Cucurbitacin I induces apoptosis in ovarian cancer cells through oxidative stress and the p190B-Rac1 signaling axis

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Abstract. Ovarian cancer is a serious threat to women's life and health, with a high mortality rate. Therefore, in addition to improving surgery for ovarian cancer, it is particularly important to develop novel drug treatments. In the present study, the anticancer effects of cucurbitacin I, a natural product, were investigated. Cucurbitacin I impaired the viability of SKVO3 cells in a concentration- and time-dependent manner. Apoptosis was involved in the process of cucurbitacin I-induced cell death, with an increase observed in cleaved-caspase 3 and BAX, and a decrease in Bcl-2. Cucurbitacin I caused a notable increase in intracellular reactive oxygen species, and regulated Kelch-like ECH-associated protein 1 and nuclear factor erythroid-derived 2-like 2 to decrease the expression of antioxidant-related genes. In addition, Cucurbitacin I induced cell shrinkage by regulating the p190BRhoGAP (p190B)-Rac1 signaling axis related to the cytoskeleton. In brief, these results suggested that cucurbitacin I induced cell death through oxidative stress and the p190B-Rac1 signaling axis in SKVO3 cells. The results may provide novel evidence for the treatment of ovarian cancer.

Introduction

Ovarian cancer is one of the most lethal types of cancers in women worldwide. It is the fifth leading cause of cancer-related deaths among women in the United States and causes >140,000 deaths annually in women worldwide (1). The pathogenesis of ovarian cancer is very complicated, making it difficult to diagnose and treat (2). Despite continuous improvements in treatment technology, the 5-year survival rate of ovarian cancer remains unsatisfactory. The standard curative treatment for ovarian cancer is anticancer treatment following surgery (3,4). Therefore, in addition to improving diagnostic technology, it is also important to develop new drugs for the treatment of ovarian cancer.

Cucurbitacin I, a natural tetracyclic triterpenoid extracted from Cucurbitaceae and Cruciferae, has been used in traditional medicine for its antipyretic, analgesic, anti-inflammatory and antimicrobial effects (5). According to recent findings, cucurbitacin I, which is 1 of 12 variants of cucurbitacins, has demonstrated a potent anticancer effect on non-small cell lung cancer cells (6). Cucurbitacin I has been reported to cause cancer cell death by inhibiting multiple signaling pathways, such as the Janus kinase (JAK)/STAT3, PI3K/AKT/p70S6K and serine/threonine-protein kinase PAK1 (PAK1)/PAK4 pathways (7-9). However, the underlying mechanism for the anticancer effect of cucurbitacin I is not yet completely understood, and further studies are required to clarify it.

A low level of reactive oxygen species (ROS) is normal in cells, as it helps maintain cell function, but excessive levels of ROS can damage cells and induce cell death. There are regulatory mechanisms that neutralize excessive ROS and provide protection against oxidative damage (10). The transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2) is an important regulator of the oxidative stress response, and plays a key role in the expression of antioxidant proteins. Nrf2 is regulated by Kelch-like ECH-associated protein 1 (Keap1). Under quiescent conditions, Nrf2 is retained in the cytoplasm after being combined with Keap1, and is degraded in the proteasome with the E3 ubiquitin ligase Cullin 3. Cellular stimuli, such as oxidative stress, induce conformational changes in Keap1, which are followed by the release of Nrf2 from Keap1. Subsequently, Nrf2 translocates to the nucleus and transactivates the expression of genes containing an antioxidant response element in their promoter regions. Nrf2 thereby upregulates phase II detoxifying enzymes and antioxidant proteins, and plays a vital role in maintaining cellular homeostasis (11,12).

p190BRhoGAP (p190B), which is a GTPase-activating protein, has been implicated in various pathological conditions, including cancer, cardiovascular diseases and developmental

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disorders (13,14). As reported by numerous studies, p190B plays an important role in regulating small GTPase activity, which influence cytoskeleton remodeling, cell adhesion, cell polarity maintenance, proliferation and division, as well as cell migration (14,15). A previous study demonstrated that p190B can regulate Ras homolog family member A (RhoA) and Rac1 to regulate cell migration (16). Meanwhile, p190B is critical in the occurrence and development of tumors, such as breast cancer, glioma and colorectal cancer (17,18).

