

Original Article

Ormeloxifene inhibits the proliferation of cervical cancer cells by suppressing Wnt/ β -catenin signaling

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Abstract: Background: Ormeloxifene, a non-steroidal selective estrogen receptor modulator used widely as an oral contraceptive, which was recently reported to play anti-cancer activity in various types of cancer. This study aimed to investigate the effect of Ormeloxifene on human cervical cancer HeLa cells and related mechanism. Material and methods: After treated with different doses of Ormeloxifene, cell viability was determined by MTT method, the cell cycle distribution and apoptosis were observed by flow cytometry (FCM), the expression of Wntless-type (Wnt), β -catenin, glycogen synthase kinase (GSK)-3 β and CyclinD1 was measured by Quantitative real-time PCR (QPCR) and Western blot analysis. Results: Ormeloxifene treatment significantly inhibited the proliferation, induced G1 arrest and apoptosis in human cervical cancer HeLa cells. More importantly, QPCR and Western blot showed that Ormeloxifene markedly down-regulated the expression levels of Wnt, β -catenin, and cyclinD1, up-regulated the expression of GSK-3 β in HeLa cells. Conclusion: Together, the results of this study reveal that Ormeloxifene significantly inhibits the proliferation of HeLa cell and is a potential drug for the treatment of cervical cancer.

Keywords: Ormeloxifene, cervical cancer, HeLa cells, Wnt, β -catenin, proliferation

Introduction

According the data from GLOBOCAN 2012, there were an estimated 527,600 new cervical cancer cases worldwide, ranks the fourth most common cancer in women worldwide, and the second in developing regions [1]. Nowadays, cervical cytology screening is useful to reduce the mortality of this cancer, however there were still 265,700 people died of cervical cancer worldwide in 2012 [1, 2]. Current treatment options of cervical cancer including surgery, chemotherapy and radiotherapy. However, surgical treatment is limited to the young patients with early stage; chemotherapy and radiotherapy often lead to drug resistance and multiple severe side effects, such as organ toxicity and immunotoxicity [3]. Therefore, an increasing number of investigators have become interested in reposition established drugs as anti-cancer agents, such as cyclooxygenase-2 inhibitors [4], lenalidomide [5, 6], as well as metformin [7], and medroxyprogesterone acetate [8].

Ormeloxifene (C30H35O3N HCl; [3,4-trans-2,2-dimethyl-3-phenyl-4-p-(b-pyrrolidinoethoxy) phenyl-7-methoxy chroman], also known as Centchroman, is a non-steroidal selective estrogen receptor modulator which is used as an oral contraceptive in many countries including India, Thailand and Russia [9]. Interestingly, previous study have demonstrated the anti-cancer activities of Ormeloxifene in several cancers including breast, head and neck cancer, prostate cancer, and ovarian cancer [10]. For example, it has been reported that Ormeloxifene induces G0/G1 arrest and apoptosis in MCF-7 and MDA MB-231 breast cancer cells [11]. Ormeloxifene has been demonstrated to display anti-proliferative activity against head and neck squamous cell carcinoma via the modulation of phosphatidylinositol-3'-kinase (PI3K)/mechanistic target of rapamycin (mTOR) pathway [12]. Moreover, Ormeloxifene effectively inhibited the growth of cisplatin resistant ovarian cancer cells by inducing cell cycle arrest and apoptosis [13]. *In vivo* study showed that Or-

Table 1. Sequences of primers used in the experiment

Gene name	Forward primer	Reverse primer
<i>Wnt</i>	5'-CTGTCCTGCCTCCTCATC-3'	5'-GGACCCAGCACAATAAATAGTT-3'
β -Catenin	5'-TGCTGAAGGTGCTGTCTGTC-3'	5'-TCGCTGACTTGGGTCTGTC-3'
GSK-3 β	5'-CACCTCTGCCTACCATCCTTA-3'	5'-ATTATTGGTCTGTCCACGGTC-3'
<i>CyclinD1</i>	5'-TCGGGAGAGGATTAGGTTCC-3'	5'-GTCAGTGGATGGTTTGTGG-3'
<i>GAPDH</i>	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-TTGCTGTTGAAGTCGCAGGAG-3'

R. China). HeLa cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

meloxifene enhanced sensitivity of Gemcitabine in pancreatic cancer [14]. These studies indicate that Ormeloxifene exerts potent anti-cancer activities. In addition, Ormeloxifene was reported safe for chronic administration. Recently, Neeraj et al reported that Ormeloxifene significantly inhibited cervical cancer cell growth and decreased mitochondrial membrane potential [15]. Yet little is known about the effect and molecular mechanism of Ormeloxifene on HeLa cells.

