Isoflavones Appeared Different Effects In The Presence of Different Concentrations of Estrogen In Ovarian And Endometrial Cancer Cells

Zhichao Shi
The People's Hospital of Lishui
Yaozhi Qi
Lianyungang Maternal and Child Health Hospital
Lili Wei
Lianyungang Maternal and Child Health Hospital
Tingting Cheng
The People's Hospital of Lishui
Fengbiao Guo (guo-fb@gdmu.edu.cn)
Affiliated Hospital of Guangdong Medical University

Research Article

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Abstract

Background: Epithelial ovarian cancer and breast cancer are common gynecological malignancies with a high mortality rate. Isoflavone is a phytoestrogen that is ubiquitous in food and Chinese herbs. Although many epidemiological studies have shown that ingestion of isoflavones can reduce the risk of ovarian and breast cancer, its cellular functions and underlying mechanisms remain unclear.

Methods: This study used isoflavones to treat SKOV-3 (ovarian cancer) and MCF-7 (breast cancer) cell lines with high estrogen levels. Cell counting and MTS assays were used to detect cell proliferation and viability. Quantitative PCR and western blotting were performed to investigate the expression levels of cell cycle factors.

Results: The results showed that isoflavones inhibited cell proliferation and viability in SKOV-3 and MCF-7 cells under high estrogen levels. After isoflavone treatment, PCNA, CDK4, and cyclin D1 expression levels were significantly decreased, but p21 and p27 expression levels were significantly increased.

Conclusion: Isoflavones could inhibit cell proliferation within high levels of estrogen in ovarian and breast cancer cells. These findings will provide a new theory for gynecological malignancy therapy.

Introduction

According to the World Health Organization/International Cancer Center team database, there were 8.6 million new cancer cases in women globally in 2018, with an incidence rate of 228 per 100,000 persons [1]. Female reproductive system malignancies are a severe threat to women's lives. The mortality rates of breast and ovarian cancers are the first and eighth among female malignant tumors, respectively. The breast is the target organ of estrogen, progesterone, prolactin, and other endocrine hormones, with estrogen and estradiol having a causal relationship with the occurrence of breast cancer. Moreover, some researchers believe that the pathogenesis of ovarian cancer may be related to the wound healing process [2, 3] and gonadotropin stimulation induced by continuous ovulation [4]. Previous studies have shown that estrogen receptor activity increases the reactive oxygen species (ROS), and the NF-κB-mediated inflammatory response may be related to the pathogenesis of ovarian cancer [5, 6]. Phytoestrogens are known to have estrogen activity and effective antioxidant activity, and it could prevent ROS production and have an anti-inflammatory effect. They also play the role of estrogen in low estrogen levels; however, they could competitively bind to estrogen receptors (ER) and inhibit the effect of estrogen under high estrogen levels presence. Isoflavones, a phytoestrogen, have been reported to inhibit prostate cancer PC-3 and LINCAP cell lines [7]. Several epidemiological studies have demonstrated that the intake of foods containing isoflavones can reduce ovarian cancer incidence [8]. Isoflavones also have high estrogen receptor β (ER-β) affinity and exert anti-cancer activity via a pleiotropic mechanism [9].

However, the relationship between phytoestrogen and the incidence rate of gynecological malignancies is controversial [9-11]. *Bandera et al.* reported no significant statistical difference between isoflavone intake from food/supplements and ovarian cancer risk at the overall level, and the negative correlation between

the highest and lowest consumers was very weak (OR=0.62, P=0.04) [12]. Nevertheless, in meta-analyses, a high intake of phytoestrogens was associated with a reduced risk of ovarian cancer. Previous studies have shown that Asian foods such as soy foods and isoflavones can reduce ovarian cancer incidence rates [13], but the mechanism still remains unclear. Therefore, the biological activities of isoflavones in ovarian and breast cancer cells need to be further studied.

