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Original Research

Mifepristone regulates the multidrug resistance via miR-758/MEPE of Hela cell lines

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Summary

*Background:*In cervical cancer, matrix extracellular phosphoglycoprotein (MEPE) plays an important role of multidrug resistance, which is associated with miR-758. Mifepristone has anti-drug resistance effects, but whether mifepristone regulates the expression of multidrug resistance proteins via the pathway mediated by miR-758/MEPE is still unclear. Hela cells were induced by mifepristone (named as Hela/MIF cells). Then, matrix extracellular phosphoglycoprotein siRNA, and miR-758 mimics were transfected into HeLa/MIF cells. The sensitivity, clone forming ability, migration, and level of apoptosis of the cells in each group were respectively measured by CCK-8 assays, clone forming assays, transwell assays, and flow cytometry analysis. The targeting of miR-758 to MEPE was verified by dual-luciferase assay. At last, the expression of MDR-1, MRP-1, and GST-π were detected by qPCR and western blot assays. *Results:* The induction by mifepristone not only decreased the sensitivity of Hela cells, but also up-regulated the expression of multidrug resistance proteins. On this basis, by increasing the expression of miR-758 or downregulating the expression of MEPE, the expression of multidrug resistance proteins decreased, while the sensitivity of Hela cells to mifepristone were improved and the level of Hela cells proliferation, colony forming, and invasion further decreased. *Conclusions*: The induction by mifepristone inhibited the proliferation of Hela cells, but the sensitivity of Hela cells increased. The mechanism may depend on the expression of multidrug resistance protein. This study shows that regulating the expression of miR-758 and MEPE can reduce the resistance of Hela cells to mifepristone, enhance the sensitivity, and further improve the inhibitory effect of mifepristone.

Key words: Cervical cancer; Multidrug resistance; Mifepristone; MicroRNA; matrix extracellular phosphoglycoprotein.

Introduction

Cervical cancer is the second most frequent tumor type and the fourth leading cause of cancer-associated mortality in females worldwide [1]. Chemotherapy is commonly used to treat cervical cancer and can improve the survival rate as well as the patients' quality of life [2]. The anticancer drug platinum plays a crucial role in the chemotherapy of this cancer type [3]. Although the efficacy of platinum has greatly improved over time, many cervical cancer patients still do not benefit from chemotherapy. Recent studies have suggested that matrix extracellular phosphoglycoprotein (MEPE) may play key roles in the development of multidrug resistance (MDR), which is the main cause of failure of chemotherapy [4]. MEPE was first identified and cloned from a cDNA library of tumor tissue obtained from patients with oncogenic hypophosphatemic osteomalacia (OHO) by *Rowe et al* [5] in 2000. In follow-up experiments MEPE was found to have important roles in the resistance to DNA damage and in the DNA repair process. In cervical cancer, the expression of MEPE is closely associated with tumor metastasis and drug resistance. *Meng et al* showed that MEPE expression in human cervical cancer tissue was significantly different to that of normal cervical tissue [6]. Their study also suggested that MEPE expression is regulated by miR-758, a microRNA associated with some MDR proteins such as multidrug resistance-associated protein (MRP-1) and glutathione S-transferase π (GST-π) [7, 8].

Mifepristone (MIF) is a progesterone antagonist with high efficacy that has found widespread clinical use since it was developed in 1981 for terminating early pregnancy and for suppressing endometrial hyperplasia [9]. Anti-tumor properties of MIF were also gradually recognized, although the mechanism for this is still unclear [10]. To date, most studies on the anti-tumor effects of MIF have been aimed at improving the sensitivity of chemotherapy and reducing the resistance to treatment [11, 12]. Few studies have been focused on MIF itself and the lack of knowledge has hindered further clinical application of this agent. Therefore, the aim of the present study was to compare the effects of different concentrations of MIF on Hela cells, a human cervical cancer cell line. Moreover, we investigated whether MIF regulates the expression of multidrug resistance protein via a mechanism involving miR-758 and MEPE.

