ζ-potential) was calculated around −7 mV which does not show high stability.

Small interfering RNA to knockdown long non-coding RNA MEG3

For the MEG3 knockdown experiments, the ovarian cancer cell lines OVCAR3 and SKOV3 were seeded into six-well plates in RPMI-1640 medium for 24h. The BLOCK-it RNA interference (RNAi) designer was used for designing of four different Small Interfering RNAs (siRNAs) that targeted MEG3 RNA with the following sequences:

1- 5-GCUGUCCCUUUACCUCAAA-3
2- 5-GCAUUAAGCCCUGACCUUU-3
3- 5-GGAAGGAUCCCUUUGGGAA -3
4- 5- GCUAGCAACUGGAGUGUU -3

and a scrambled siRNA control were purchased from Life Technologies. The MEG3 siRNAs stored at -20°C after dissolving in RNase free-water, the cells were transfected with these siRNAs (oligonucleotides encoding 19-mer hairpin sequences) with lipofectamine 3000 (Invitrogen) specifically targeting MEG3, according to the manufacturer's instructions. Transfection efficacy was checked with scramble for 24, 48 and 72h with GFP florescent. 48h after transfection was a best time of transfection efficacy. Briefly, cells were plated in 6-well culture dishes and allowed to attachment. Our condition were DNC, DNC+MEG3 siRNA, OXA+MEG3 siRNA, co-treated of DNA and OXA MEG3 siRNA and the untreated cells attended as
controls, respectively. The cells were treated with DNC, OXA or Combination of them 48h before MEG3 siRNA. The efficiency of siRNA knockdown was determined by real-time PCR analysis.

**MEG3 expression analysis by real-time PCR**

Total RNA was extracted from OVCAR3 and SKOV3 cells using TRizol® reagent ((Invitrogen Life Technologies) followed by DNase I digestion (Thermo Fisher Scientific, Waltham, MA, USA). The quantity and quality of the isolated RNA were determined by Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA) and agarose gel electrophoresis (1% agarose; Gibco/BRL), respectively. 500 ng of extractive RNA was used to synthesize the cDNA by PrimeScript™ RT reagent kit (Takara Bio Inc., Shiga, Japan). Real-time PCR was performed by IQ5 (Bio-Rad, Germany) using 2µl of the synthetized cDNA and SYBR green master mix (Biofact, Corea) in a total reaction volume of 10µl. PCR was performed in triplicate. GAPDH, as the internal control, was used to normalize the gene expressions. The sequences of specific primers were illustrated in Table 1. The relative expressions were calculated according to $2^{-\Delta \Delta Ct}$.

**Table 1. List of specific primers used in real-time polymerase chain reaction assay**

<table>
<thead>
<tr>
<th>GENE</th>
<th>Designed oligonucleotide</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEG3</td>
<td>TCCGTCACCTCCTTTGCT</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td>TAGGGCATTTGTTTAAGTCTTTAG</td>
<td></td>
</tr>
<tr>
<td>BCL2</td>
<td>GGGATGCGGGAGATGTGG</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>GTAGCGGGGAGAAGTTC</td>
<td></td>
</tr>
<tr>
<td>BAX</td>
<td>AAGAAGCTGAGCGAGGTGTCT</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>GTTCTGATCAGTCCGGGAC</td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>TTGATGGCATCGCTCAGATC</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>TTGTCACTGGTCGTCACAGT</td>
<td></td>
</tr>
<tr>
<td>MMP9</td>
<td>GACCGAGACATCGTCATCCA</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>CACAACCTCGTCATCGTCGAAA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAGTCAACGGATTTGGTCGT</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>TTGATTTTGAGGGATCTCG</td>
<td></td>
</tr>
</tbody>
</table>

**Colony formation assay**

Colony formation assay was conducted to evaluate the role of MEG3 in the cell proliferative potential of OVCAR3 and SKOV3 cells. For this assay, the cells were seeded into 6-well plates at 500 cells/well and transfected with MEG3 siRNA in optimum and lipofectimin. Then, the cells were treated with DNC, OXA or combination of them at concentrations lower than its IC50 values for 48h then cells were transfected with
MEG3 siRNA for 48h [18]. After that, the cells were cultured at 37°C and 5% CO2 in incubator. After 10
days, the dishes were washed twice in PBS, fixed by methanol and stained with 0.1% crystal violet and
then air dried. The total number of colonies was counted in a microscope. The percentage of colonies
was defined with the number of colonies formed in treated plate divided by number of colonies formed in
control groups with no treatment.