Therefore, this present study aimed to investigate the potential anti-cancer effects of Ormeloxifene on human cervical cancer HeLa cells, and to explore the underlying molecular mechanisms involved in this process.

Material and methods

Reagents

Polyvinylidenedifluoride (PVDF) membranes and enhanced chemi-luminescence (ECL), were purchased from Merck Millipore (Millipore, Billerica, MA, USA). Antibodies of Wingless-type (Wnt), glycogen synthase kinase (GSK)-3 β , phosphor (p)-GSK-3 β , β -catenin, CyclinD1 as well as glyceraldehyde-3-phosphate (GAPDH) were obtained from Santa Cruz (Santa Cruz, CA). Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Gibco, Life technologies (Carlsbad, CA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Serva (Heidelberg, Germany) and was dissolved in PBS at a concentration of 5 mg/ml. Propidium iodide and the Annexin-V FITC apoptosis detection kits were purchased from Beyotime (Shanghai, P. R. China). Ormeloxifene was obtained from Sigma-Aldrich Shanghai Trading Co., Ltd. (Shanghai, P. R. China) and prepared in Dimethyl Sulphoxide (DMSO) and stored at -20°C.

Cell culture

Human cervical cancer cells HeLa were purchased from Shanghai Cell Bank (Shanghai, P.

Cell proliferation and viability assays

HeLa cell proliferation was evaluated using a MTT assay according to the manufacturer's protocol. Briefly, cells were seeded in 96-well plates at the concentration of 5 $\times$ 10⁴ cells/well and added various doses of Ormeloxifene at the final concentrations of 5, 10, 20, or 40 μ M. After incubation for 48 h, 20 μ L of MTT was added to each well, followed by an incubation of the plates at 37°C for 4 h. The medium was then removed and 150 μ L of 10% DMSO was added to each well. Absorbance at 450 nm was measured by a 96 well plate reader (Bio-Tek, Burleigh, QLD, Australia). Cell viability was calculated as: cell viability (%) = (corrected treated sample OD/corrected control sample OD) $\times$ 100%.

Cell apoptosis assays

Hela cell apoptosis was measured by using an Annexin-V FITC Apoptosis Detection Kit (Beyotime, Shanghai, P. R. China) following the manufacturer's instruction. Briefly, Hela cells at a concentration of 3 $\times$ 10⁶ cells per in 6-well plates were cultured in DMEM medium with 10 or 20 μ M Ormeloxifene for 48 h, respectively. The medium was removed and the cells were trypsinized, then centrifuged at 1,000 $\times$ g for 5 min to remove trypsin. Next, 50 μ g/mL of Annexin-V-FITC and 10 μ L of propidium iodide from the detection kit were added to each well and the plate was incubated at room temperature for 10 min in the dark, followed immediately by flow cytometric analysis.

Cell cycle assays

After treatment with and without Ormeloxifene for 48 h, Hela cells were trypsinized and centrifuged at 1,000 $\times$ g for 5 min. The supernatant was removed and the cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol at 4°C for overnight. The fixed cells were washed and stained with 50 μ g/mL