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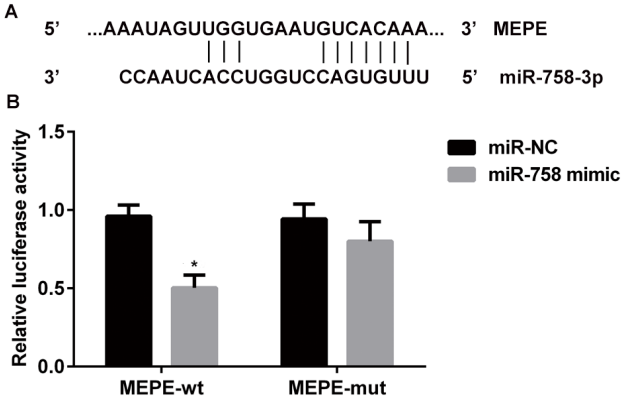

Figure 1. Targeted regulatory relationship between miR-758 and MEPE. (A) The sequence and predicted binding sites of miR-758 and MEPE. (B) Relative luciferase activities of MEPE-WT 3'UTR and MEPE-MUT 3'UTR. Values are represented as mean ± SD (n = 3). *p value of the relative luciferase activity of miR-758 mimic compared with the miR-NC was < 0.05.

Materials and Methods

Hela cell lines were purchased and cultured in RPMI-1640 medium containing 10% fetal bovine serum as the regular medium in a humidity-saturated incubator with 5% CO_2 at 37 °C. When the cell density reached approximately 80–90%, cells were detached from the culture plate using trypsin and passaged. A portion of the cells was cultured in regular medium with 0.125 μg/ml mifepristone for 24 h when in the logarithmic growth phase, then cultured in regular medium without MIF. When the cell density reached approximately 80%, the cells were passaged again. The cycle of cell culture and passage was repeated, while the concentration of MIF was doubled in each cycle until it reached 2 μg/ml. After that, cells were cultured in RPMI-1640 medium containing 10% FBS with 2,000 μg/ml MIF. The cells induced by this treatment were referred to as Hela/MIF.

MEPE siRNA, miR-758 mimics and negative controls were synthesized and the recombinant plasmids were extracted from positive clones for cell transfection. Hela/MIF cells were seeded into 6-well plates at a density of 4 × 105 cells/well and transfected in Opti-MEM with 100 nM siRNA or 50 nM miRNA plasmid and an optimal volume of Lipofectamine 2000. Cells were cultured in regular medium for 8 h following transfection and the expression of miR-758 and MEPE were analyzed 48 h after transfection.

The CCK-8 assay was used to determine the sensitivity to MIF. Five groups of cells (Hela, Hela/MIF, MEPE siRNA + Hela/MIF, miR-758 mimics + Hela/MIF, mimic control + Hela/MIF) in the logarithmic growth phase were seeded into 96-well plates at a density of 104 cells/well. Plates with no cells in PBS were used as a control group. The regular culture medium was removed after 24 h and 100 μl of medium containing MIF at a concentration of 8 μg/ml was added and the cells cultured for a further 24 h. The cells were then grown in regular medium for another 24 h period. Ten μl of CCK8 was added for 90 min. The blank and culture medium without the drug were used as controls. Three replicates per sample were evaluated and the experiment was repeated three times. The half maximal inhibitory concentration (IC50) was estimated by measuring the absorbance at 450 nm using a microplate reader. The resistance index, or RI, was defined as drug-resistant cell IC50/parental cell IC50.

The cells were seeded in fresh 24-well plates (200 cells/well) and maintained in RPMI-1640 medium containing 10% FBS. After 14 days they were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted and each experiment was performed in triplicate.

Cell migration assays were carried out in transwell chambers. For the cell migration assay, 104 cells in 200 μl of RPMI-1640 culture medium without FBS were added to the upper chamber, while 750 μl RPMI-1640 medium containing 20% FBS was added to the lower chamber. After 24 h incubation, the chamber was washed with PBS and filtered cells were fixed and then stained with 0.5% crystal violet. Cell migration was measured under an inverted microscope in five random fields per transwell chamber.

Hela cells were seeded in 6-well plates for 24 h before being exposed to MIF at 8 μg/ml for 24 h. The cells were then harvested, washed twice with pre-chilled PBS and prepared as a single-cell suspension. Apoptosis was measured using a flow cytometer and FITC-conjugated Annexin V and propidium iodide (PI) double staining.

