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Original Article

Down-regulation of anti-apoptosis protein livin promotes HMR1275 (flavopiridol)-induced apoptosis of endometrial carcinoma cell line ishikawa

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Abstract: HMR1275 (flavopiridol) is one of the commonly used drugs for anti-cancer treatment, and plays a pivotal role in breast cancer and lung cancer. The treatment of endometrial carcinoma is always delayed due to misdiagnosis for gynecological inflammation. In addition, it remains unclear how HMR1275 (flavopiridol) attenuates lesion of endometrial carcinoma. Our study was focused on the exploration of molecular mechanism underlying anti-cancer effect of HMR1275 (flavopiridol) via Livin. Endometrial carcinoma cell line Ishikawa was treated with HMR1275 (1 μmol/L). MTT assay and flow cytometry examination were performed to assess the cell growth, proliferation and apoptosis of Ishikawa cells. Western blot was performed to examine the protein expression of Livin. siRNA was used for HMR1275 (1 μmol/L) treated Ishikawa cells to inhibit the expression of Livin, and apoptosis was also examined by MTT assay and flow cytometry. HMR1275 (1 μmol/L) treatment significantly reduced the growth rate of ishikawa cells, resulted in plasma membrane translocation of phosphatidylserine and activation of caspase. Protein expression of Livin was remarkably decreased after HMR1275 (1 μmol/L) treatment. Moreover, down-regulation of Livin further enhanced apoptosis of ishikawa cells after HMR1275 (1 μmol/L) treatment. Down-regulation of anti-apoptosis protein Livin promotes HMR1275 (flavopiridol)-induced apoptosis, and HMR1275 was possible to alleviate cancer via decreasing the expression of Livin.

Keywords: HMR1275 (flavopiridol), livin, endometrial carcinoma, apoptosis, ishikawa

Introduction

Endometrial carcinoma is a kind of epithelial cancer [1]. Although mortality of endometrial carcinoma varies in different regions, global analysis data showed that its mortality ranked the third among common gynecologic malignancy [2]. Mortality of endometrial carcinoma gradually increases yearly in China, and is ranked second among genital malignancy [3].

Surgery is the main treatment for endometrial carcinoma [4]. For endometrial carcinoma patients at early stage, surgery can be used for many purposes, including operation-pathologic staging, judgement of disease extent, prognosis estimation, uterectomy, removal of metastatic lesion and choice of adjuvant therapy. Routine protocols of surgery were performed as follows: peritoneal lavage, epifacial panhysterectomy, bilateral salpingo-oophorectomy, pelvic lymph node dissection and para-ortic lymphadenectomy. Although most of early-stage patients could be cured by standard surgery, efficacy of current therapies is not promising for late-stage patients or patients with high recurrence risk according to operation-pathologic staging. Thus such high-risk patients always need adjuvant therapy. In addition, endometrial carcinoma patients are always complicated with multiple diseases, including hypertension, diabetes, obesity and other cardiovascular or cerebrovascular diseases. Accordingly, combined therapy should be individualized for late-stage patients [3].

Chemotherapy drugs were developed for cancer therapy, such as platinum drugs, taxol and adriamycins. Flavonoids are novel anti-cancer drugs with flavone structure. As a member of

flavonoids, HMR1275 (flavopiridol) was proved to have promising efficacy in breast cancer and lung cancer. However, the anti-cancer mechanisms of HMR1275 remain unclear [5].

Apoptosis is a programmed cell death regulated by genes to maintain homeostasis. Previous studies demonstrated apoptosis was feasible to alleviate tumor lesions. Anti-apoptosis proteins are potential targets for cancer treatment, and some have been used for drug design, including P53, CrmA, IAPs, FLIPs and Bcl-2. Recent studies demonstrated that Livin was a protein kinase influencing apoptosis [3] and programmed necrosis [6]. Inhibition of Livin promoted apoptosis of cancer cells, which provided a potential mechanism for HMR1275 (flavopiridol).

Our study is focused on the exploration of molecular mechanism underlying anti-cancer effect of HMR1275 (flavopiridol), and Livin could be regulated by HMR1275 (flavopiridol).