In the present study, cucurbitacin I, a botanical extract, induced death in SKVO3 cells in a time- and concentration-dependent manner. Cucurbitacin I promoted cell apoptosis and induced an increase in the intracellular ROS level, with decreased antioxidant-related gene expression by the Keap1-Nrf2 signaling axis. The expression of p190B and Rac1 was also found to be altered, which was associated with changes in the cell morphology. The present study may have provided novel evidence for the treatment of ovarian cancer.

Materials and methods

Reagents. Cucurbitacin I and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA. Cell Counting Kit-8 (CCK-8) was purchased from Bimake. ROS assay kit was obtained from Beyotime Institute of Biotechnology. Anti-BAX (cat. no. 2772) and anti-cleaved-caspase-3 (cat. no. 9664) were from Cell Signaling Technology, Inc. Anti-Bcl-2 (cat. no. ET1603-11) was from Hangzhou HuaAn Biotechnology Co., Ltd. Anti-Keap1 (cat. no. 10503-2-AP), anti-Nrf2 (cat. no. 16396-1-AP), anti-Heme oxygenase-1 (HO-1; cat. no. 10701-1-AP), anti-p190B (cat. no. 55165-1-AP) and anti-Rac1 (cat. no. 24072-1-AP) were purchased from ProteinTech Group, Inc. Anti-GAPDH (cat. no. GB11002) was from Wuhan Servicebio Technology Co., Ltd., and all secondary antibodies (cat. nos. ZB-2301 and ZB-2305) were from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Other chemicals and reagents were analytically pure.

Cell culture. SKOV3 human ovarian carcinoma cells (ATCC HTB-77) were obtained from American Type Culture Collection. SKVO3 cells (70-80%) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml streptomycin and 100 U/ml penicillin. SKVO3 cells were incubated in a humidified incubator containing 95% air and 5% CO_2 with the temperature stabilized at 37˚C. The cell culture media was refreshed every 3 days.

Cell viability assays. Cell viability assays were performed according to a previous study (19). SKVO3 cells were seeded into 96-well plates at a density of 10,000 cell per well. The experimental groups were treated with 0.075, 0.15, 0.3 and 0.6 μM cucurbitacin I (dissolved in DMSO) for 12, 24 and 48 h, and the control group was treated with DMSO. Finally, a CCK-8 assay was performed, according to the manufacturer's protocol, to detect cell viability using a microplate reader (BioTek). As 0.3 μM cucurbitacin I treatment for 24 h could effectively reduce cell viability by ~50%, this concentration and time was chosen for subsequent experiments.

ROS assay. The ROS assay was performed according to a previous study (20). ROS were measured using a fluorescent probe DCFH-DA (Beyotime Institute of Biotechnology). SKVO3 cells ($2x10^5$ per well) were seeded into 6-well plates, and then when cells reached ~80% confluence they were treated with 0.3 μM cucurbitacin I for 24 h at 37˚C (control cells were treated with DMSO). All cells were further incubated with 10 μM DCFH-DA for 20 min, and then washed with serum-free DMEM 3 times. The level of ROS was detected using a fluorescence microscope.

Western blot analysis. Western blotting was performed as previously described (21,22). SKVO3 cells were lysed on ice for 20 min, centrifuged at 10,000 x g for 10 min at 4˚C and the cell supernatant was collected. Protein concentration was measured by the BCA method, and 35 μg protein per lane was loaded onto 10 or 12% gels and separated via SDS-PAGE. The separated proteins were subsequently transferred onto polyvinylidene fluoride membranes. The membranes were incubated with 5% skimmed milk diluted by TBS with 0.1% Tween-20 (TBST) for 1 h at room temperature, and then probed with primary antibodies (1:1,000) at 4˚C overnight. The membranes were washed with TBST 3 times and incubated with the secondary antibodies (1:3,000) for 1 h at room temperature to amplify the chemiluminescence. Bands were semi-quantified and analyzed using ImageJ Software (V1.8.0.112; National Institutes of Health).

Statistical analysis. All data are expressed as the mean ± SEM. Comparisons were performed using an unpaired Student's t-test and groups of ≥3 were analyzed by one-way ANOVA, followed by Tukey's post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cucurbitacin I causes SKVO3 cell death in a concentration- and time-dependent manner. To evaluate the cytotoxicity of cucurbitacin I on SKVO3 cells, the cell viability of SKVO3 was detected by a CCK-8 assay. As shown in Fig. 1A, cucurbitacin I treatment induced cell death in a concentration- and time-dependent manner. Briefly, the cucurbitacin I concentration used was 0.075-0.6 μM. The higher the concentration, the higher the death rate and the lower the survival rate. Cells were treated with cucurbitacin I for 12, 24 and 48 h. The longer the treatment time, the higher the cell death rate and the lower the survival rate. Cell death is always accompanied by changes in cell morphology. As shown in Fig. 1B, following cucurbitacin I treatment for 24 h, there was a decreased number of SKVO3 cells and cell surface shrinkage; the higher the concentration, the more severe this was.