Cell apoptosis assay

Cell apoptosis assay was performed to detect the apoptosis rate by using Annexin V-FITC/ PI apoptosis
detection kit (Roche Applied Science, Penzberg, and Germany.) was used to detect the apoptosis rate
according to the manufacturer’s instruction. Briefly, the OVCAR3 cells were seeded in 6-well plates
overnight, then treated similar to above. After washing twice with PBS, treated cells were stained with
Annexin V-FITC/PI (Roche Applied Science, Penzberg, Germany cat number: 858777001), finally incubated for 10–15 min at 15–20 °C and analyzed by FACSCalibur flow cytometer immediately.

Transwell cell invasion assays

Cell invasion of ovarian cancer cells was determined by Transwell Filter with 8 μm pore size coated with
Matrigel (BD Biosciences, San Jose, CA, USA). Briefly, poly vinyl pyrolidone-free polycarbonate filters
(Millipore) (8μ M pore size) were coated with matrigel (15 μg/filter). 48h after treatments and MEG3
siRNA transfection, OVCAR3 cells in 200μl of serum-free DMEM were seeded into the upper chamber,
while medium supplemented with 10% FBS was added to the lower chamber which acts as a chemo
attractant. After 48 h incubation, the non-invading cells remaining in the upper chamber were removed
with a cotton-tipped swab. The invaded cells on the lower surface of the membrane were fixed and
stained by methanol and 1% crystal violet, respectively. Then, data were recorded through direct
observation and the cells were photographed under a microscope. Finally, the number of invasive cells
was counted using a light microscope in at least 10 random fields for each well.

Transwell migration assays

The migration assay was identical to above invasion assay except the inserts were not coated with
Matrigel. A 24-well transwell chamber with 8.0-μm pore size membrane was used according to the
manual Instructions. In brief, 48h after treatments and MEG3 siRNA transfection, the cells were
suspended in culture media and were seeded into the upper chamber of the transwell plates. After 24h
incubation in at 37 °C, the non-migrated cells on the upper surface of the membrane were removed with a
cotton swab. The migrated cells to the lower surface of the membrane were then fixed and stained with
methanol and 1% crystal violet, respectively. The cells were counted in 10 randomly separate fields per
membrane using a microscope

Cell cycle assay
Cell cycle was detected by flow cytometry assay. For the cell cycle assessment, 1.5×10^5 cells/well of OVCAR3 were seeded 6-well plates overnight at 37 °C and 5% CO2. After overnight of incubation, the cells were treated with DNC, OXA and combination of them at the proper concentration. All experiments were repeated three times. 48h after treatments, cells were transfected with MEG3 siRNA. Finally, after 48h, the cells were washed twice with PBS, harvested and then trypsinesed and fixed in 70% cold ethanol and incubated at 20 °C. After washing the cells twice with PBS, they were suspended in a solution containing 10 mg/ml propidium iodide (Molecular Probes, Invitrogen, UK) and 0.2 mg/ml RNAase-DNAase free (Sigma) and incubated for 30 min at 37°C in the dark. The percentage of cells in the G0/G1, S, and G2/M phases were determined by a flow jo software for Windows 64-bit (Beckman Coulter, USA). Gating strategy was done to exclude cell doublets, clumps and debris.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed by SPSS 16.0 software (SPSS Inc., USA). One-way ANOVA and Tukey’s test were performed for multi-group comparison. All the data are presented as mean ± SD and value of P<0.05 was considered statistically significant.

Results

**DNC nanoparticles and Oxaliplatin increased MEG3 expression in ovarian cancer cells**

In this study, we observed MEG3 expression was increased in OVCAR3 and SKOV3 cell after all 3 treatments with DNC, OXA and combination of them at 24-48h. Also, this MEG3 over-expression was in a time-dependent manner and higher after 48 h treatment with OXA (P<0.01) in OVCAR3 cell lines. Notably, the higher MEG3 expression in OVCAR3 cell line was significant only in combination treatment compared to the DNC treatment alone (P <0.001) (Fig.1). We also assessed the role of siRNA-mediated knockdown of MEG3 on induced apoptosis, anti-metastatic, invasion and cell cycle arrest effects of DNC treatment alone, also in combination with OXA in OVCAR3 cell lines.