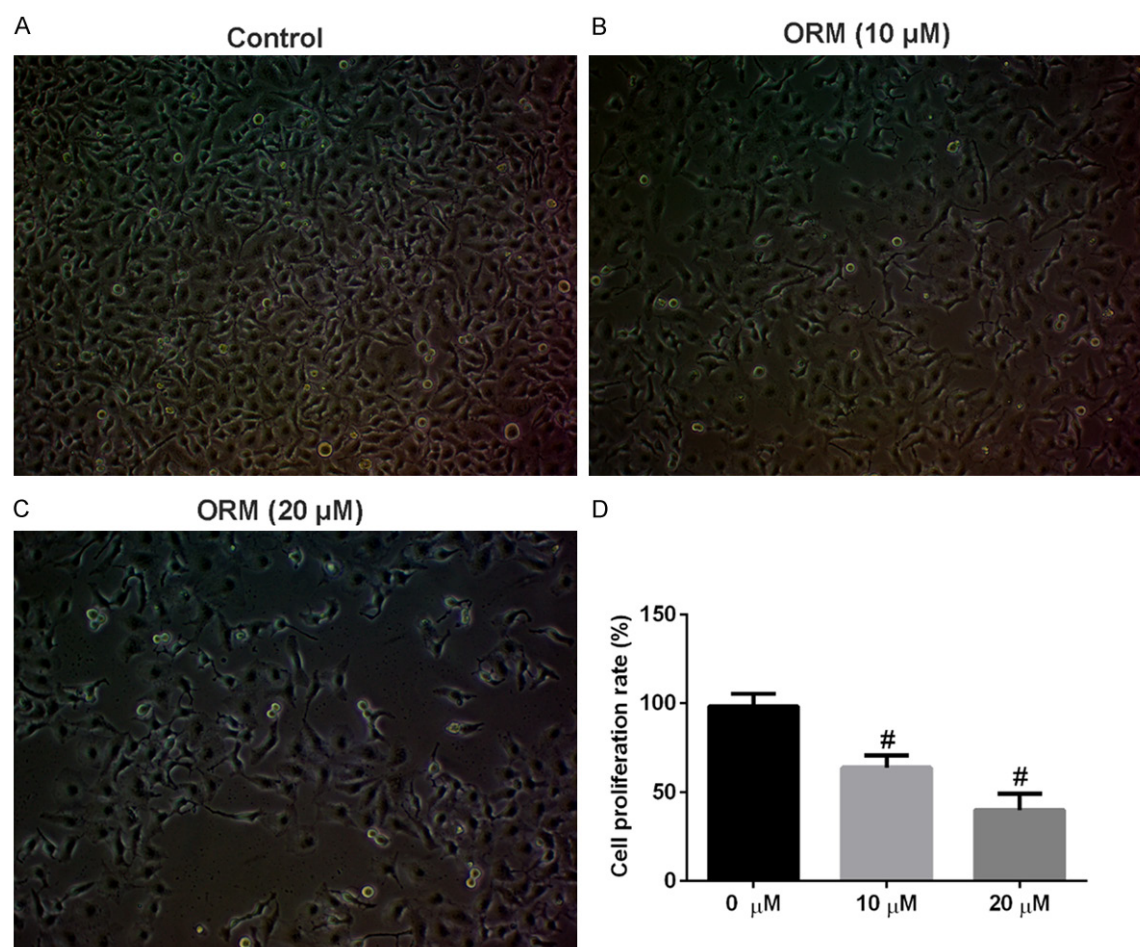


Figure 1. Effects of Ormeloxifene on proliferation of HeLa cells. Cells were incubated with 0, 10, 20 μM of Ormeloxifene for 48 h (A-C) and MTT assay (D) was used for the measurement of cell viabilities. Data were expressed as mean ± SD. #, $P < 0.05$, compared with the control group. Control, control group; ORM, Ormeloxifene-treated group.

of PI, 50 mg/mL of RNase A at 37°C for 30 min. The cellular DNA content and cell cycle phases were analyzed with flow cytometer (Becton Dickinson, SanJosé, CA).

Quantitative real-time PCR

Total RNA was extracted from cultured cells with TRIzol reagent (Invitrogen, Carsbad, CA) and DNA was removed by DNase (Promega) according to the manufacturer's instructions and mRNA expression levels were measured by Quantitative real-time PCR (QPCR) using an iQ5 multicolor real-time PCR Detection System (Bio-Rad) with SYBR Green I staining (Takara). Reverse transcription was performed with the PrimeScript RT reagent Kit (Perfect Real Time; Takara) according to the manufacturer's instructions. For mRNA analysis, GAPDH mRNA

levels were used as internal normalization control. Fold changes were calculated and normalized using the CT method. Primers used were listed in **Table 1**.