Cucurbitacin I induces apoptosis in SKVO3 cells. To determine whether cucurbitacin I induced cell apoptosis, a concentration of 0.3 μM was selected as it was shown to kill ~50% of SKVO3 cells. It was observed that treatment with 0.3 μM cucurbitacin I for 24 h induced a significant decrease in Bcl-2 (37.3%, $P<0.05$) and a significant increase in BAX (80.2%, $P<0.05$). The expression level of cleaved-caspase-3, an index of apoptosis, was also examined, and it was found to

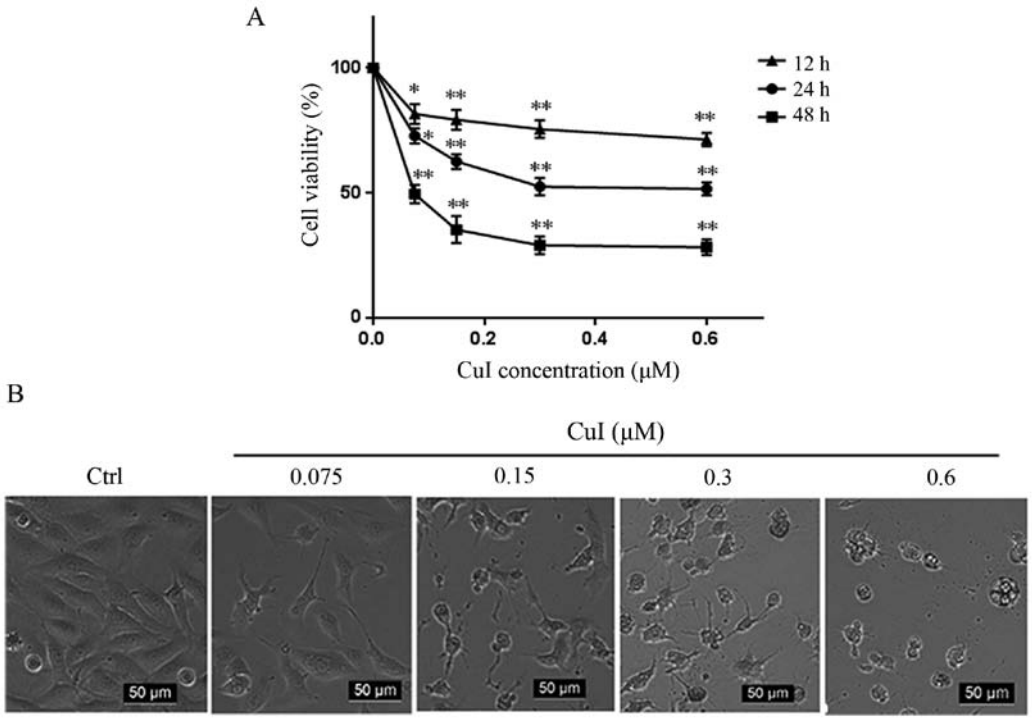

Figure 1. CuI causes SKVO3 cell death in a concentration- and time-dependent manner. (A) SKVO3 cells were treated with 0.075, 0.15, 0.3 and 0.6 μM CuI for 12, 24 and 48 h, whereas the control group was treated with DMSO alone. Cell viability was then measured by Cell Counting Kit-8 assay (n=3/group). (B) Cells were visualized using an inverted microscope. Scale bars, 50 μm; magnification, x200. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test and are presented as the mean ± SEM. *P<0.05, **P<0.01 vs. DMSO-treated group (control). Ctrl, DMSO-treated cells; DMSO, dimethyl sulfoxide; CuI, cucurbitacin I.

