Effect of *Momordica charantia* protein on proliferation, apoptosis and the AKT signal transduction pathway in the human endometrial carcinoma Ishikawa H cell line *in vitro*

HANG-ZHI GU[*], RONG-RONG LIN[*], HAN-CHU WANG, XUE-JIE ZHU, YAN HU and FEI-YUN ZHENG

Department of Gynecology, The First Affiliated Hospital of
Wenzhou Medical University, Wenzhou, Zhejiang 325000, P.R. China

Abstract. Endometrial carcinoma (EC) is one of the most common female malignancies, and there is an urgent requirement to explore new therapeutic strategies. In the present study, Ishikawa H cells were treated with *Momordica charantia* protein (MCP30). The cell morphology, growth inhibition rate, cell cycle distribution, and expression of phosphate and tensin homolog, P-AKT and AKT were measured. DNA fragmentation analysis and Annexin V-fluorescein isothiocyanate/propidium iodide double staining assay were used to analyze cell apoptosis. MCP30 decreased the viability of Ishikawa H cells in a dose- and time-dependent manner. The early apoptotic rates of Ishikawa H cells treated with MCP30 at 666.67 pM reached to 16.07±0.15%, following 72 h of treatment. DNA ladder was observed in cells treated with 333.33 and 666.67 pM MCP30 following 72 h of treatment. MCP30 blocks Ishikawa H cells from progressing between the S-phase and the G2/M-phase in a time- and concentration-dependent manner. Western blotting revealed that MCP30 treatment decreased the levels of P-AKT in a dose-dependent manner. It was revealed that MCP30 decreases cell proliferation, and induces apoptosis and S-phase cell cycle arrest through the AKT signaling pathway in Ishikawa H cells.

Correspondence to: Professor Fei-Yun Zheng, Department of Gynecology, The First Affiliated Hospital of Wenzhou Medical University, South Baixiang Street, Wenzhou, Zhejiang 325000, P.R. China
E-mail: zfy5710@163.com

[*]Contributed equally

Abbreviations: MCP30, *Momordica charantia* protein; PTEN, phosphatase and tensin homolog; FITC, fluorescein isothiocyanate; PI, propidium iodide; EC, endometrial carcinoma; MC, *Momordica charantia*; RIPs, ribosome inactivating proteins

Key words: endometrial carcinoma, AKT, cell cycle arrest, apoptosis, MCP30

Introduction

Endometrial carcinoma (EC) is one of the most common female pelvic malignancies; it develops in ~142,000 women worldwide and is responsible for ~42,000 mortalities each year (1). The 5-year survival rate is 95, 67 or 16%, if the cancer is diagnosed at a local, regional or distant stage, respectively (2). In China, the number of women with newly diagnosed endometrial cancer has also significantly increased annually (3).

Current treatments for EC comprise surgery, hormonal therapy, radiotherapy and chemotherapy. Young patients (age, ≤40 years) who suffer from endometrial atypical hyperplasia or well-differentiated EC classified as Federation of Gynecology and Obstetrics stage IA (intramucous) may choose hormonal treatment if they decide to preserve fertility (4). However, the risk of non-response, tumor progression and recurrence remain (5). The cornerstone of treatment for EC is surgery (6); early-stage patients can achieve a satisfying outcome, but the outcomes of high-risk patients are not positive, and the patients also require adjuvant therapy, such as radiotherapy and/or chemotherapy. Radiotherapy, including vaginal brachytherapy and pelvic external beam radiotherapy, is the main method of postoperative adjuvant treatment, and can decrease the local recurrence rate (7), but no overall survival rate improvement can be found in the high-risk group (8). The cytotoxic therapies available for the treatment of advanced-stage, progressive and recurrent disease have shown limited success (9-11). Therefore, exploration of new therapeutic strategies continues to be urgently required.

Phosphate and tensin homolog (PTEN) is a tumor suppressor gene, and loss of function mutations are common and appear to be important in the pathogenesis of EC (12). Silencing of PTEN is frequently associated with advanced EC and is likely to play a critical role in promoting AKT activation (13).

Epidemiological evidence strongly suggests that diets rich in fruit and vegetables are associated with reduced risks of cancers (14). *Momordica charantia* (MC), often termed bitter melon, grows in tropical Asia. The fruit has been widely used as food and herbal medicine in China for centuries. However, little is known about the mechanism of the effect of MC, which

limits the use of MC worldwide. Recently, scientists have elucidates that MC is capable of controlling plasma glucose, and has anti-viral, anti-fertility, immunomodulatory and antitumor effects (15-21). Our previous study successfully extracted a new protein with a molecular weight of 30 kDa from MC seeds and termed it MC protein (MCP30) (22). MCP30 is a ribosome inactivating protein (RIP), which is a type of protein that can inhibit protein synthesis in cell system or cell-free system (23,24).