Isoflavone is a phytoestrogen that mainly exists in legumes. Soybean isoflavones are secondary metabolites that are formed during soybean growth. It is also called phytoestrogen because it is extracted from soybean and other plants, and its structure is similar to that of estrogen [7]. *Fukutake et al.* demonstrated that the low daily intake of soy products by Europeans and Americans might be related to the high incidence rate of colon, prostate, and breast cancers [14]. Other foods such as garlic, chickpeas, licorice, and asparagus are also rich in isoflavones. *Wu et al.* conducted a large-scale investigation of Chinese herbal formulas containing coumarins, isoflavones, and daidzein used by women in Taiwan. They found that approximately 20% of the prescriptions and 7% of Chinese herbal medicines contain phytoestrogens, suggesting that the isoflavones consumed by women in Taiwan may have come from Chinese herbal medicine. However, this has raised concerns about the safety of phytoestrogens in endocrine function [15].

We speculated whether the opposite results of isoflavones in different studies might be related to individual differences. But the reason of this case needs to be further studied. Therefore, it is of great clinical significance to elucidate the biological functions of phytoestrogens, especially the effects of isoflavones on ovarian and breast cancer cells. Thus, we designed and performed relevant studies to explore the effects and mechanisms of isoflavones on the growth of ovarian and breast cancer cell lines SKOV-3 and MCF-7, respectively. This study could provide more information for further studies on the therapeutic effects and specific mechanisms of isoflavones in ovarian and breast cancer.

Materials And Methods

Cell culture

Ovarian and breast cancer cell lines, SKOV-3 and MCF-7, respectively, were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 and DMEM medium, respectively, both including 10% fetal bovine serum (FBS) (with high estrogen levels) and 1% penicillin/streptomycin (Mediatech, Inc., Manassas, VA, USA), cultured in a cell incubator with 5% CO_2 at 37°C. RPMI-1640 and DMEM were purchased from HyClone Laboratories, Inc. (Logan, UT, USA).

Cell counting and MTS assays

SKOV-3 and MCF-7 cells were seeded into 96-well plates and treated with 1 nM 17β-estradiol (E_2) and 10 μM isoflavones (Merck KGaA, Darmstadt, Germany), the cells were collected at every 24-h time point. Then, 0.4% trypan blue solution was added to the suspended cells, and the solution was mixed well before cell counting using a hemocytometer. Cell viability was assessed using MTS assays; cells were

treated with 10 μM isoflavones, and 20 μl of CellTiter 96 AQueous One Solution Reagent (Cat. No. G5430, Promega, Madison, WI, USA) was added to the cell culture plates every 24 h. After incubation for 1 h, 100 μl of culture medium was transferred to a new 96-well plate, and the absorbance was measured at 490 nm using a 96-well plate reader (Modulus Microplate Multimode Reader, Turner BioSystems, USA).

BrdU incorporation assay

SKOV-3 and MCF-7 cells were maintained in a two-well chamber and treated with 10 μM isoflavones for 48 h. BrdU reagent (Cat. No. 000103; Invitrogen, Rockville, Maryland, USA) was added to the two-well chamber before incubation for 2 h. Cells were washed with cold PBS thrice for 5 min each time, and then the cells were fixed for 30 min using 70% alcohol at 4°C and permeabilized for 30 min using 0.2% Triton X-100. After repeating the washing step, BrdU antibody was added to the slides and incubated for 1 h. Color development was performed using the BrdU kit (Cat. No. 933943; Invitrogen, Rockville, MD, USA) following the manufacturer's instructions. The BrdU-positive cells and total cells were counted using a microscope, and the BrdU-positivity rate was calculated.

RNA extraction and real-time quantification PCR

After treating SKOV-3 and MCF-7 cells with 10 μM isoflavones for 48 h, cells were washed with cold PBS, and RNeasy plus kit (Cat. No. 74136, QIAGEN, Valencia, CA, USA) was used to extract the total RNA. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Fisher Scientific, Waltham, MA, USA). Then, 1 μg of total RNA was used for cDNA synthesis using the High-Capacity RNA Transfer cDNA Kit (Cat. No. 4387406, Applied Biosystems, Foster, CA, USA). Primer 3 program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) was used to design the real-time PCR primers (Table 1). An ABI 7900HT fast real-time PCR machine (Applied Biosystems, Dublin, Ireland) was used for real-time quantitative PCR. Each PCR reaction contained 6 μl real-time PCR reaction mix (Cat. No. 75600, Affymetrix, Cleveland, OH, USA), 2 μl of 1:5 diluted cDNA, 1 μl forward and reverse primers (10 μM each), and 2 μl RNase free water. After initial denaturation at 95°C for 10 min, 40 amplification cycles were performed: denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and amplification at 72°C for 1 min. *GAPDH* was used as an internal reference gene to standardize the results.