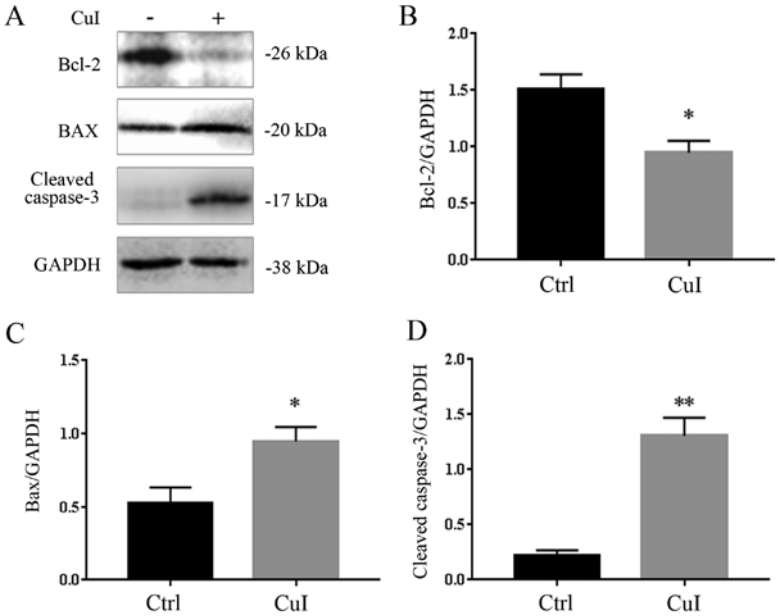

Figure 2. CuI induces apoptosis in SKVO3 cells. (A) Representative protein expression of apoptosis markers, Bcl-2, BAX and cleaved-caspase-3, in SKVO3 cells treated with 0.3 μM CuI for 24 h. The control group was treated with DMSO alone. (B-D) Corresponding histograms of the protein expression of apoptosis markers in CuI-treated SKVO3 cells (n=3/group). All data were analyzed with an unpaired Student's t-test and are presented as the mean ± SEM. *P<0.05, **P<0.01 vs. Ctrl. Ctrl, control; CuI, cucurbitacin I.

have increased by 380% (P<0.01) compared with the control group treated with DMSO alone (Fig. 2).

Cucurbitacin I increases cellular oxidative stress via the Keap1-Nrf2 pathway. To evaluate the effect of cucurbitacin I on oxidative stress, the level of ROS in SKVO3 cells was measured. As shown in Fig. 3A, when cells were treated with 0.3 μM cucurbitacin I, the level of intracellular ROS markedly increased, whereas that of the control group was very low. Cells have protective mechanisms against oxidative stress, including a