In the present study, the effects of MCP30 on proliferation, cell cycle arrest, apoptosis and the AKT signal pathway in the human endometrial carcinoma Ishikawa H cell line were investigated *in vitro*.

Materials and methods

Reagents. RPMI-1640 medium, penicillin and streptomycin were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fat-free milk (5%) was obtained from Bright Dairy (Shanghai, China). Fetal bovine serum (10%; FBS) was purchased from ZhengJiang High Technology (Tianjin, China). Tween-20, rabbit primary anti-p-AKT (dilution, 1:1,000; SAB4301414), anti-AKT (dilution, 1:1,000; SAB4500797) and anti-PTEN (dilution, 1:1,000; SAB4300337) polyclonal antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:10,000; A0545) and GAPDH antibody (dilution, 1:1,000; G9545) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). The chemiluminescent substrate for HRP was obtained from Pierce (Thermo Fisher Scientific, Inc.). MCP30 was extracted from bitter melon seeds, prepared by Xiong *et al*, as previously described (22).

Cell culture. The Ishikawa H cell line was kindly provided by the Women's Hospital, School of Medicine, Zhejiang University (Hangzhou, China), and was cultured in RPMI-1640 medium, which was supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin, at 37°C in a fully humidified incubator containing 5% CO_2.

Cell viability assay. Cell viability in various concentrations of MCP30 (0, 8.33, 16.67, 33.33, 166.67, 333.33 and 666.67 pM; these concentrations were selected due to preliminary experiments) for 24, 48 and 72 h was assessed using the Cell Counting Kit-8 (CCK-8 kit; Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol. In brief, 10 μl of CCK-8 solution and 100 μl of cell culture supernatants (5x10³ cells, log phase) were added to each well of the 96-well plate (Corning, Inc., Corning, NY, USA). The reaction system was incubated at 37°C for 1 h. The absorbance was detected at a 450-nm wavelength using a microplate reader. Cell growth inhibition was measured using the following formula: Cell growth inhibition rate (%)=[1-(value of experimental group-value of blank group)/(value of control group-value of blank group)]x100.

DNA fragmentation assay. The DNA of cells treated with a series of concentrations of MCP30 for 72 h was extracted using the selected DNA Ladder Extraction kit from Aidlab Biotechnologies (Beijing, China), according to the manufacturer's protocol. The DNA fragmentation was assayed by electrophoresis on a 1.5% agarose gel and its pattern was examined on the images obtained under ultraviolet illumination. Images were captured by Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining assay. The cell apoptosis assay was performed using flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and was detected with the Annexin V-FITC/PI Apoptosis Detection kit (BD Biosciences). Subsequent to culture of cells in 666.67 pM MCP30 for 72 h at 37°C, apoptotic cells were treated with the agents of the Annexin V-FITC/PI Apoptosis Detection kit (composed of Annexin V binding buffer, Annexin V-FITC and PI staining solution), according to the manufacturer's protocol. In brief, cells were resuspended in 200 μl Annexin V binding buffer and subsequently incubated with 5 μl Annexin V-FITC and 10 μl PI for 15 min in room temperature. Subsequently, 200 μl Annexin V binding buffer was added. After 1 h, the FITC/PI double staining reaction system was detected using flow cytometry (excitation wavelength, 488 nm; emission wavelength, 530 nm).

Cell cycle analysis. Cells were seeded in 12-well plates at a density of 4x10⁴ cells per well in 2 ml of complete culture medium. Subsequent to culturing with MCP30 (166.67, 333.33 or 666.67 pM) for 48 or 72 h, cells were analyzed using Flow Cytometry Analysis of Cell Cycle kit (GenMed; Seisa, Plymouth, MN, USA) with a FACSCalibur Flow Cytometer (BD Biosciences) and distribution of the cell-cycle phases was determined using CellQuest Software (BD Biosciences).