Hela cells were seeded into 12-well plates and grown to approximately 70% confluence. The cells were then co-transfected with recombinant reporter plasmids (pmir GLO-MEPE-WT 3'UTR or pmir GLO-MEPE-MUT 3'UTR, 0.5 μg of each), pRL-TK (20 ng), and miR-758 mimics (100 nM) or the mimic controls using Lipofectamine 2000. After 24 h the cells were harvested and lysed. Luciferase activities were determined using a dual luciferase assay reporter system according to the manufacturer's instructions. Firefly luciferase activity was normalized to that of renilla luciferase.

TRIzol lysis solution was used to extract total RNA from the cells of each group in accordance with manufacturer instructions and cDNA synthesis was performed using a reverse transcription kit. cDNA samples were processed with a qPCR amplification kit and real-time PCR was performed under the following reaction conditions: 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and amplification at 60 °C for 34 s. Each sample was tested three times in triplicate. Data collection and analysis were performed using the QuantStudio 7 Flex System. Data were analyzed using the relative quantification method ($\Delta\Delta$Ct) and statistical analysis was performed using GAPDH as an internal control for data conversion ($2^{-\Delta\Delta Ct}$).

Cell samples were treated with a lysis buffer and the pro-

tein concentration was then quantified using a BCA protein assay kit. Twenty μg of protein from each sample was separated using 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes prior to incubation with primary antibodies overnight at 4 °C. Primary antibodies included anti-MDR1 (1:2000, ab170904), anti-MRP1 (1:500, ab32574), anti-GST-π (1:100, ab135535). The membranes were then incubated with corresponding HRP-conjugated secondary antibodies for 2 h at 25 oC. The blot signals were visualized using ECL western blotting substrate and the gray scales of protein bands were quantified using ImageLab software.

SPSS 22.0 was used for statistical analysis. The quantitative data for each group was tested for normality. Normally distributed data were expressed as the mean ± standard deviation (x ± s). The means of multiple groups that satisfied homogeneity of variance were compared using one-way analysis of variance (ANOVA). Pairwise comparisons were performed using the least significant difference (LSD) test. In the case of heterogeneity of variance, the Dunnett T3 method was used for analysis. A value of $p <$ 0.05 indicated the differences were statistically significant.

Results

Online prediction from targetscan.org showed the existence of complementary binding sites between miR-758 and the 3'-UTR of MEPE mRNA (Figure 1). Dual luciferase gene reporter assay showed that transfection with the miR-758 mimic significantly suppressed the relative luciferase activity in Hela cells transfected with pmir-GLO-MEPE-WT 3'UTR plasmid. However, there was no significant effect on the relative luciferase activity in Hela cells with pmir-GLO-MEPE-MUT 3'UTR plasmid transfection, indicating targeted regulation of MEPE by miR-758.

As shown in Figure 2, induction by MIF resulted in increased expression of MEPE and reduced expression of miR-758. MiR-758 mimics not only significantly increased the expression of miR-758 in Hela/MIF cells but also decreased the expression of MEPE at both the mRNA and protein levels. As expected, transfection with miR-758 mimics increased the expression of miR-758 in Hela/MIF, while transfection with MEPE siRNA decreased the expression of MEPE at both the mRNA and protein levels. The expression of MEPE in the Hela/MIF+miR-758 mimics group also declined significantly.

Mifepristone inhibited the proliferation and colony formation of Hela cells and this inhibition became more pronounced by reducing MEPE. The trends for invasion and migration of Hela cells was also suppressed by mifepristone (Figure 3).

As shown in Figure 4, cell apoptosis in the Hela and mimic control group showed a decrease following the induction of MIF resistance. However, there was no significant difference in the percentage of apoptotic cells between the MEPE siRNA group and the miR-758 mimic group, regardless of MIF induction.