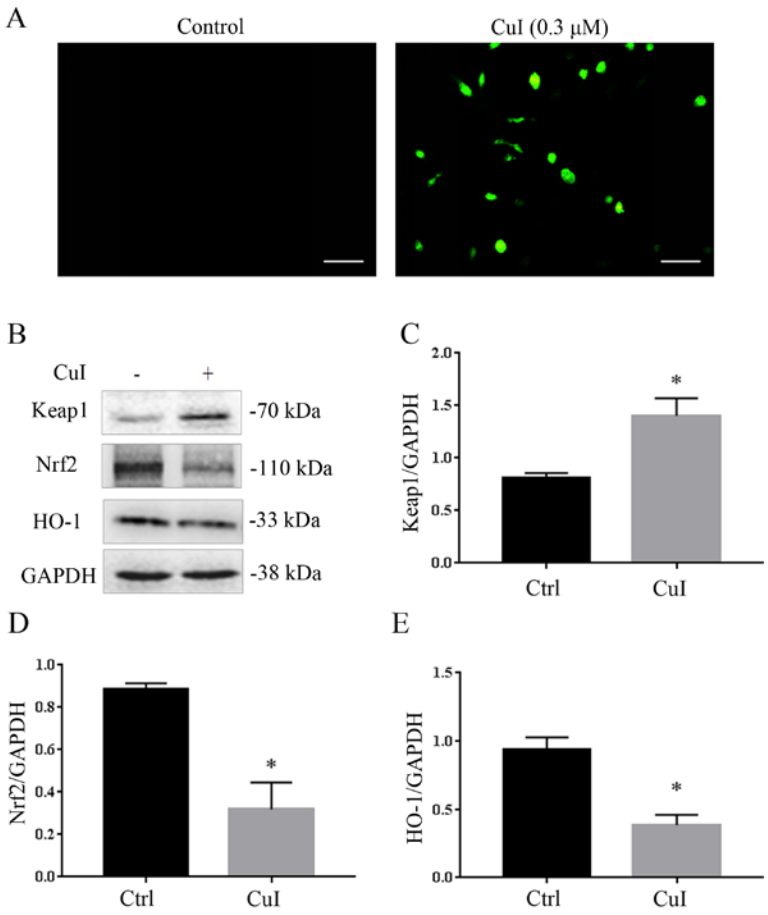

Figure 3. CuI increases cellular oxidative stress via the Keap1-Nrf2 pathway. (A) Representative fluorescence images of ROS in SKVO3 cells were captured using a fluorescent microscope with DCFH-DA stain. Scale bar, 50 μm. (B) Representative protein expression of Keap1, Nrf2 and HO-1 in SKVO3 cells. (C-E) Corresponding histograms of the protein expression of Keap1, Nrf2 and HO-1 (n=3/group). All data were analyzed with an unpaired Student's t-test and are presented as the mean ± SEM. *P<0.05 vs. Ctrl. Ctrl, control; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid-derived 2-like 2; ROS, reactive oxygen species; HO-1, Heme oxygenase-1; CuI, cucurbitacin I.

number of antioxidant proteins and signaling pathways, of which the Keap1-Nrf2 pathway is one of them. Therefore in the present study, the expression of Keap1, Nrf2 and HO-1, which is downstream of Nrf2, was measured. As shown in Fig. 3B-E, the protein expression of Nrf2 decreased by 63.9% (P<0.05), HO-1 decreased by 58.7% (P<0.05) and Keap1 increased by 72.5% (P<0.05) compared with the control group. These results indicated that that cucurbitacin I increased the level of intracellular oxidative stress and therefore regulated the Keap1-Nrf2 pathway.

Cucurbitacin I alters cell morphology by regulating the p190B-Rac1 pathway. It has been reported that the expression of p190B is increased in ovarian cancer, which may affect the cell cytoskeleton, morphology and migration (23). As shown in Fig. 1B, the number of cells decreased and the cell surface had shrunk with the increase in cucurbitacin I concentration. Therefore, the expression levels of cytoskeleton-related protein p190B and its downstream protein Rac1 were detected. The results showed that, as compared with the control group, the expression of p190B decreased by 42.8% (P<0.01) and Rac1 expression increased by 66.3% (P<0.05) in cells treated with 0.3 μM cucurbitacin I for 24 h (Fig. 4). Thus, suggesting that cucurbitacin I regulated the p190B and Rac1 pathway to regulate cell morphology.

Discussion

Ovarian cancer is one of the most common malignant tumors in female reproductive organs. The incidence of ovarian cancer is second only to that of cervical cancer and uterine body cancer, and is a serious threat to women's lives (2-4). The etiology of ovarian cancer is still unclear, but may be related to genetic and endocrine factors. At present, in addition to surgery, the development of antitumor drugs is also very important. Cucurbitacin I has a strong antitumor activity (6,7). Cucurbitacin I has been reported to induce death in various types of tumor cells; for example it can induce apoptosis in non-small cell lung cancer cells and osteosarcoma cells by inhibiting the PI3K/AKT/p70S6K and STAT3 signaling, respectively. Zhang *et al* (24) observed that cucurbitacin I modulated the balance between autophagic and apoptotic modes of cells to cause cancer cell death. In the present study, the SKVO3 cell line was selected to investigate ovarian cancer via various experiments. The present findings confirmed that cucurbitacin I could kill SKVO2 cells in a concentration- and time-dependent manner, indicating that its antitumor effect was enhanced as the concentration increased. As shown in Fig. 1, a noteworthy phenomenon was found, after cucurbitacin I administration, some of the shrunk cells that had attached to the culture dish still had cell viability and did not

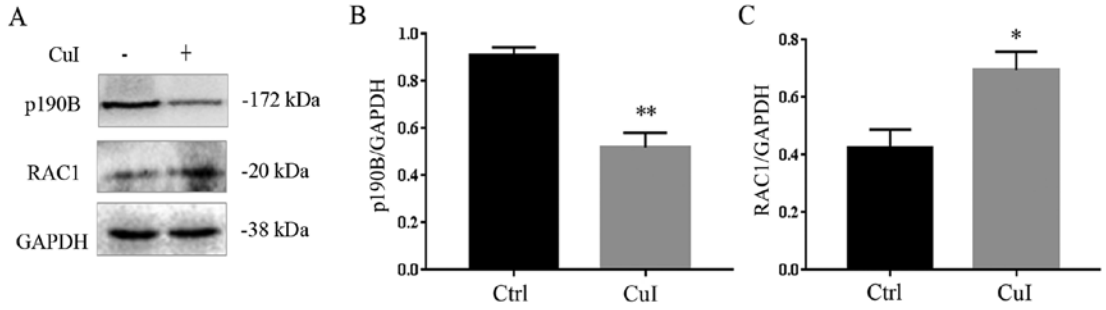

Figure 4. CuI regulates the p190B-Rac1 pathway. (A) Representative protein expression of p190B and Rac1 in SKVO3 cells. (B and C) Corresponding histograms of the protein expression of p190B and Rac1 (n=3/group). All data were analyzed using an unpaired Student's t-test and are presented as the mean ± SEM. *P<0.05, **P<0.01 vs. Ctrl. Ctrl, control; CuI, cucurbitacin I; p190B, p190BRhoGAP.

die completely. The morphology of SKVO3 cells changed in a concentration-dependent manner.

