Marchantin M Induces Apoptosis of Prostate Cancer Cells Through Endoplasmic Reticulum Stress

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Data Collection B
Statistical Analysis C
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Background: Apoptosis is mediated by the endoplasmic reticulum (ER) stress pathway, mitochondrial pathway, and death receptor. Data herein suggested an inhibitory effect of marchantin M on tumor formation in nude mice as well as the impact on CHOP and GRP78 expression.

Material/Methods: The role of marchantin M on proliferation and apoptosis of DU145 cells were measured by MTT and flow cytometry, respectively. Western blot was applied to detect the expression of GRP78 and CHOP. The mice received abdominal injection at 1 time/2 d and 2 ml/time. Tumor volume was measured every 6 days. The mice were euthanatized 30 days after marchantin injection and tumor weight was measured. Cell apoptosis was determined by TUNEL. The expressions of CHOP and GRP78 were detected by immunohistochemistry.

Results: Tumor size and weight in marchantin groups were significantly lower than in the control group (A, B) (P<0.05), and the inhibitory rate presented a dose-dependent increase. Compared with controls, the levels of CHOP and GRP78 expression elevated obviously following the treatment with marchantin (P<0.05). It showed statistically significant difference among groups C, D, E, with different levels of apoptosis indexes incremented in groups of marchantin H, M, L, compared with groups A and B (P<0.05).

Conclusions: Overall, this study shows that marchantin M circumvents the growth of prostate cancer PC-3 tumor and up-regulates expressions of CHOP and GRP78. Our data also indicate that marchantin M limits the proliferation and favors apoptosis of DU145 cells in a time- and dose-dependent manner.

MeSH Keywords: Aspergillosis, Allergic Bronchopulmonary • Calcitonin Gene-Related Peptide • Prostatic Neoplasms

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Background

Prostate cancer is a common malignant tumor of the male reproductive system; it has a high mortality rate. Endocrine treatment is the main therapy for advanced prostate cancer. It usually develops to hormone-independent prostate cancer in most of the patients during the course of therapy, and hormone-independent transformation poses a serious obstacle to the efficacy of the treatment [1,2]. In malignant tumor cells, proteasome activity is increased, with disordered protein metabolism and dysregulated ubiquitin-proteasome. Evidence demonstrated that proteasome is a novel target for tumor treatment [3,4]. Tumor malignancy degree, including prostate cancer and colon cancer, correlates with proteasome activity up-regulation. Proteasome inhibitor decreases antiapoptotic proteins and up-regulates proapoptotic pathways, which leads to tumor cell apoptosis by blocking cell cycles. The proteasome exhibits potent induction of apoptosis in comparison to standard cytotoxic drugs. As a sensitization agent for chemoradiotherapy, it generates litter toxicity to normal tissue with selectivity and low resistance [5,6]. As a novel proteasome inhibitor, marchantin M is a type of double benzyl compound obtained from bryophytes. Priming studies illustrated a significant inhibitory role of marchantin in prostate cancer cell proliferation, which blocks PC-3 in G0/G1, induces tumor cell apoptosis, down-regulates antiapoptotic protein Bcl-2, and up-regulates proapoptotic protein Bax expression. However, its antitumor mechanism has not been fully elucidated [7,8]. Apoptosis is mediated by the endoplasmic reticulum (ER) stress pathway, mitochondrial pathway, and death receptor. ER stress appears in the early stage of apoptosis and CHOP and GRP78 are the markers of ER stress [9,10]. This study aimed to investigate the effect of marchantin M on tumor formation in nude mice and impact on CHOP and GRP78 expression.

Material and Methods

Human prostate cancer cell line PC-3 and DU145 were gifts from the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, and were maintained in RPMI1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin-streptomycin at 37°C and 5% CO_2. Marchantin M was provided by Sigma (purity over 95%). Dimethyl sulfoxide was bought from Xi'an Tianzheng Co., LTD. TUNEL apoptosis detection kit, CHOPm and GRP78 monoclonal antibody, and CHOP and GRP78 immunohistochemistry kits were supplied by the Wuhan Boster Biotechnology Co., LTD.

Animals

Fifty male SPF BALB/c nude mice weighing 18–20 g at 4–6 weeks were provided by the Institute of Chinese Academy of Medical Sciences Tumor Animal Center and fed according to experimental animal standards. The experimentation with animals was governed by the Regulations of Experimental Animals of Hunan Authority and approved by the Animal Ethics Committee of Southern Medical University.