Western blot assay

Cells were collected and suspended in 250 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Equal amounts of protein (20 μg) from different samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA) for Western blot analysis. After transfer, the membranes were blocked in non-fat milk (5% in Tris-buffered saline with TWEEN-20, TBST) and then incubated with antibodies against Wnt (1:500 dilution), β-caten-

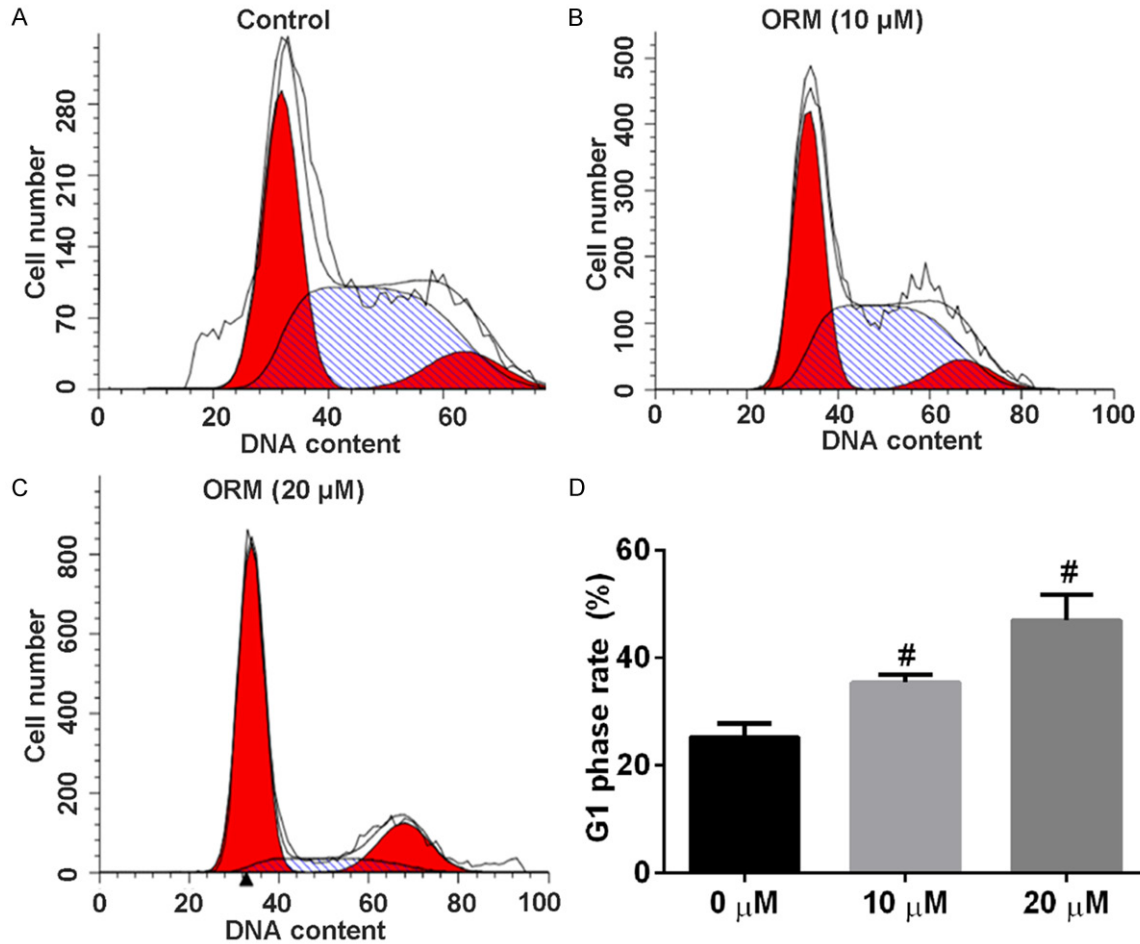


Figure 2. Effects of Ormeloxifene on cell-cycle phase distribution of HeLa cells. Cells were incubated with 0, 10, 20 μ M of Ormeloxifene for 48 h and Cell cycles were determined using PI staining of the nuclei. A: Control; B: HeLa cells were treated with 10 μ M Ormeloxifene; C: HeLa cells were treated with 20 μ M Ormeloxifene; D: G1 phase rate of control and sample. Data were expressed as mean $\pm$ SD. #, $P < 0.05$, compared with the control group. Control, control group; ORM, Ormeloxifene-treated group.

in (1:500 dilution), CyclinD1 (1:500 dilution), p-GSK-3 β (1:500 dilution), GSK-3 β (1:500 dilution) and GAPDH (1:500 dilution) at 4°C overnight. After two washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Thermo Fisher Scientific, New York, NY, USA). Then the bands were detected by using an enhanced chemiluminescence reagent (Millipore, MA, USA) and exposed by autoradiography. GAPDH was used as an internal loading control.