**MEG3 down regulation arrested anti cell proliferative effect of DNC nanoparticles and Oxaliplatin in ovarian cancer cells**

Based on previous study, we had shown a significant decrease in cell proliferation and colony formation in ovarian cancer cell lines after treatment with DNC, OXA and combination of them compared to control untreated cells. This significantly reduction in cell proliferation was higher in combination therapy (P<0.001) than DNC (P<0.05) or OXA (P<0.01) treatment alone. Our data demonstrated that MEG3 knockdown could significantly decline anti proliferative effect of all treatments. After MEG3 siRNA the colony formation in ovarian cancer cell line was increased from 0.55 to 1.1 in DNC (p<0.01), 0.5 to 0.95 in OXA (p<0.05) and 0.3 to 0.6 in combination treatment (p<0.05). The above results suggested that inhibitory effect of all treatments on colony formation in ovarian cancer cell lines was influenced by MEG3 downregulation (Fig.2).
MEG3 down regulation inhibited apoptotic activating effect of DNA and Oxaliplatin treatment in ovarian cancer cells

As our previous result [18,19], we observed a significantly induced apoptosis in OVCAR3 cell lines treated with DNC, OXA and a combination of them compared with untreated control group (P <0.001). We further explored that OXA treatment combined with MEG3 downregulation could significantly decrease the percentage of the apoptotic cell from 75% to 50% compared with OXA treatment alone (P <0.01). However, this synergistically effect was not observed in the other two treatments. In other words, OXA-induced apoptosis is partly influenced by the MEG3 downregulation in ovarian cancer (Fig.3).

MEG3 down regulation inhibited anti-migrate effect of DNC nanoparticles and Oxaliplatin in ovarian cancer cell

Transwell chamber migration assay was employed to investigate the possible role of MEG3 knockdown (down regulation) in migration of ovarian cancer cell lines after DNC, OXA and combination treatment. Matrigel as membrane model of barrier was used to determine the cell migration capacity by counting the number of cells which able to pass through a porous membrane down to the lower chamber and the degree of their permeability in matrigel in all three treatments after MEG3 knockdown. The density of the cell passed through the matrigel to the other side of the chamber is shown in (Fig.4). Transwell assay results in previous study had exhibited that all OXA, DNC and combination treatments had a significant inhibitory effect on cell migration in ovarian cancer cell lines compared to untreated control cells.

While, MEG3 knockdown notably increased the cell migration from 40% to 78% in DNC treatment (p <0.001), 45% to 60% in OXA treatment and 25% to 50% in combination treatment (p <0.001%). According to these results, it is suggested that the inhibitory effects of DNC and combination treatment on migration capacity of OVCAR3 cells was influenced by MEG3 downregulation. Moreover, the effect of MEG3 downregulation on cell migration in combination treatment was greater than DNC or OXA treatment alone (Fig.4).

MEG3 down-regulation arrested anti-invasion effect of DNC and Oxaliplatin treatment in ovarian cancer cell

As our previous results, we found that cell invasion was decreased after all 3 treatments in ovarian cancer cell lines. These results revealed that MEG3 knock down (down – regulation) also increased cell invasion from 43% to 58% (p <0.05) in DNC treatment, 55% to 65% (p <0.01) in OXA treatment and 40% to 53% (p <0.05%) in combination therapy. This finding revealed the significantly effect of MEG3 gene on sensitivity of OVCAR3 cell lines to invasion after all treatments (Fig.5).

Antagonistic effects of MEG3 knockdown and DNC on cell cycle analysis in OVCAR3 cancer cells

In terms of cell cycle analysis, similar to our previous results, all 3 treatments had a significant effect on the cell cycle regulation of OVCAR3 cells (P <0.001). Notably, our present study found that knockdown of
MEG3 remarkably decreased the effect of DNC treatment after 48 hr on promoting the G1 phase (P <0.001) (Fig.6).

**The effect of MEG3 knockdown on the expression of apoptosis and metastasis-associated genes after DNC, OXA treatment and their combination treatment.**

We evaluated the function of MEG3 knockdown on associated – apoptosis genes of Bcl2 and BAX as well as matrix metalloproteinase (MMPs) involved in cell metastasis such as MMP-2 and MMP-9. As our previous study, our results revealed reduced expression of MMP2 in OVCAR3 and SKOV3 ovarian cancer cell lines treated with DNC (P <0.01), OXA (P <0.001) and combination of them (P <0.01). While after MEG3 knockdown, MMP2 expression was significantly increased only with DNC (P <0.01) and combination treatment (P<0.001). This finding indicated that DNC and combination treatments effect on expression of MMP2 in ovarian cell lines influenced by MEG3 expression. However, MMP9 expression was significantly decreased only after OXA (P<0.05) and combination treatment (P <0.05), but not with DNC treatment. After MEG3 knockdown, we found the same results and observed a significant difference in MMP9 expression after OXA treatment, alone (P <0.01). This result suggested the possible role of MEG3 gene in the treatment of ovarian cancer with OXA which inhibit MMP9-dependent metastasis. In addition, we found no significant difference in the expression of apoptosis-related proteins Bcl-2 and BAX after MEG3 knockdown, suggesting MEG3 didn’t influence the apoptosis that was induced by OXA or combination of them in OVCAR3 cells and only Bcl-2 expression increased after treatments and MEG3 knockdown (p<0.05) (Fig.7). Considering all these results, we used transwellwss chamber migration and invasion assay to more investigation of the effect of vector–mediated MEG3 over expression. The results further confirmed the remarkable role of the MEG3 over expression in sensitivity of OVCAR3 cells to all 3 treatments (Fig.8).