Experimental methods

Subcutaneous prostate cancer PC-3 nude mice model

We subcutaneously injected 0.1 ml PC-3 cells in logarithmic phase at 1×10^7/ml to the left anterior axillary inoculation of nude mice. Mice phenotype and survival were observed daily, and tumor diameter was measured to draw the tumor growth curve.

Animal grouping and administration

When the tumor volume increased to 300 mm³, the mice were randomly divided into group A (saline), group B (DMSO), and group marchantin C, D, E (marchantin M 200, 100, 50 µmol/kg) with 10 animals in each group. The mice received abdominal injection at 1 time/2 d and 2 ml/time. Marchantin M was given to the mice at 200, 100, 50 µmol/kg in group C, D, E, respectively, according to previous results [11,12]. Tumor volume was measured every 6 days, $V=\pi/6(a \times b^2)$. The mice were euthanatized at the 30th day after injection and tumor weight was measured. Tumor inhibitory rate (%)=(mean weight in control – mean weight in test)/mean weight in control ×100%. Cell apoptosis was determined by TUNEL according to manufacturer's instructions. We observed 100 nucleuses in each field with 5 fields chosen in each slide. Apoptosis index AI=apoptotic cell number/cell number × 100%.

CHOP and GRP78 expression detection in tumor tissue

Immunohistochemistry SP (streptavidin-peroxidase) method was applied to detect CHOP and GRP78 expression. The paraffin section was prepared after PBS washing, primary antibody incubation, repairmen, DAB colorization (1 drop of DAB was added into 1ml water, before adding 1 drop of H_2O_2, and then mixed with 1 drop of phosphate buffer. The mixture was dripped to the section, and was shaken well for staining 5 to 10 min controlled by microscope), and dehydration. Result determination: positive staining of GRP78 and CHOP was found in cytoplasm and nucleus, respectively. Yellow or tan particles were observed in positive cell nuclei or cytoplasm. Dyeing strength was quantitatively analyzed and the average integral absorbance of positive area was determined. Dyeing strength and the percentage of positive cells were scored together for the evaluation. The score of the percentage of positive cells: 0 point, negative, 1 point, 0–25%, 2 points, 26–50%, 3 points, 51–75%, 4 points, ≥76%. The score of dyeing strength is determined by

color depth: 0 point, colorless, 1 point, canary yellow, 2 points, brown yellow, 3 points, brown. Evaluation: negative, 0 point, weakly positive (+), 1–3 points, moderately positive (++), 4–5 points, strongly positive (+++), 6-7 points. The measurement of optical density was performed by using Image-Pro Plus 6.0 for immunohistochemical staining analysis.

DU 145 cells proliferation measurement by MTT

DU 145 cells were seeded into 96-well plate (5×10^3/well) followed by treatment with Marchantin M (5, 10, 20 μmol/L) after 24h culture for 24 h, 48 h and 74 h. At each time point, cell proliferation was measured.

DU 145 cells apoptosis measurement by flow cytometry

DU 145 cells were seeded into 96-well plate (1×10^6/well) followed by treatment with marchantin M (5, 10μmol/L) after 24 h culture. After that, cells were washed twice with PBS followed by fixation with 70% cold ethanol and subsequent flow cytometric analysis.

Western blot

Cell supernatants were isolated from lysed cells with lysis buffer, and protein concentration was measured by Bradford method. We loaded mg proteins into 8% SDS-PAGE followed by transferring into cellulose nitrate film and block for 1 h at room temperature. After that, primary antibody anti-β-actin, CHOP, or GRP78 was added and incubated overnight at 4°C, followed by addition of HRP-conjugated secondary antibody and incubated for 1 h. The protein band was visualized by enhanced chemiluminescence.

Statistical analysis

All statistical analyses were performed using SPSS19.0 software. Numerical data were presented as means and standard deviation ($\bar{\chi} \pm S$). Differences between multiple groups were analyzed using one-way ANOVA. $P < 0.05$ was considered as significant difference.