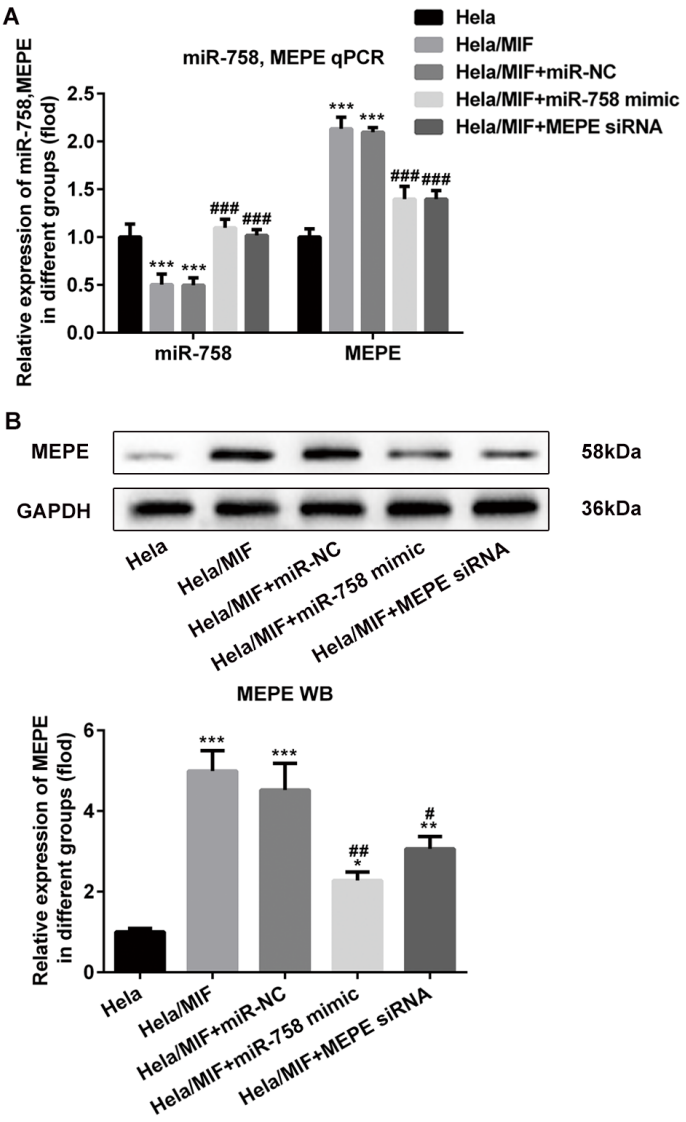

Figure 2. Expression of miR-758 and MEPE in each group with different processes on Hela cells. (A) mRNA expression of miR-758 and MEPE were analyzed by qPCR. (B) Western blotting to measure the protein expression of MEPE. The relative density was quantified relative to GAPDH. Values are represented as mean ± SD (n = 3 per group). Compared with the group of Hela, *p $<$ 0.05, **$p <$ 0.01, ***$p <$ 0.001; Compared with the group of Hela/MIF, #$p <$ 0.05, ##$p <$ 0.01, and ###$p <$ 0.001.

MRP-1, MDR-1 and GST-π are three important factors that reflect multidrug resistance. As shown in Figure 5, the expression of all three of these factors after treatment with MIF in Hela cells was significantly greater compared to cells without treatment. In contrast, the expression of these factors in Hela cells transfected with miR-758 mimic or MEPE siRNA was reduced.