Western blot analysis. Total proteins from the cells were prepared by cell lysis buffer (Applygen Technologies Inc., Beijing, China) and phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck Millipore) inactivated protease. The protein concentration was determined using the bicinchoninic acid method. Protein extracts were fractionated on 12% polyacrylamide SDS gel and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% fat-free milk in Tris-buffered saline with Tween-20 (0.1%), followed by incubation with rabbit anti-rat primary anti-p-AKT (dilution, 1:1,000), anti-AKT (dilution, 1:1,000) and anti-PTEN (dilution, 1:1,000) polyclonal antibodies at 4°C for 20 h. Subsequent to washing the membrane with TBST, the membrane was treated with HRP-conjugated goat anti-rat secondary antibody IgG-HRP (dilution, 1:10,000) for 1 h at room temperature via agitation. The enhanced HRP-DAB substrate solution was added to the membrane and incubated for 5 min. Bands were visualized by chemiluminescence and exposed to X-ray. The GAPDH antibody (dilution, 1:1,000) was used as an internal control. The relative optical density (ratio to GAPDH) of each blot band was quantified by Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Each experiment was repeated in triplicate. All data were analyzed using SPSS Statistics 19.0 (IBM Co., Armonk, NY, USA), and data were expressed as the mean ± standard deviation. For comparisons among groups,
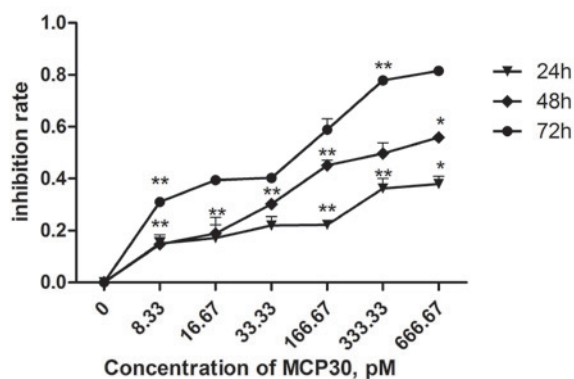
Figure 1. Inhibition rate of cells treated with various concentrations MCP30 for three periods. MCP30 significantly decreased the cell viability in a dose- and time-dependent manner. All data are expressed as the percentage change in comparison with the control group, which was the cells treated with 0 pM MCP30 and assigned a 0% inhibition rate. The data are expressed as the mean ± standard deviation of 3 independent experiments performed in triplicate. *$P<0.05$ and **$P<0.01$ compared with next lowest concentration group. MCP30, *Momordica charantia* protein.

independent-samples t-test and two-way analysis of variance were performed, as appropriate. If the test of homogeneity of variances was satisfied, then Tukey pairwise comparison was used for post hoc analysis. If not, Dunnet's T3 test was selected. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MCP30 decreased the viability of Ishikawa H cells in a dose- and time-dependent manner. The effect of different concentrations of MCP30 on cell viability was shown in Fig. 1. MCP30 significantly decreased the cell viability in a dose- and time-dependent manner (two-way analysis of variance; time, $F=89.529$, $P<0.001$; concentration, $F=56.119$, $P<0.001$). Following a 72 h incubation with 33.33 pM MCP30, the viability of the cells was reduced by 40.26% (33.33 pM group vs. control group). Additionally, 166.67 pM MCP30 reduced cell viability by 58.84% (166.67 pM group vs. control group). The half-maximal inhibitory concentration of MCP30 for 72 h was ~62.00 pM (data not shown). These results indicated that treatment with MCP30 decreased the viability of Ishikawa H cells.

MCP30 induced early apoptosis in Ishikawa H cells. The results of Annexin FITC/PI staining revealed that cell viability decrease was associated with early apoptosis. The early apoptotic rates of Ishikawa H cells treated with MCP30 at 666.67 pM reached to 16.07±0.15% following 72 h of treatment. By contrast, the control cells showed early apoptosis rates of only 5.08±0.19% ($t=76.589$; $P<0.001$) (Fig. 2). Furthermore, DNA ladder was observed in cells treated with 333.33 pM and 666.67 pM MCP30 following 72 h of treatment (Fig. 2D; lanes 3 and 4), while no DNA ladder was found in the blank control and 8.33 pM groups (lanes 1 and 2).