Results

Tumor growth and nude mice general information

No animals died during the test. The mass of tumor could be felt by touch after 1 week, and all the mice had a tumor of about 0.5-0.6 cm after 2 weeks. Tumor formation rate was 100%. Nude mouse activities, hair, and eating showed no significant abnormality among each group. Seven days after abdominal injection, tumor size increased gradually in group A
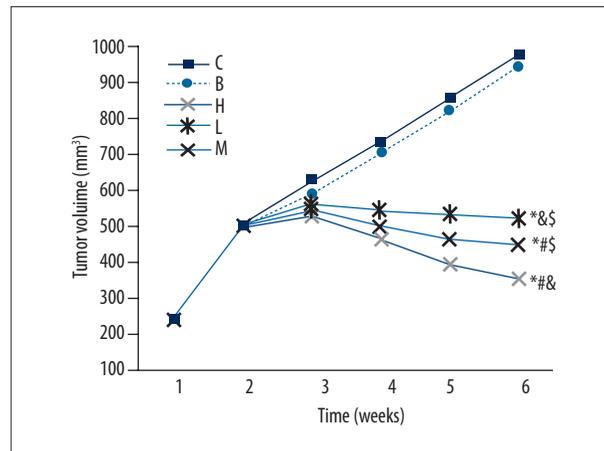
Figure 1. PC-3 tumor growth curve in each group. 0.1 ml PC-3 cells in logarithmic phase at the concentration of 1×10^7/ml were subcutaneous injected to the left anterior axillary inoculation of nude mice. Tumor diameter was measured to evaluate tumor volume. * $P < 0.05$, compared with group A and B; # $P < 0.05$, compared with group E; & $P < 0.05$, compared with group D; $ $P < 0.05$, compared with group C. A: Control (Saline); B: DMSO; C: Higher dose of marchantin (200 μmol/kg); D: Medium dose of marchantin (100 μmol/kg); E: Lower dose of marchantin (50 μmol/kg).

and B, but it grew more slowly in group C, D, E. After administration for 14 days, tumor size in group C, D, and E began to decrease, while it kept increasing in group A and B. The tumor size showed significant difference among different groups after 30 days ($P < 0.05$). The nude mice in group A and B had less hair, no activity and less eating. Their health status in group C, D, E was dose-dependent (Figure 1).

Marchantin M's impact on tumor weight and tumor inhibitory rate

The nude mice were euthanized after intraperitoneal injection at 30 days. The tumor weight and size were significantly lower in groups of marchantin treatment with higher tumor inhibitory rates than those in group A and B ($P < 0.05$). In addition, the tumor inhibitory rates presented statistical differences among different marchantin groups ($P < 0.05$). Group H showed most significant inhibitory effect, in a time- and dose-dependent manner (Figure 2).

Marchantin M's effect on CHOP and GRP78 protein expression

GRP78 is mainly expressed in the cytoplasm, while CHOP is mostly expressed in the nucleus. CHOP and GRP78 conferred no noticeable increased expression in group A and B, but the expression increased significantly in group C, D, and E ($P < 0.05$). They presented statistical differences among different
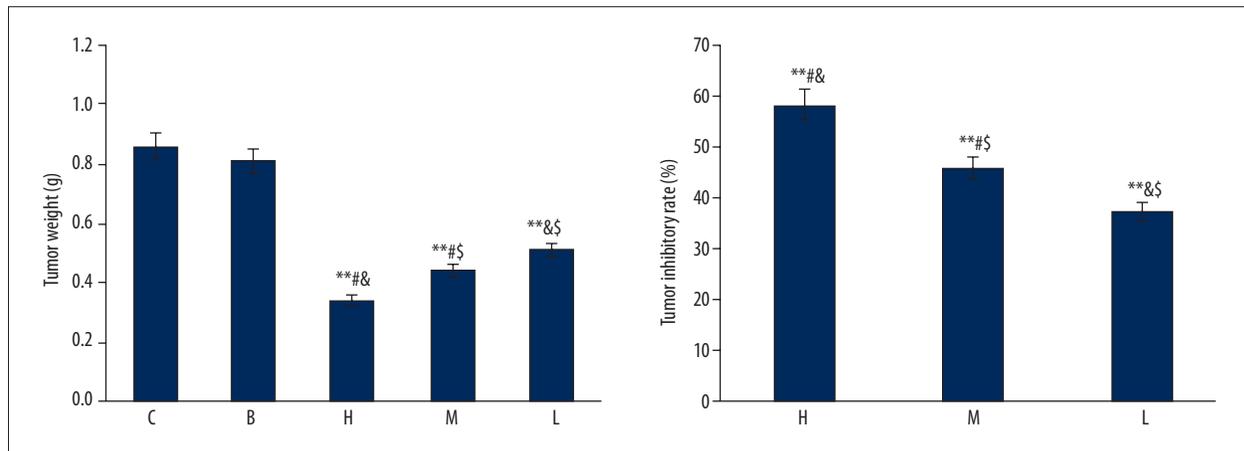
Figure 2. Marchantin impact on tumor weight and tumor inhibitory rate. Thirty days after the treatment of Marchantin, mice were sacrificed and tumor was isolated for measurement of tumor weight. * P<0.05, ** P<0.01, compared with group A and B; # P<0.05, ## P<0.01, compared with group E; & P<0.05, && P<0.01, compared with group D; $ P<0.05, $$ P<0.01, compared with group C. A: Control (Saline); B: DMSO; C: Higher dose of marchantin (200 µmol/kg); D: Medium dose of marchantin (100 µmol/kg); E: Lower dose of marchantin (50 µmol/kg).