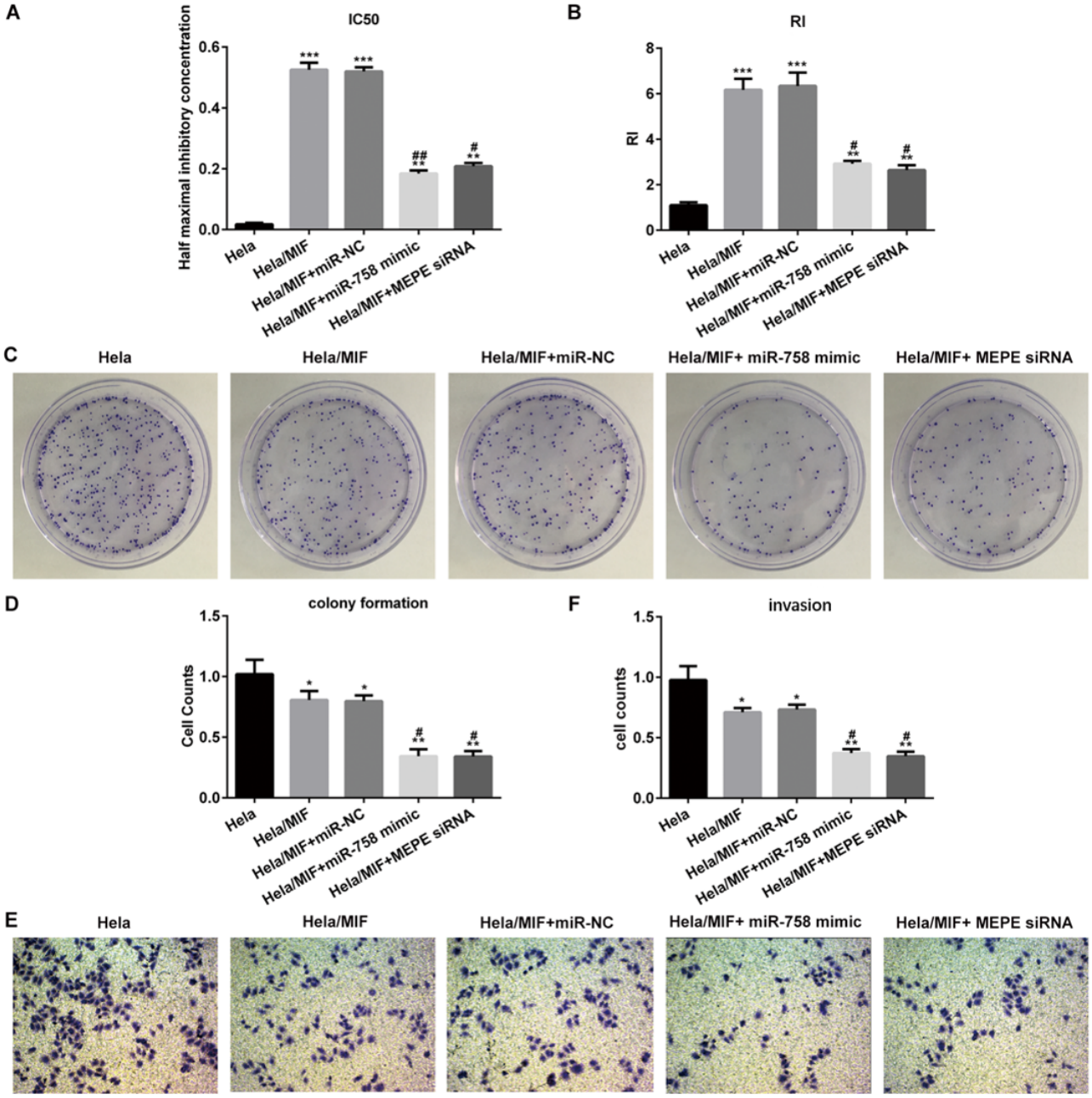

Figure 3. Proliferation, colony formation, migration, and invasion of the Hela cells with different processes. (A) and (B) Half maximal inhibitory concentration (IC50) detected by CCK-8 assay and Resistance Index (RI) of each group. RI = IC50 (Test group) /IC50 (Hela group). (C) and (D) Performances and statistical results of colony formation assays in each group. (E) and (F) Cell migration in each group detected by Transwell assay. Values are represented as mean ± SD (n = 3 per group). Compared with the group of Hela, *$p <$ 0.05, **$p < 0.01$, and ***$p < 0.001$; compared with the group of Hela/MIF, #$p < 0.05$ and ##$p < 0.01$.

Discussion

In the present study, proliferation of the Hela cells induced by mifepristone were decreased significantly. However, long-term induction also reduced cells sensitivity and even caused the multidrug resistance in these cells, which may limit the anti-tumor effect of mifepristone and the role in reversing the resistance of platinum drugs. Therefore, if those side effects of mifepristone can be reduced, its anti-tumor effect will effectively be improved.