Apoptosis is a form of programmed cell death that brings about the orderly and efficient removal of damaged cells, such as those resulting from DNA damage or during development (25,26). The mechanism of apoptosis is very complex and numerous signaling pathways are involved in this process. However, caspases, a family of cysteinyl-aspartate-specific proteases, are central to the mechanism of apoptosis (27,28). The activity of caspases is responsible for the hallmarks of apoptosis, so the expression level of cleaved-caspase-3 was detected. As shown in Fig. 2A, cucurbitacin I caused a significant increase in the expression level of cleaved-caspase-3 and pro-apoptotic protein BAX in SKVO3 cells, whereas the expression of anti-apoptotic protein Bcl-2 decreased and the ratio of Bcl-2/BAX decreased. In general, these results indicated that cucurbitacin I induced SKVO3 cell apoptosis.

Cancer development is characterized by the uncontrolled growth and proliferation of transformed cells. A tumor environment is characterized by low levels of oxygen and glucose, which can induce ROS. At moderate concentrations, ROS activates the cancer cell survival signaling cascade. At high concentrations, ROS can cause cancer cell apoptosis (29). In the present study, the mechanism through which cucurbitacin I induced cell death in ovarian cancer was further explored. It was found that cucurbitacin I increased the level of ROS in cells and decreased the expression of antioxidant genes. Briefly, cucurbitacin I decreased the expression of Nrf2, but increased that of the Keap1 protein, which can combine with Nrf2 to further prevent Nrf2 from translocating into the nucleus. Next, the expression of HO-1, an antioxidant gene downstream of Nrf2, was detected, and it was found to have decreased by 58.7%. Generally speaking, cucurbitacin I increased the level of ROS and decreased the expression of antioxidant genes via the Keap1-Nrf2 signaling axis, resulting in cell death.

When ovarian cancer occurs, the expression of cytoskeleton-related proteins increases. Earp *et al* (23) suggested that the expression of the p190B protein increased significantly during the development of ovarian cancer. Therefore, reducing the expression of p190B could be a potential target for treatment. In the present study, following the treatment of SKVO3 cells by cucurbitacin I, the expression of p190B, a regulatory protein associated with the cytoskeleton, decreased significantly, and that of the negative regulatory protein Rac1 increased. This was associated with the morphological changes of cells in Fig. 1B.

The anti-tumor signaling pathway of cucurbitacin I is very complex. Although, it has been reported by Li *et al* (30) that cucurbitacin I causes ovarian cancer cell death via endoplasmic reticulum stress, the present study demonstrated for the first time, to the best of the our knowledge, that cucurbitacin I can also activate oxidative stress by regulating the Keap1-Nrf2-HO1 signaling pathway, and regulate the p190b-Rac1 signal axis to alter cell cytoskeleton shape, so as to achieve the anti-tumor effect. A limitation of the present study is that only one cell line was used and so there was a lack of dose responses in this experiment, in future experiments other cell lines, as well as primary cells, will be used to confirm our findings. Additionally, further experiments to determine concentration gradient are needed, and a variety of research methods, such as flow cytometry and TUNEL fluorescence detection, will be performed to further explore the findings of the present study.

In conclusion, cucurbitacin I, a newly identified potential chemotherapeutic drug, induced ovarian cancer cell death in a concentration- and time-dependent manner. Cucurbitacin I induced cell apoptosis by increasing BAX and cleaved-caspase-3, and decreasing Bcl-2. In addition, cucurbitacin I increased the intracellular ROS level and decreased antioxidant gene expression via the Keap1-Nrf2 signaling pathway. In addition, the p190B-Rac1 signaling axis was inhibited by cucurbitacin I. These insights may be used to develop novel therapeutic approaches to treat ovarian cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RL and FL performed the experiments and drafted the manuscript. JX, XL, HX and ST participated in the experiments. MY and BH contributed to the final data analysis and edited the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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