MCP30 affected the cell cycle distribution of Ishikawa H cells in a time- and concentration-dependent manner. Cell cycle
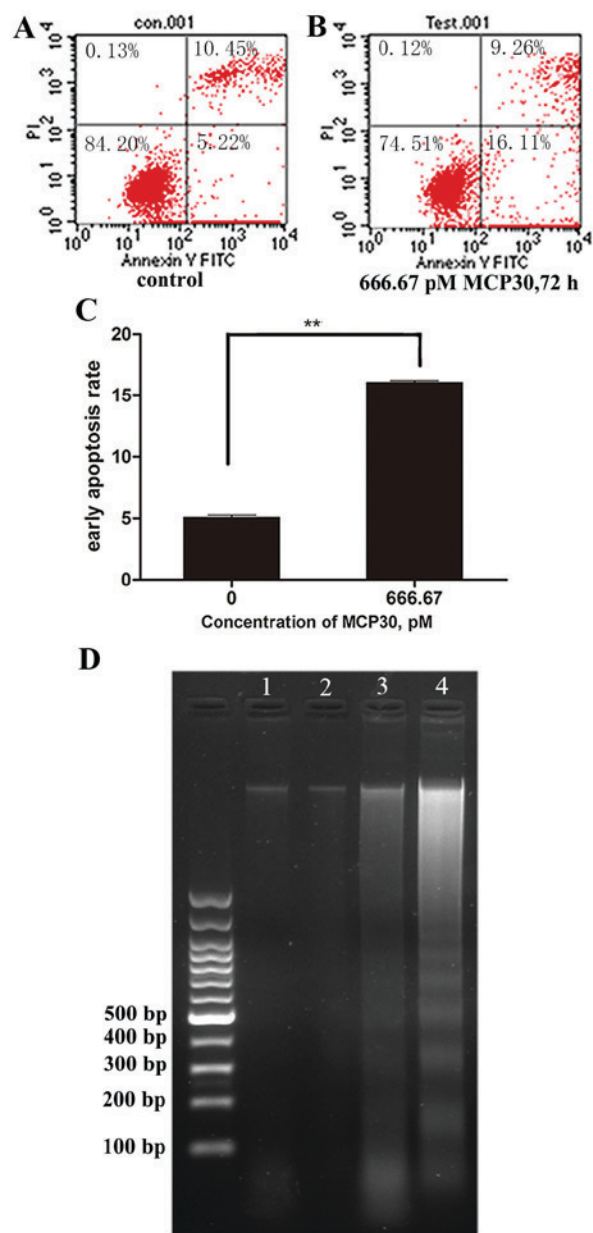
Figure 2. (A) Flow cytometry analysis of apoptotic Ishikawa H cells in the control group and (B) cells treated with 666.67 pM MCP30 for 72 h. The upper left region shows necrotic cells, the upper right region shows necrotic cells and late-apoptotic cells, the lower left region shows normal live cells, and the lower right region shows early-apoptotic cells. (C) Early-apoptosis rates in the control and 666.67 pM MCP30 groups. Data is expressed as the mean ± standard deviation of 3 independent experiments performed in triplicate. **$P<0.01$. (D) DNA fragmentation assay. Lanes 1-4 show DNA fragmentation from cells treated with 0 (lane 1), 8.33 (lane 2), 333.33 (lane 3) and 666.67 pM (lane 4) of MCP30 for 72 h. DNA ladder was observed in lane 3 and lane 4. MCP30, *Momordica charantia* protein.