Statistical analysis

Data was analyzed with Graph pad Prism software (Graph pad version 6.01). Statistical dif-

ferences between groups were determined using one-way ANOVA followed by the post-hoc LSD. Statistically significant differences were considered for p values < 0.05 .

Results

Ormeloxifene inhibits proliferation of HeLa cells

The potential of Ormeloxifene to inhibit the proliferation of HeLa cells was evaluated by MTT assay. As showed in **Figure 1**, after treatment with Ormeloxifene for 48 h, the cell viability of HeLa cells was significantly decreased (10 and 20 μ M: $P < 0.05$) in comparison of control group, suggesting that Ormeloxifene was effective in inhibiting the proliferation of HeLa cells.

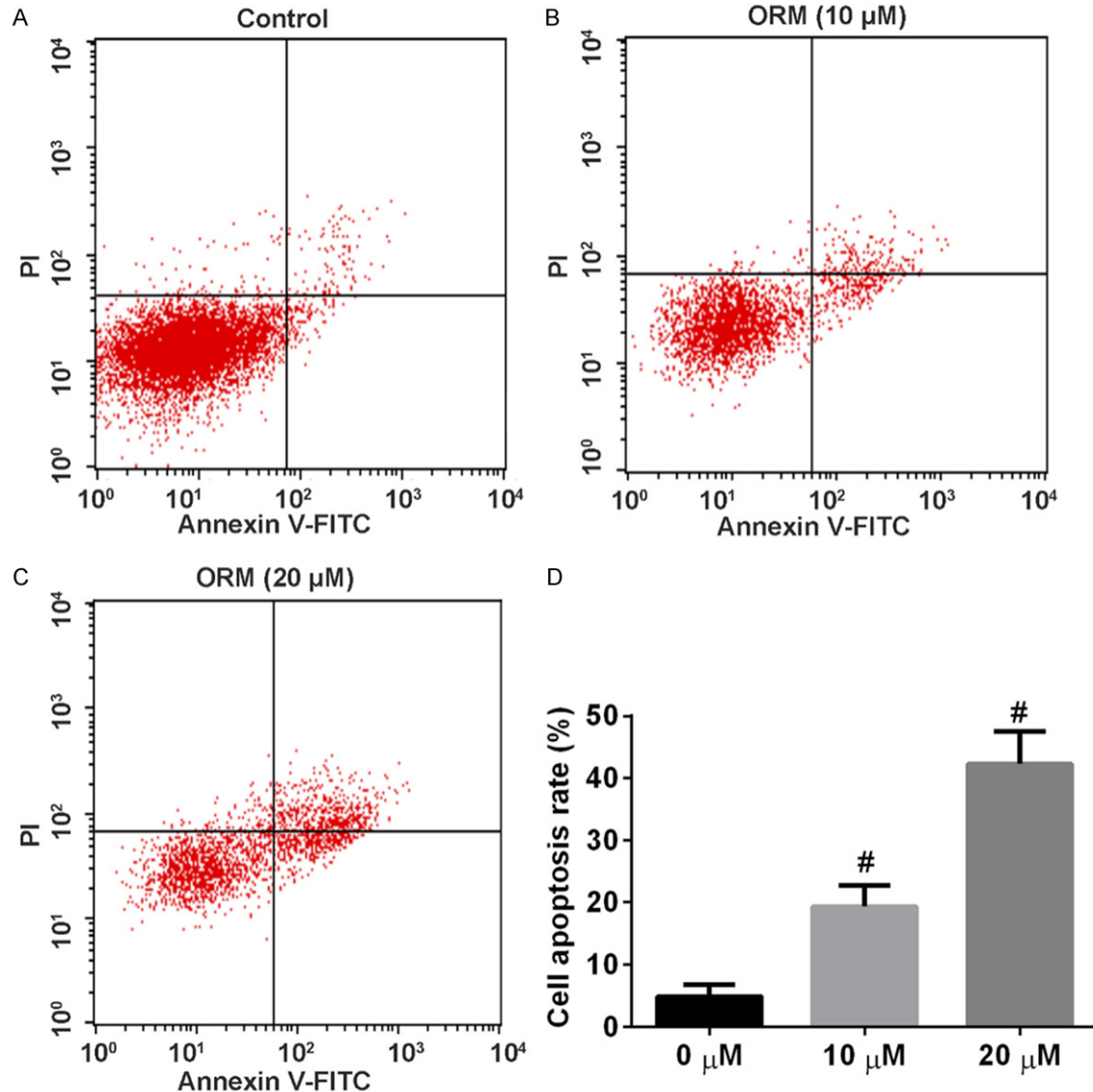


Figure 3. Effect of Ormeloxifene on the apoptosis of HeLa cells. Apoptosis analysis of HeLa cells induced by Ormeloxifene for 48 h, using a flow cytometer with Annexin V-FITC/PI binding assay. A: Control; B: HeLa cells were treated with 10 μM Ormeloxifene; C: HeLa cells were treated with 20 μM Ormeloxifene; D: Apoptosis rate of control and samples. Data were expressed as mean ± SD. #, P<0.05, compared with the control group. Control, control group; ORM, Ormeloxifene-treated group.

Ormeloxifene induced G1 phase arrest in HeLa cells