Materials and methods

Reagents and cells

MTT assays, lipidosome and Livin antibody were purchased from Sigma (USA). Reagents for examination of apoptosis were purchased from Beyotime (China), including tetramethylrhodamineethylester (TMRE), FITC-Annexin-V and caspase Kit. DMEM and fetal bovine serum were purchased from Dingguo (China). siRNA for inhibition of Livin expression was designed and synthesized by GenePharma (China), and sequences are as follows: 5'-TTCCGGGGGATTGAATTT-3' and 5'-ATTCCAATAGAATAATTT-3'.

Cell culture

Ishikawa cell line was purchased from ATCC and stored at -80°C. Cell resuscitation was performed with routine protocol. Resuspended Ishikawa cells into DMEM (5% fetal bovine serum). Add 1 μmol/L HMR1275 (flavopiridol) into experimental group, and the same amount of DMEM into control group. Ishikawa cells were treated for 24 hours.

Lipidosome transfection

Transfer Ishikawa cells into 24-well plate. Dilute Escort™ Transfection Reagent and siRNA at room temperature. Add lipidosome into 24-well plate. Detailed protocols were performed as follows: Add 400 μL ddH_2O into reaction liquid with Ishikawa cells. Maintain reaction at vibration for 10 seconds to dissolve flocculent precipitation. Store reaction liquid at -20°C. Transfect reaction liquid with Ishikawa cells at an appropriate mixing ratio (lipidosome: DNA = 1:1-1:2). Store reaction liquid at room temperature for 10 to 15 minutes. Discard culture medium in 24-well plate, and wash gently with PBS or serum-free medium. Add reaction liquid into 24-well plate, and maintain culture for 1 hour. Discard culture medium in 24-well plate, add complete medium and maintain culture for 24 to 48 hours [7].

MTT assays

MTT assays were performed to examine the survival and growth of Ishikawa cells with routine protocols [8]. Transfer Ishikawa cells into 24-well plate. Add MTT reagents to terminate culture. Data of absorbance value (560 nm) was analyzed for establishing the growth chart.

Flow cytometry test

As reported previously, TMRE (cytochemical stains for membrane potential) and FITC-Annexin-V (cytochemical stains for phosphatidylserine) were used for flow cytometry test [9, 10].

Detailed protocols of TMRE stain were performed as follows: Resuspend appropriate amount of Ishikawa cells with 0.5 ml cell culture fluid. Add 0.5 ml TMRE treatment fluid, and incubate at 37°C for 20 minutes. Prepare TMRE buffer solution: mix TMRE treatment fluid with ddH_2O (1:4) and store in ice-bath. Centrifuge (600×g) at 4°C for 3 to 4 minutes after incubation. Discard supernatant.

Wash cells twice with TMRE buffer solution, details were as follows: Resuspend cells with 1 ml TMRE buffer solution. Centrifuge (600 g) at 4°C for 3 to 4 minutes after incubation. Discard supernatant. Add appropriate amount of TMRE buffer solution and analyze with flow cytometry.

Western blot

Western blot are performed according to previously reported protocols [11]. Expression of Livin and β-actin were examined, respectively.

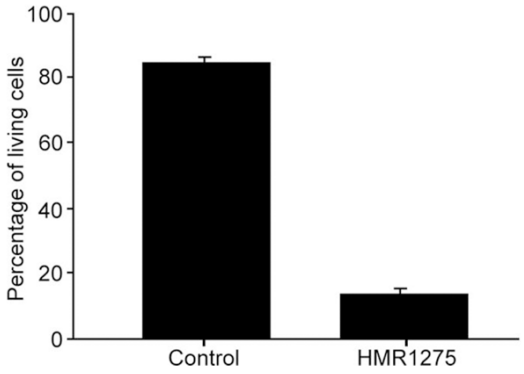

Figure 1. Analysis of cell growth in two groups. *P < 0.05 versus control group.