**Discussion**

During recent years, many studies focused on the role of IncRNA as main regulators of signaling pathways and genes involved in a variety of cancers (28–30). Furthermore, some cancer-related IncRNAs have been found to modulate the drug-resistance by regulating related genes and signaling pathways involve in the apoptosis, proliferation, metastasis, cell cycle arrest in ovarian cancer (11). Therefore, further investigation of function of these IncRNAs in response to agents such as DNC and OXA in chemo-resistance cells may supply novel prognostic biomarkers or therapeutic targets for ovarian cancer therapy. In this study we assessed the role of MEG3 expression on induced apoptosis, anti-metastatic, invasion and cell cycle arrest effects of DNC, OXA and combination treatment in Ovarian cancer cell lines. Our previous study confirmed the synergetic effect of DNC and OXA combination treatment on cell death and on the expression of cancer-associated long non-coding RNAs in OVCAR3 cells (11). In this study we also observed an increase in MEG3 expression at all 3 treatments (P ≤ 0.001). However, this elevated MEG3 expression was significant between 24h and 48h with DNC and OXA (P ≤ 0.001, P ≤ 0.01 respectively) in OVCAR3 cells, also only OXA had significant effect compare to combination (P ≤ 0.001). Furthermore, our earlier study also showed altered expression of apoptosis-associated genes of Bcl-2 and
BAX only after DNC treatment in MEG3 siRNA transfected cells (11). As expected, these results suggested the role of MEG3 in DNC-induced apoptosis in line with Shi et.al. that showed an increase in expression of the apoptosis-associated markers of BAX, Bcl-2 and Bcl-XL in ovarian cancer cells after treatment with curcumin (31). Therefore, we hypothesize a targeting relationship between upregulation of MEG3 and anti-cancer effects of DNC and OXA treatment in OVCAR3 and SKOV3 cell lines. Interestingly, we found no significant difference in the expression of Bax as a proapoptotic gene in MEG3 siRNA transfected cells at the present study, suggesting it may MEG3 downregulation influence the anti-cancer effect of DNC and OXA through other crucial cancer-associated genes in OVCAR3 cells also, we showed Bcl-2 expression increased after MEG3-siRNA In treatment with DNC and there is not synergistic effect of DNC and OXA in combination therapy after MEG3 knockdown. Accordingly, we further evaluated the expression of metastasis – associated MMP-2 and MMP-9 genes after MEG3 knockdown (down-regulation) in ovarian cell lines. Notably, our results implied that MEG3 knockdown significantly increased MMP-2 only after DNC (P < 0.01) and combination treatment (P < 0.001) and MMP-9 expression levels only after OXA (P < 0.05) which might be partly attributed to promoting cell migration. We further assessed the role of MEG3 knockdown on colony formation, cell cycle arrest and migration in ovarian cell lines. Our evaluation about the effect of MEG3 knockdown on cell cycle arrest confirmed that MEG3 siRNA remarkably suppress the effect of DNC treatment on subG1 phase cell cycle progression while increasing subG1 phase was found before MEG3 knockdown in all of the treatments. Also an increasing cell numbers in subG1 phase in all of the treatments was observed compare to control (11). This might be partly attributed to main role of MEG3 knockdown on the activation of cyclin-dependent kinases (CDKs) and cyclins through regulate the expression level of cell cycle regulatory factors such as f Cdc2, Cdc25A (32). In addition, the effect of MEG3 knockdown on cell migration in combination treatment was significantly greater than DNC or OXA treatment alone. Taken together, our results showed that the effect of DNC, OXA and combination treatments on colony formation, apoptosis, cell cycle, trans-well invasion and migration assay in ovarian cancer cell lines was influenced by MEG3 downregulation.

The process of tumor metastasis and invasion requires degradation of extracellular matrix (ECM) and most components of basement membrane by Matrix metalloproteinase (MMPs), a zinc-dependent endonuclease protease family (33). Elevated MMP-2 and MMP-9 levels have been strongly correlated with the development of metastases in several types of cancers (33). Considering all these findings, we supposed MEG3 downregulation facilitate metastasis progression and cell invasion through regulation expression of MMP-2 and MMP-9 genes in ovarian cancer cell lines.