Multidrug resistance (MDR) in tumors is thought to be due to multiple factors and mechanisms. Extensive research has revealed that overexpression of ATP-binding cassette (ABC) transporters is one of the most critical and direct factors in producing MDR. Examples of these transporters are p-glycoprotein (p-gp) encoded by ABCB1, breast cancer

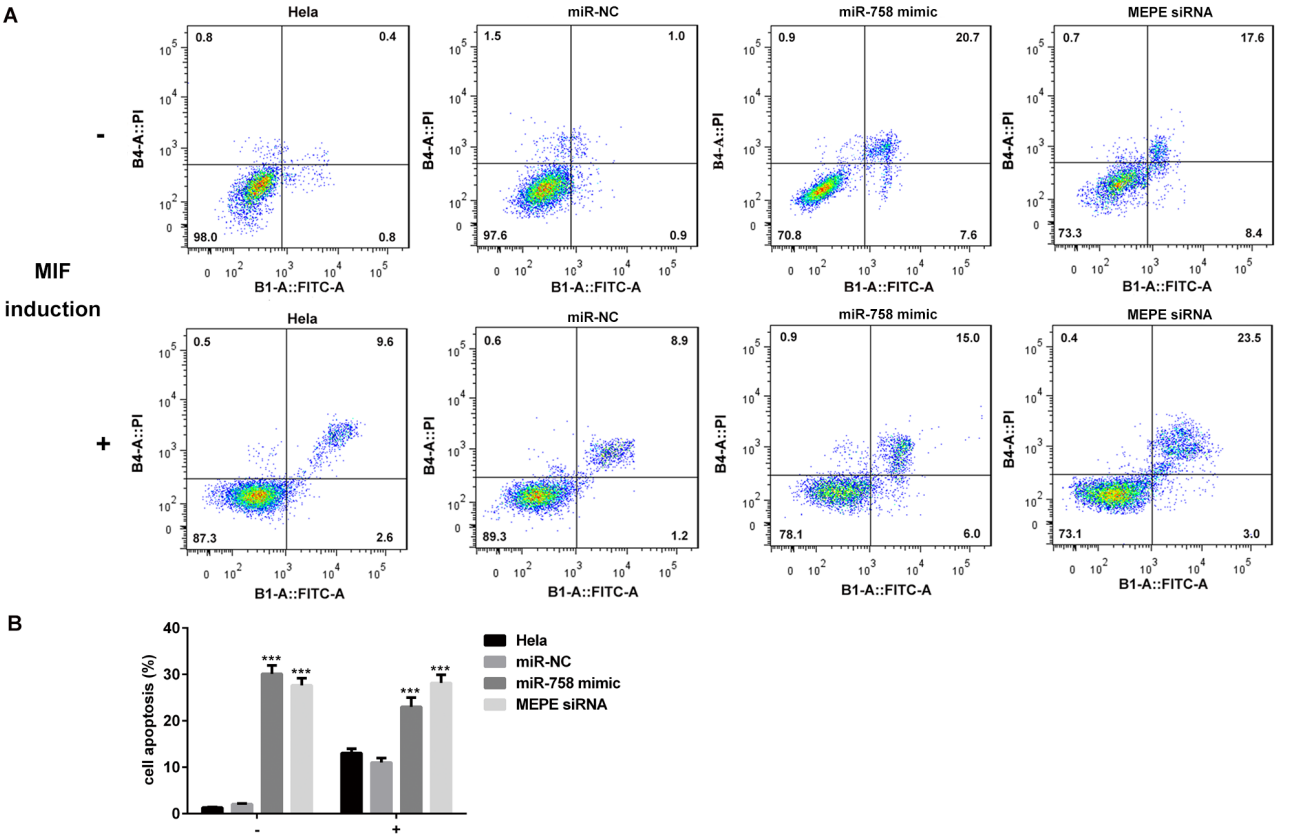

Figure 4. Cell apoptosis analysis by flow cytometry. (A) With or without mifepristone induction, the performance of cells apoptosis in four groups. (B) The statistical results of cells apoptosis in four groups. Values are represented as mean ± SD (n = 3 per group). Compared with the group of Hela, $***p < 0.001$.

resistance protein (BCRP) encoded by ABCG2, and multidrug resistance protein (MRP) encoded by ABCC [13-15]. The drug resistance mechanisms that underlie these proteins are similar and involve the transport of drugs away from their cellular targets. The proteins p-gp and MRP are directly relevant to cervical cancer. Both cause the sequestration of drugs into unrelated organelles, thus reducing their concentration at target sites in cervical cancer cells. MRP also changes the permeability of cell membranes and the pH within cells, both of which are important factors leading to MDR. Furthermore, both p-gp and MRP can alter the sensitivity of drug-resistant cells to chemotherapy by impacting DNA repair activity [16, 17].