marchantin groups (P<0.05). Their expression showed time- and dose-dependence (Figure 3).

Marchantin M's effect on PC-3 cell apoptosis

Apoptosis indexes in group marchantin H, M, and L increased to varying extents compared with group A and B (P<0.05) (Figure 4).

Effect of Marchantin M on DU145 cell proliferation

After treatment with Marchantin M, DU145 cell proliferation wa dose- and time-dependent. Marchantin M inhibited the growth of DU 145 cells, which was obvious 24 h after treatment of Marchantin M at a dose of 10 µmol/L. Furthermore, 20 µmol/L Marchantin M could significantly block the proliferation of DU145 cells (Figure 5).

Effect of Marchantin M on DU145 cell apoptosis

After treatment with Marchantin M, there were an increased number of apoptotic DU145 cells (Figure 6), suggesting Marchantin M is a promoter of cancer cells apoptosis.

Discussion

Currently, effective drugs are insufficient for clinical treatment of prostate cancer. As a double-benzyl drug, marchantin M is a proteasome inhibitor that has a variety of pharmacological activities. *In vivo* and *in vitro* studies showed that marchantin M presents a potential antitumor effect on numerous tumor cells, especially for prostate cancer PC-3 cell line. Marchantin

M functions to induce p21 protein expression, block the cell cycle, and inhibit cell proliferation. By regulating the expression of apoptosis-related genes, it imposes cell apoptosis [13,14]. Studies reported that mRNA of XBP-1, as well as the expression of CHOP and GRP78, in marchantin M-treated DU145 cells increased in a time- and dose- dependent manner. The ER stress-initiated IRE1 pathway and XBP-1 was changed to XBP-1s, which stimulated the transcription of GRP78, indicating that marchantin M is as a direct inducer of ER and in turn promotes the apoptosis of prostate cancer cells [19,20].

Apoptosis is mediated by the endoplasmic reticulum (ER) stress pathway, mitochondrial pathway, and death receptor pathway. ER stress appears in the early stage of apoptosis [15,16]. GRP78 is a major molecular chaperone of ER stress, located on the ER, and is known to be an anti-apoptotic factor. It is overexpressed in prostate cancer and plays an essential role in the blockade of tumor cell invasion and tumor proliferation [17,18], possibly due to conferring nutrient deprivation or hypoxia in the microenvironment of tumors ([23]). CHOP is an ER stress-specific transcription molecule with normally low expression. In the context of the expressions of ER associated chaperon, GRP78 and CHOP, we found that the levels were elevated in marchantin M-treated prostate cancer cells. Sustained ER stress was reported to contribute to activation of CHOP and induce tumor cell apoptosis [19,20]. Our data are consistent with previous studies and illuminates an inhibitory effect of marchantin M on tumor formation in nude mice and CHOP and GRP78 expression.

The results from recent work now indicate that ubiquitin – proteasome activity is up-regulated in the tumor cells [16,17]. Tumor cells are unraveled to be more sensitive to proteasome