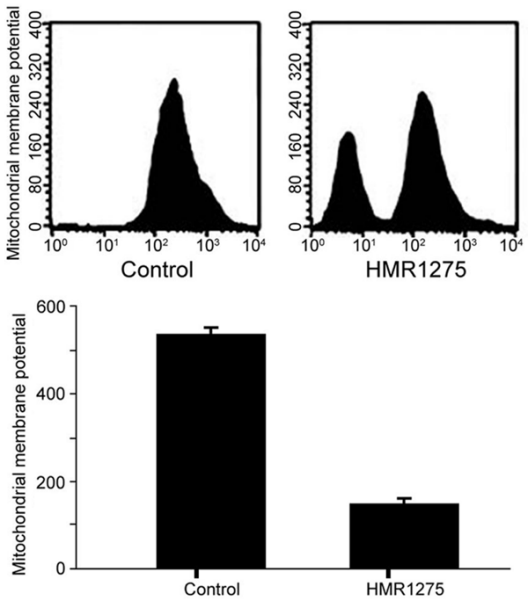

Figure 2. Analysis of mitochondrial membrane potential in two groups.

Examination of Caspase-3 activation

As reported previously, examination of Caspase-3 activation was performed according to routine protocols [12]. Data of absorbance value (560 nm) was analyzed for different groups.

Statistical analysis

SPSS 12.0 software was used for data processing. Measurement data were represented as mean ± standard deviation (SD). One-Way ANOVA was performed for analysis of the statistical significance. *P* value < 0.05 was considered to be statistically significant.

Results

HMR1275 (flavopiridol) inhibited growth of Ishikawa cells

As showed in **Figure 1**, compared with control group (DMSO treated), the cell growth of Ishikawa cells in HMR1275 group was significantly decreased.

HMR1275 (flavopiridol) induced decrease of membrane potential

HMR1275 (flavopiridol) treatment significantly decreased the mitochondrial membrane potential of Ishikawa cells, suggesting HMR1275 possibly regulated apoptosis of Ishikawa cells (**Figure 2**).

HMR1275 (flavopiridol) enhanced apoptosis of Ishikawa cells

HMR1275 (flavopiridol) treatment significantly increased the percentage of phosphatidylserine translocation, suggesting HMR1275 indeed enhanced apoptosis of Ishikawa cells (**Figure 3**).

HMR1275 (flavopiridol) activated caspase-3 in Ishikawa cells

As showed in **Figure 4**, compared with control group (DMSO treated), activation of caspase-3 in Ishikawa cells was enhanced in HMR1275 group, confirming HMR1275 increased the apoptosis of Ishikawa cells.

HMR1275 (flavopiridol) down-regulated expression of livin in ishikawa cells

As showed in **Figure 5**, compared with control group (DMSO treated), the expression of Livin in Ishikawa cells was decreased in HMR1275 group, suggesting HMR1275 regulated apoptosis via Livin.

Down-regulation of livin promoted HMR1275 (flavopiridol)-induced apoptosis

siRNA indeed decreased the expression of Livin, while inhibition of Livin alone did not increase the apoptosis. Moreover, inhibition of Livin promoted apoptosis of HMR1275-treated Ishikawa cells (**Figure 6**), suggesting down-regulation of Livin promoted HMR1275 (flavopiridol)-induced apoptosis.

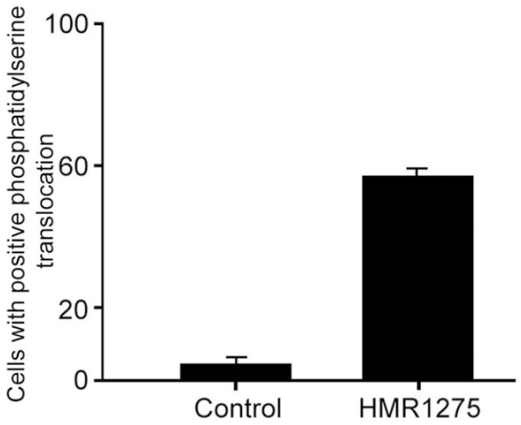

Figure 3. Analysis of percentage of phosphatidylserine translocation in two groups.

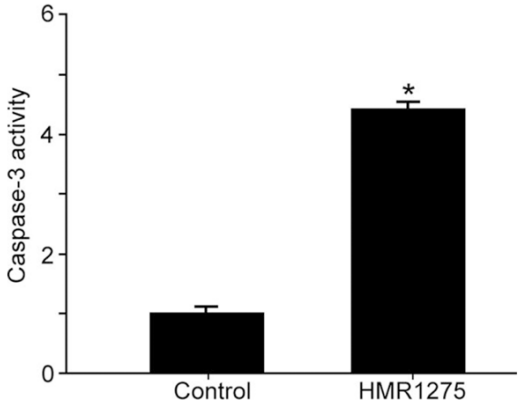

Figure 4. Analysis of activation of caspase-3 in Ishikawa cells. *P < 0.05 versus control group.