In order to determine whether the cell-growth suppressive effect of Ormeloxifene attributed to inhibit proliferation, the cell cycle distribution was detected by flow cytometric analysis. As illustrated in **Figure 2**, Ormeloxifene treatment obviously increased the percentage of cells at the G1 phase compared with the control group (10 and 20 μM: P<0.05).

Ormeloxifene induces apoptosis of HeLa cells

We then investigated the effects of Ormeloxifene on cell apoptosis by using an Annexin-V FITC Apoptosis Detection Kit. Cells were treated with 10, 20 μM of Ormeloxifene for 48 h, **Figure 3** shows the representative results of flow cytometry. Apoptosis rate was significantly higher in the Ormeloxifene treatment groups compared to the control group (10 and 20 μM: P<0.05). These results above indicated that Ormeloxifene could induce the apoptosis of HeLa cells.

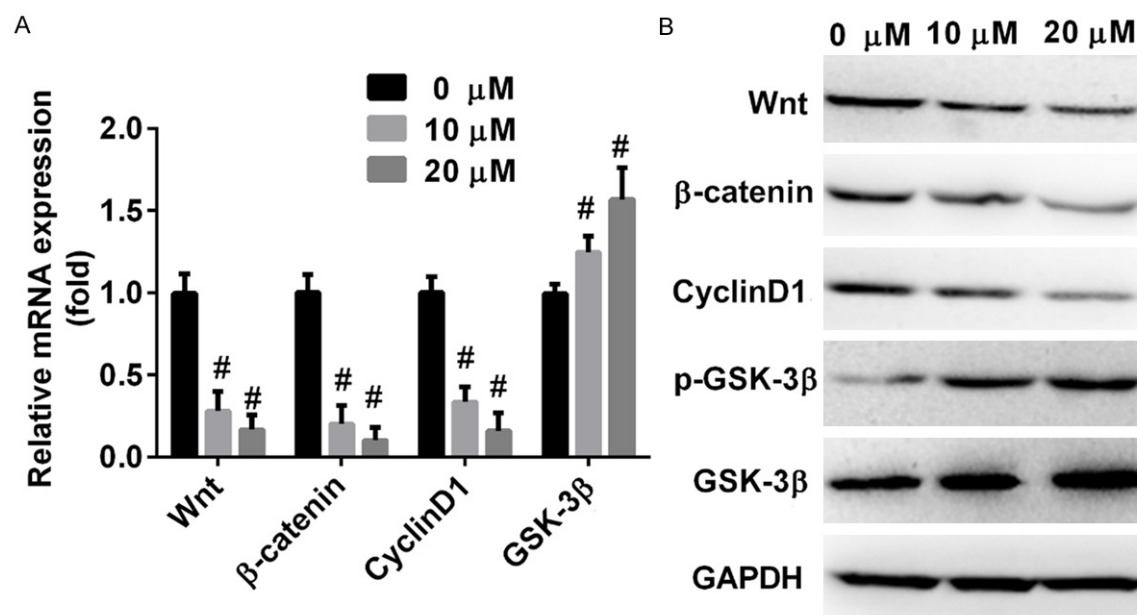


Figure 4. Effect of Ormeloxifene on expression of Wnt/β-catenin signaling in HeLa cells. The mRNA (A) and protein (B) levels of Wnt/β-catenin signaling were detected by QPCR and Western blot analysis, respectively. Data were expressed as mean ± SD. #, $P < 0.05$, compared with the control group. Control, control group; ORM, Ormeloxifene-treated group.