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Figure 3. Marchantin effect on CHOP and GRP78 protein expression. Thirty days after the treatment of Marchantin, mice were sacrificed and tumor tissue was isolated for immunohistochemical analysis of the expression of CHOP and GRP78. * $P<0.05$, ** $P<0.01$, compared with group A and B; # $P<0.05$, ## $P<0.01$, compared with group E; & $P<0.05$, && $P<0.01$, compared with group D; $ $P<0.05$, $$ $P<0.01$, compared with group C. GRP78 protein mainly express in cytoplasm. Compared with group A and B, its expression increased in marchantin groups in a dose dependent manner. CHOP protein mainly express in nucleus. Compared with group A and B, its expression increased in marchantin groups with dose dependence. A: Control (Saline); B: DMSO; C: Higher dose of marchantin (200 µmol/kg); D: Medium dose of marchantin (100 µmol/kg); E: Lower dose of marchantin (50 µmol/kg).
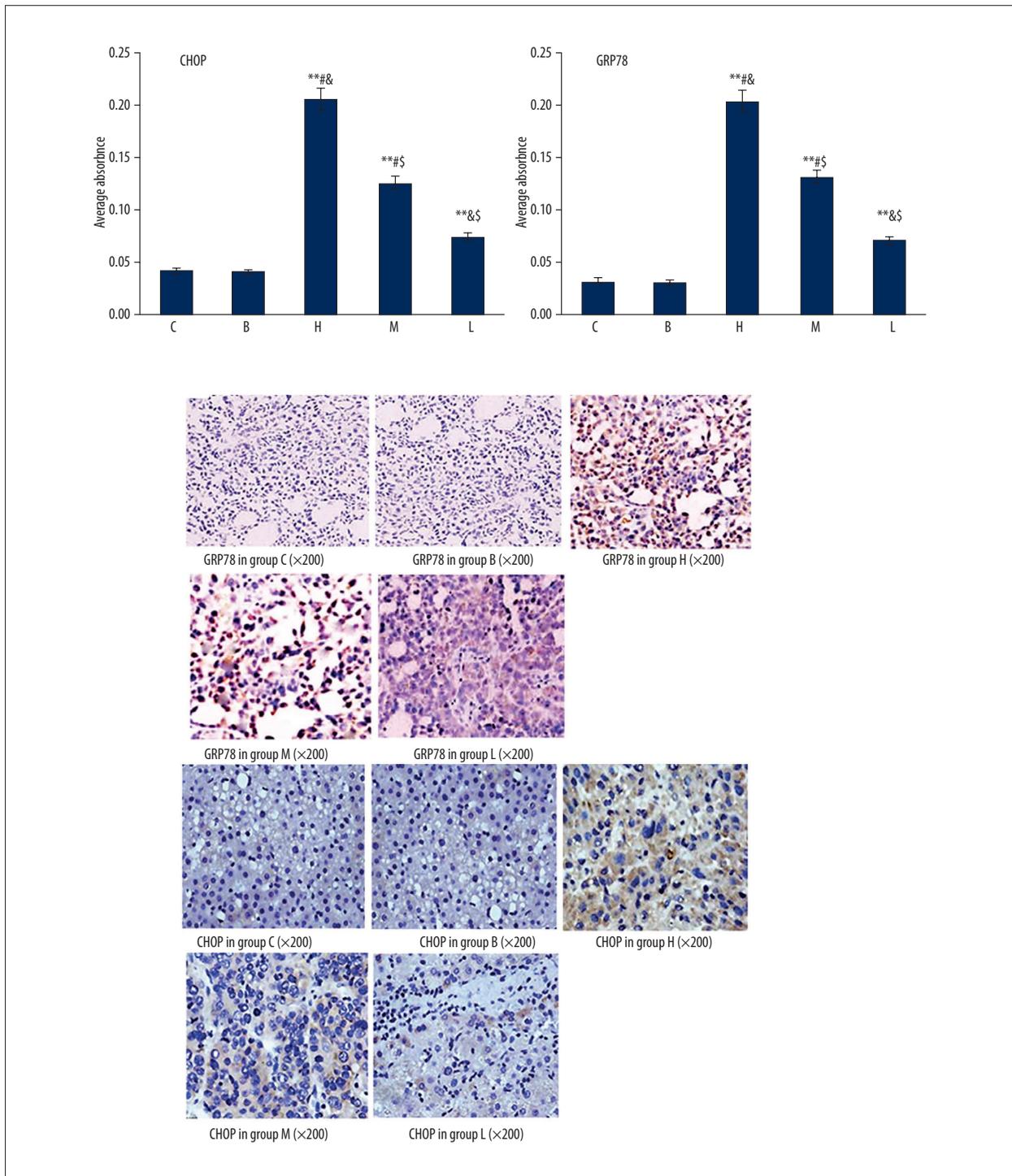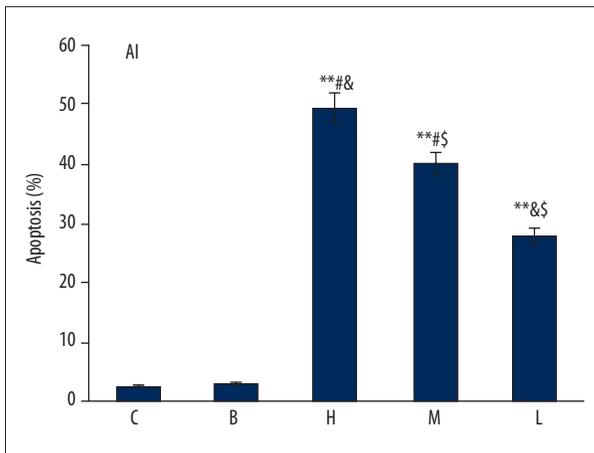
Figure 4. Marchantin effect on PC-3 cell apoptosis. Thirty days after the treatment of Marchantin, PC-3 cell apoptosis was measured by flow cytometry. * P<0.05, ** P<0.01, compared with group A and B; # P<0.05, ## P<0.01, compared with group E; & P<0.05, && P<0.01, compared with group D; $ P<0.05, $$ P<0.01, compared with group C. A: Control (Saline); B: DMSO; C: Higher dose of marchantin (200 µmol/kg); D: Medium dose of marchantin (100 µmol/kg); E: Lower dose of marchantin (50 µmol/kg).