The membrane protein MRP transports a variety of drugs and derivatives, especially glutathione-coupled drug derivatives. It also affects the movement of cytoplasmic vesicles, resulting in a decrease in the effective concentration of the drug at its target. Glutathione S-transferase (GST) is a key enzyme that catalyzes the initiation of glutathione-binding reactions [18]. GST-π is the most highly expressed member of the GST family and a reduction in its expression has been associated with MDR. Annereau et al reported that GST-π showed the strongest decrease in expression amongst more than 20,000 genes studied in resistant derivatives of KB-3-1 parental cells selected in colchicine (KB-8-5), whereas the ABC transporters showed dramatic overexpression.

Although MIF has been used for many years to treat tumors, there are still only a few studies on the mechanism of action of this drug in cervical cancer. These focused on the expression of proteins that are related to cell apoptosis, immune suppression, and the interstitial environment. B-cell lymphoma-2 (Bcl-2), Bcl2-sssociated X (Bax) and the caspase family are the most widely studied apoptotic factors in cervical cancer. Studies by Chen et al and by Jurado et al both reported that MIF could reverse the drug resistance of HeLa cells by increasing Bcl-2 protein expression and reducing Bax protein expression. In addition, the overexpression of glucosylceramide synthase may play a crucial role in the development of MDR in cervical cancer cells and can be induced by MIF [19, 20]. Additional studies have shown that by altering the expression of Bcl-2 and Bax, MIF changes the mitochondrial permeability and causes release of Omi/HtrA2 protein, which subsequently antagonizes inhibitor of apoptosis proteins (IAPs). Finally, MIF has been shown to induce the apoptosis of cervical cancer cells [21, 22]. Recent studies have reported that MIF can increase the sensitivity to chemotherapeutics of cervical cancer cells by reducing expression of nuclear transcription factors, including NF-κB, p53, p65 and ki-67 [23-26]. Secretion of