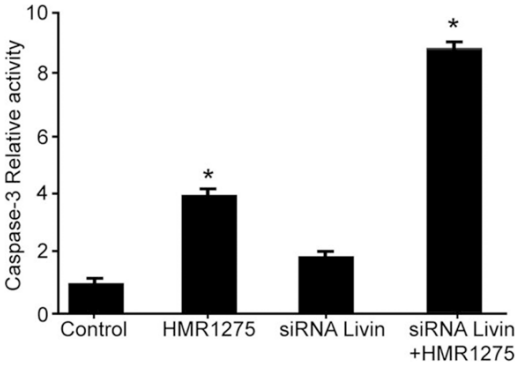

Figure 5. Protein bands of Livin and β-actin in two groups.

Discussion

HMR1275 (flavopiridol) has been proved as an important anti-cancer drug [1, 2]. Our study demonstrated that down-regulation of Livin enhanced HMR1275 (flavopiridol)-induced apoptosis of endometrial carcinoma cell Line Ishikawa, which was consistent with previous report [6].

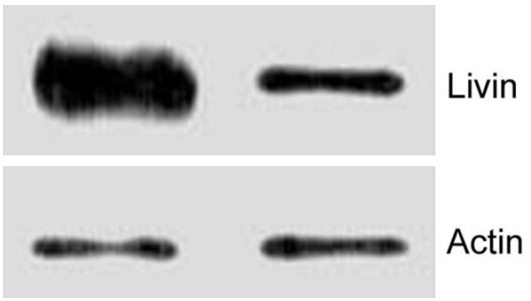

Figure 6. Down-regulation of Livin promoted HMR-1275 (flavopiridol)-induced apoptosis. *P < 0.05 versus control group.

We explored the effect of HMR1275 (flavopiridol) on growth and survival of Ishikawa cells, and found that HMR1275 (flavopiridol) indeed inhibited the growth of Ishikawa cells. Furthermore, HMR1275 (flavopiridol) enhanced the apoptosis of Ishikawa cells, verified by significant decrease of mitochondria membrane potential and increase of Caspase-3 activity. All above were consistent with previous results [13, 14]. What's more, although previous study reported HMR1275 (flavopiridol) also resulted in apoptosis of breast cancer cells, Ishikawa cells seemed to be more vulnerable at the same concentration of HMR1275 (1 μmol/L), verified by higher levels of apoptosis than breast cancer cells. This suggested HMR1275 (flavopiridol) was more effective in the treatment of endometrial carcinoma, which could be explained by different chemo-sensitivities among cells [15-17].

We further explored the mechanisms underlying anti-cancer effect of HMR1275 (flavopiridol). Activation of caspase-3 induced by HMR-1275 (flavopiridol) suggested HMR1275 (flavopiridol) enhanced apoptosis of Ishikawa cells via mitochondrial signaling pathway, which is consistent with previous studies [18-20]. As a novel biomarker in mitochondrial [4], Livin was possibly regulated by multiple kinds of microRNAs. Our study demonstrated that HMR-1275 enhanced apoptosis via down-regulating Livin, verified by siRNA experiment. This suggested inhibition of Livin increased the sensitivity of Ishikawa cells treated with HMR1275 (flavopiridol).

Four aspects should be focused in the future studies. 1) Clinical specimens should be collected for examination of Livin expression. 2) Analogue of HMR1275 (flavopiridol) will be studied for better understanding of the pharmacological action. 3) Mice models with Livin gene knock-out should be established to confirm our findings [21]. 4) The relationship between Livin and HMR1275 (flavopiridol) should be confirmed *in vivo*.

In conclusion, down-regulation of anti-apoptosis protein Livin promotes HMR1275 (flavopiridol)-induced apoptosis via increasing the drug sensitivity, and HMR1275 was possible to alleviate cancer via decreasing the expression of Livin.

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Disclosure of conflict of interest

None.

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