Figure 5. Effect of marchantin on DU145 cell proliferation. Thirty days after the treatment of Marchantin, DU145 cell proliferation was measured by MTT.

inhibitors. Marchantin M, but not trypsin, reverses tumor drug resistance, and inhibits peptidyl glutamine peptide hydrolyase and protease activities. ER stress initiates the expression of GRP78, and then activates IRE1, PERK, and AFT6 signaling pathways. As an upstream regulatory protein of PI3K, GRP78 affected the expression of AKT/PKB via PI3K/PKB signaling pathway. Abnormal regulation in the upstream signaling
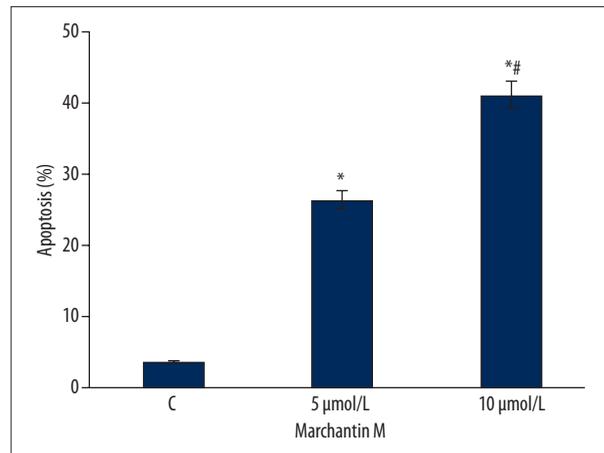
Figure 6. Effect of marchantin on DU145 cell apoptosis. Thirty days after the treatment of Marchantin, DU145 cell apoptosis was measured by flow cytometry. * P<0.05, compared with group A; # P<0.05, compared with 5µmol/L group. A: Control (Saline).

pathway leads to higher levels of KT/PKB activity in tumors. At the end of ER stress, CHOP, a small-molecule protein in the nucleus, is produced to induce cell apoptosis [17,18]. CHOP-induced apoptosis may be related to Akt phosphorylation reduction and TRB3 protein up-regulation. CHOP is likely to mediate the death receptor pathway through up-regulating DR5 expression. Ischemia and hypoxia caused by tumor cell excessive proliferation activate ER stress and induce cell apoptosis [21,22]. Once the degradation pathway of ER-associated proteins is impaired, misfolded proteins and unfolded proteins are prone to be accumulated in the endoplasmic reticulum lumen, leading to ER stress. Higher expression of CHOP and GRP78 was observed after marchantin M treatment, suggesting marchantin M could induce apoptosis of prostate cancer cells through ER stress. Through binding to cAMP response element protein, up-regulated CHOP is able to be involved in the regulation of Bcl-2, including up-regulation of Bax/Bak, down-regulation of Bcl-2 (causing imbalance of Bcl-2 and Bax), and subsequent increase of cytosolic calcium concentration (leading to apoptosis). However, whether marchantin M-induced cell apoptosis is involved in other pathways still needs to be determined.

Conclusions

We propose that marchantin M facilitates CHOP and GRP78 expression to suppress prostate cancer PC-3 tumor growth, and it also induces apoptosis of DU145 cell through ER stress.

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