Altogether, our findings showed that downregulated MEG3 indirectly acts as a mediator between the cancer genes expression, signaling pathways and anti-cancer effect of DNC and OXA treatment. This results propose MEG3 as a probable therapeutic target which inhibit MMP9-dependent metastasis and enhance the chemo-response in ovarian cancer (33).

Conclusion
In conclusion, our findings support the role of MEG3 on anti-tumor effects of DNC and OXA treatment via regulating a number of genes. The mechanisms underlying the resistance to these chemotherapeutic agents in ovarian cancer treatment is remain unclear. Further investigation on function of cancer-associated IncRNAs combined with DNC and OXA combination treatment and other crucial factors and related genes, may support its use as a chemotherapeutic or a chemopreventive agent in ovarian cancer. In addition, MEG3 may be also considered as a potential biomarker and therapeutic target in ovarian cancer therapy. However, this study also has limitations. Therefore, in vivo experiments need to be conduct in the future studies for a comprehensive analysis of our results.

**Declarations**

**Ethics approval and consent to participate:**

Not applicable.

**Consent to publish:**

Not applicable.

**Data Availability Statement:**

All the authors confirm the availability of data and materials.

**Conflicts of Interest:**

The authors declare that they have no competing interests.

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**Author Contributions:** The original research described in this paper is part of the research project of ESH. All experiments were performed by ESH, MAZ and H.N. provided direction and guidance. ESH, N.R.K, MAZ, HHK and HN conducted the literature and prepared the first draft of the manuscript. NRK and HHK thoroughly reviewed and made significant revisions to the manuscript. All authors read and approved the final version.

**References**


Figures
Figure 1

Effect of DNC, OXA and combination therapy on MEG3 expression 24h and 48h.

Notes: Data expressed as mean ± standard deviation; ***$P \leq 0.001$ treats compared to control, ### $P \leq 0.001$ treats compared to combination and $$ P \leq 0.01; $$$ P \leq 0.001$ between 24h and 48h.

![Figure 1](image)

Figure 2

Effect of MEG3 siRNA on DNC, OXA and combination therapy to inhibit colony formation of OVCAR3

Notes: Data expressed as mean ± standard deviation; *$P \leq 0.05$, **$P \leq 0.01$; ***$P \leq 0.001$ treats compared to control, # $P \leq 0.05$, ## $P \leq 0.01$ compared to MEG3 siRNA
Figure 3

Effect of MEG3 siRNA on DNC, OXA and combination therapy in apoptosis induction of OVCAR3 ovarian cancer cell line.

Notes: Data expressed as mean ± standard deviation; **P ≤ 0.01; ***P ≤ 0.001 treats compared to control.
Figure 4

Effect of MEG3 siRNA on DNC, OXA and combination therapy in migration of OVCAR3 ovarian cancer cell line.

Notes: Data expressed as mean ± standard deviation; **P ≤ 0.01; ***P ≤ 0.001 treats compared to control ### P ≤ 0.001 treats compare to treats+SiRNA.
Figure 5

Effect of MEG3 siRNA on DNC, OXA and combination therapy in invasion of OVCAR3 ovarian cancer cell line.

Notes: Data expressed as mean ± standard deviation; **P ≤ 0.01; ***P ≤ 0.001 treatments compared to control.
# P ≤ 0.05, ## P ≤ 0.01 treatments compared to treatments+SiRNA.
Figure 6

Effect of MEG3 siRNA on DNC, OXA and combination therapy in cell cycle arrest of OVCAR3 ovarian cancer cell line. Notes: Data expressed as mean ± standard deviation; **P, 0.01; ***P, 0.001 compared to control ## P ≤ 0.01 treats compare to treats+SiRNA.
Figure 7

Effect of DNC, OXA and combination therapy on BAX, BcL2, MMP2 and MMP9 in 48h.

Notes: Data expressed as mean ± standard deviation; **$P$, 0.01; ***$P$, 0.001 compared to control \# $P \leq 0.05$, ## $P \leq 0.01$ treats compare to treats+SiRNA
Figure 8

The effect of MEG3 over expression on DNC, OXA and combination therapy in A) migration and B) invasion of OVCAR3 ovarian cancer cell line.

Notes: Data expressed as mean ± standard deviation; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 treats compared to treats+MEG3 plasmid # P ≤ 0.05, ##P ≤ 0.01, ### P ≤ 0.001 MEG3 plasmid compare to treats+ MEG3 plasmid.