analysis was performed by flow cytometry (Fig. 3). Treatment with different concentrations (166.67, 333.33 and 666.67 pM) of MCP30 for 48 h resulted in the distribution of the cell phase changing so that the higher the concentration added, the lower the G0/G1-phase rate and the higher the S-phase rate (G0/G1-phase rate, $F=106.866$, $P<0.001$; S-phase rate, $F=99.686$, $P<0.001$). The G2/M-phase rate remained consistent. A similar result was obtained when the time of treatment was prolonged to 72 h (G0/G1-phase rate, $F=169.836$, $P<0.001$;
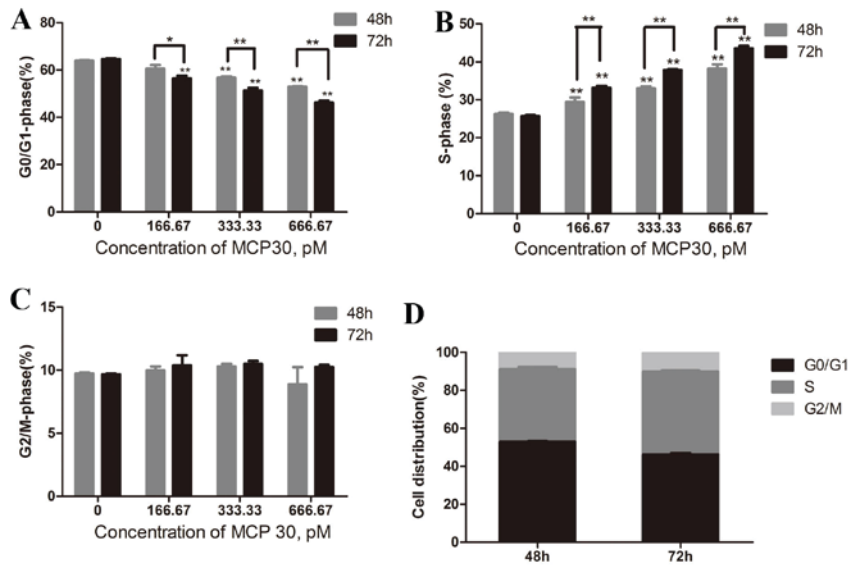
Figure 3. (A) G0/G1-phase rate of cells treated with 166.67, 333.33 and 666.67 pM for 48 and 72 h. **P<0.01, compared with the control group between different concentrations. *P<0.05, **P<0.01, compared between different timepoints within the same concentration. (B) S-phase rate of cells treated with 166.67, 333.33 and 666.67 pM for 48 and 72 h. **P<0.01 compared with the control group between difference concentrations. *P<0.05, **P<0.01 compared between different timepoints within the same concentration. (C) G2/M-phase rate of cells treated with 166.67, 333.33 and 666.67 pM for 48 and 72 h. Data is expressed as the mean ± standard deviation of 3 independent experiments performed in triplicate. (D) Cell cycle distribution of cells treated with 666.67 pM MCP30 between 48 and 72 h. These findings indicate that MCP30 induced S-phase arrest. MCP30, *Momordica charantia* protein.
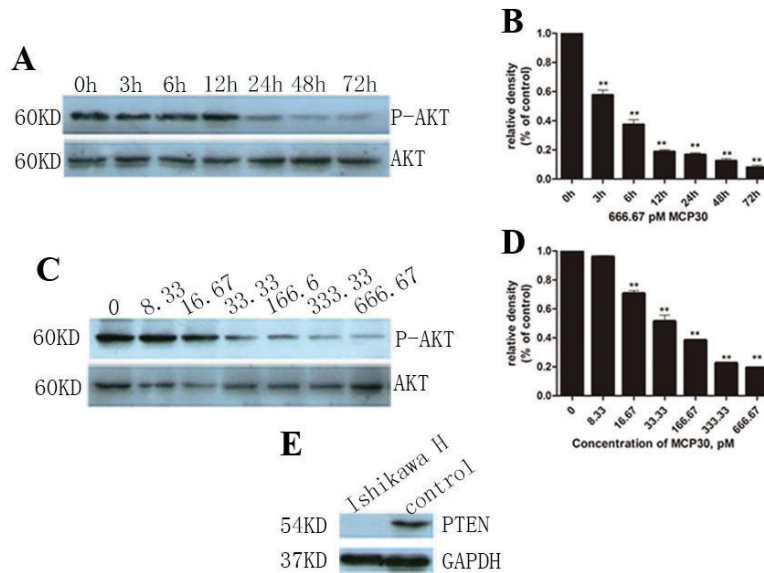
Figure 4. (A) MCP30 decreased total P-AKT expression in a time-dependent manner. (B) The relative density (P-AKT/AKT) compared to the control group at different treatment times. **P<0.01 compared with the control group. (C) MCP30 decreased total P-AKT expression in a dose-dependent manner. (D) The relative density (P-AKT/AKT) compared to the control group at various concentrations of MCP30. (E) Ishikawa H cells lost PTEN expression. P-AKT, phosphorylated AKT; PTEN, phosphate and tensin homolog; MCP30, *Momordica charantia* protein.

S-phase rate, F=742.190, P<0.001). This indicated that MCP30 blocks Ishikawa H cells from progressing between the S-phase and the G2/M-phase in a time- and concentration-dependent manner.

MCP30 induced Ishikawa H cell apoptosis and S-phase arrest through the AKT signaling pathway. Finally, in order to evaluate the effect of culture time on the P-AKT expression level, 666.67 pM MCP30 was added to the cell culture system for various time points (0, 3, 6, 12, 24, 48 and 72 h). With increased culture time, the P-AKT level decreased (F=286.582, P<0.001), but this change stopped when the time reached 12 h, and no subsequent decrease was observed (Fig. 4A and B). Thus, it was hypothesized that 12 h is the best culture time for cells with MCP30. The levels of P-AKT were detected by western blot analysis following incubation with MCP30 for 72 h (Fig. 4C and D). MCP30 treatment decreased the levels of P-AKT in a dose-dependent manner (F=975.799; P<0.001).

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