Ormeloxifene attenuates the Wnt/β-catenin pathway

To determine whether Wnt/β-catenin pathway involve in the anti-cancer effect of Ormeloxifene, QRT-PCR and Western blot were used to detect the expression levels of Wnt/β-catenin pathway in human cervical cancer HeLa cells. In this study, QPCR analysis showed that Wnt, β-catenin and CyclinD1 mRNA levels were significantly decreased, while pGSK-3β mRNA levels were remarkably increased in Ormeloxifene-treated HeLa cells (**Figure 4A**). According, Western blot analysis suggested that Ormeloxifene significantly down-regulated Wnt, β-catenin and CyclinD1 protein levels, up-regulated pGSK-3β protein levels in HeLa cells (**Figure 4B**).

Discussion

Ormeloxifene, a non-steroidal selective estrogen receptor modulator, which has been widely used as an oral contraceptive. Recent preclinical, and clinical studies have shown that Ormeloxifene is a potential anticancer drug as well [10, 16]. However, most studies were focused on breast and ovarian cancers. There are very few reports examining the role of Ormeloxifene in cervical cancer. To gain insight into the pos-

sible role of Ormeloxifene in cervical cancer, we investigated the effect of Ormeloxifene on cervical cancer cell line HeLa cells. We found that Ormeloxifene effectively of HeLa cells. The Ormeloxifene-induced apoptosis might be mediated by the suppression of Wnt/β-catenin pathway in HeLa cells. To our knowledge, the present study firstly demonstrated that Ormeloxifene treatment exerts anti-cancer effect on HeLa cells.

Up to date, Ormeloxifene has been reported to inhibit cell growth in various types of cancer including ovarian cancer [13], head and neck [12]. In a recent study, Ormeloxifene significantly inhibited cell proliferation in cervical cancer cell lines Caski, SiHa, C33A and HT3 cells [17]. In the currently study, MTT analysis showed that the proliferation of HeLa cells were significantly inhibited by the treatment of Ormeloxifene, suggesting the anti-cancer activity of Ormeloxifene in cervical cancer HeLa cells. Moreover, investigations of the mechanism showed that cell cycle arrested at G0/G1 phase. Similar effects in cell-cycle phase distribution were observed in human breast cancer cells [11, 18], pancreatic cancer [14] and chronic myeloid leukemia [19]. What's more important, apoptosis rate was significantly higher in Or-

meloxifene-treated cells compared to the control group. These results above suggest that Ormeloxifene induces G1 arrest and apoptosis, thereby inhibits human cervical cancer HeLa cells proliferation.

Wnt signaling transduces evolutionarily conserved pathways which play important roles in initiating and regulating biological processes including cell proliferation, migration, differentiation and apoptosis [20, 21]. Wnt pathway plays a pivotal role in the development of multiple types of cancer [22, 23], including lung cancer [24], ovarian cancer [25] and cervical cancer [26]. Furthermore, aberrant activation of Wnt/ β -catenin signaling is widespread in human cervical cancer, a secreted Wnt antagonist WIF1 induces cell cycle arrest and apoptosis in human cervical cancer cells [26]. Therefore, inhibition of Wnt effects may have major therapeutic potential. A previous study suggested that Ormeloxifene treatment effectively attenuated Wnt/ β -catenin signaling in human colon cancer cells [27]. According to this study, we found that treatment with 10, 20 μ M Ormeloxifene significantly decreased the expression of Wnt both in mRNA and protein levels. In addition, Wnt pathway target gene, CyclinD1 is a nuclear protein plays a key role in cell cycle progression in G1 [28]. In the present study, we found the expression levels of CyclinD1 were significantly down-regulated by Ormeloxifene treatment in HeLa cells, which is consistent with the observations in cell cycle analysis. Collectively, these data suggest that Ormeloxifene induces cell cycle arrest and apoptosis in HeLa cells may via the inhibition of the Wnt/ β -catenin signaling.

In conclusion, we showed evidence that Ormeloxifene effectively inhibits the proliferation, induces apoptosis and cell cycle arrest in cervical cancer HeLa cells. In addition, the results in the present study indicated that Ormeloxifene might exert its anti-cancer effect by inhibiting Wnt/ β -catenin signaling in human cervical cancer HeLa cells. However, the anti-tumor effect of Ormeloxifene on cervical cancer *in vivo* and the possible underlying molecular mechanism remain to further study.

Disclosure of conflict of interest

None.

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