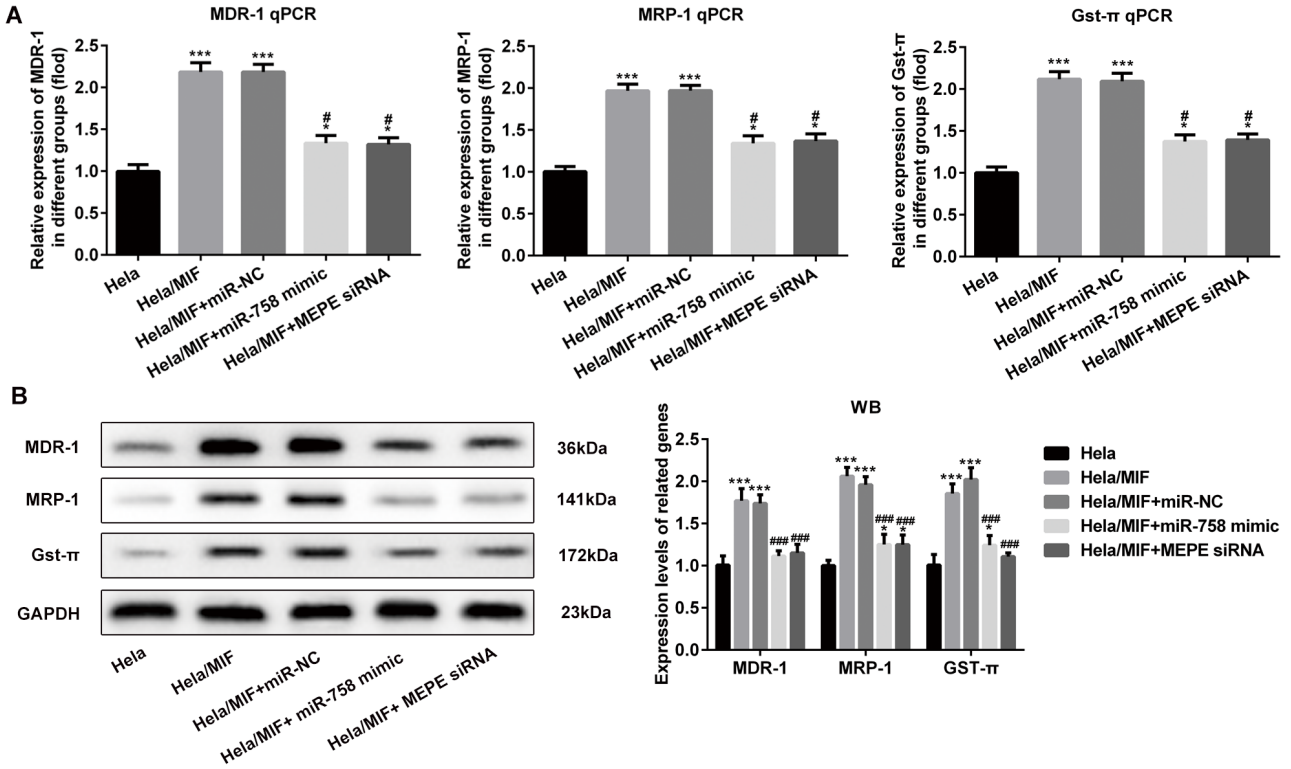

Figure 5. Expression of MDR-1, MRP-1, and GST-π in each group with different processes on Hela cells. (A) mRNA expression of MDR-1, MRP-1, and GST-π were analyzed by qPCR. (B) Western blotting to measure the protein expression of MDR-1, MRP-1, and GST-π. The relative density quantified relative to GAPDH. Values are represented as mean $\pm$ SD (n = 3 per group). Compared with the group of Hela, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; compared with the group of Hela/MIF, #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$.

exosomes from cervical cancer cells are reduced by MIF [27], which is also known to be an important mechanism for inhibiting tumor metastasis.

MEPE has important roles in the resistance to DNA damage and in the DNA repair processes. The expression of MEPE has been closely associated with tumor metastasis and drug resistance in cervical cancer [5]. In the present study we observed that the proliferation and clonality of Hela/MIF cells were further inhibited by direct knock down of MEPE mRNA, or indirectly by reducing mRNA expression through the up-regulation of miR-758. At the same time, the expression levels of MDR1, MRP1, and GST-π were significantly reduced. Together, these results indicate that the sensitivity of Hela/MIF cells to MIF may be altered by regulating the expression of MDR proteins in these cells.

It is well established that human papillomavirus (HPV) can induce squamous metaplasia of cervical cells, leading to cervical cancer. Among the numerous subtypes of this virus, the most common and important high-risk type is HPV16. At least 50% of cervical cancers are associated with HPV16 [28]. HPV16 E6 protein is one of the major proteins regulating the life cycle of the virus and plays an important role in tumor cell development. Previous studies have demonstrated that MIF may inhibit the transcription and expression of HPV16 E6 in Hela and C4-1 cell lines, as well as reducing the rate of clone formation in vitro. MIF also reduces the proliferation of Hela cells in a dose-dependent fashion. The lowest dose of MIF used in the present study, 2.5 μmol/L, was effective in reducing cell proliferation. Based on the relevant studies to date, it is clear that MIF significantly inhibits the growth of progesterone receptor positive cervical cancer cells in vitro [29].

In summary, the present authors demonstrated that mifepristone regulates the expression of multidrug resistance factors and the activity of relevant enzymes, of which the mechanism is up-regulating miR-758 and then down-regulating MEPE. Interestingly, mifepristone itself also showed a characteristic of resistance. Given the characteristics of multidrug resistance and reversal of drug resistance, future research can be designed and carried out about the contradiction of mifepristone.

Conflict of Interest

The authors declare no competing interests.

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