Western blotting

SKOV-3 and MCF-7 cells were treated with 10 μM isoflavones for 48 h. Cells were washed twice with cold PBS and lysed on ice with cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride. The cell lysate was collected and centrifuged at 14,000 rpm at 4°C for 30 min. Protein (20 μg) was mixed with the sample loading buffer, boiled for 5 min at 95°C, and subjected to gel electrophoresis using 8%–12% SDS-polyacrylamide. The protein was transferred to a PVDF membrane and blocked with TBS buffer containing 0.1% Tween-20 and 3% BSA for 1 h at room temperature. The membrane was incubated at room temperature for 1 h or overnight at 4°C with the primary Anti-PCNA mouse monoclonal antibody (1:1,000; Cat. No. SC-56, Santa Cruz Biotechnology Co., Ltd., Dallas, TX, USA), Anti-cyclin D1 rabbit monoclonal antibody (1:1,000; Cat. No. ab16663, Abcam Cambridge, MA, USA), Anti-CDK4 rabbit

monoclonal antibody (1:1,000; Cat. No. ab108357, Abcam Cambridge, MA, USA), Anti-p21 mouse monoclonal antibody (1:1,000; Cat. No. SC-397, Santa Cruz Biotechnology Co., Ltd., Dallas, TX, USA), Anti-p27 rabbit polyclonal antibody (1:1,000, Cat. No. 3686S, Cell Signaling, MA, USA), Anti-β-actin mouse monoclonal antibody (1:1,000; Cat. No. EG-262, Excellgen, Rockville, MD, USA). The membrane was washed with TBST buffer thrice for 5 min each time. Then, the membrane was incubated with the Goat anti-rabbit IgG-HRP (Cat. No. SC-2301, Santa Cruz Biotechnology Co., Ltd., Dallas, TX, USA), Goat anti-mouse IgG-HRP (Cat. No. Thermo Scientific, Waltham, MA, USA) for 1 h at room temperature and washed three times with TBST buffer for 5 min each. Color development was performed using an ECL system (Cat. No. 32106, Fisher Scientific, Rockford, IL, USA).

Statistical analysis

All experiments were repeated three times. All data in this study are quantitative data and are expressed as mean ± standard deviation (SD). SPSS software (version 19.0; SPSS, IBM, Chicago, IL, USA) was used to analyze and process the experimental data, and statistical differences were calculated using the Student's *t*-test. Statistical significance was set at $P \leq 0.05$.

Results

Isoflavone inhibited SKOV-3 and MCF-7 cell growth and viability

To detect the effect of isoflavones on cell growth, SKOV-3 and MCF-7 cells were treated with 10 μM isoflavones under E_2 (final concentration 1 nM), and the number of cells was counted every 24 h (Fig 1). The number of SKOV-3 and MCF-7 cells was significantly increased with E_2 or isoflavones treatment alone at every time point, However, the cell numbers of treated with E_2 combine isoflavones was significantly decreased compared to E_2 or isoflavones treatment alone at 72 and 96 h time point (SKOV-3: Fig 1A; MCF-7: Fig 1B). Consistently, the MTS assay results showed that the cell viability of SKOV-3 (Fig 1C) and MCF-7 (Fig 1D) was significantly reduced after 72 h of treatment. These indicate that isoflavones can inhibit the proliferation and viability of SKOV-3 and MCF-7 cells.

Isoflavone reduces DNA synthesis

SKOV-3 and MCF-7 cells were treated with 10 μM (final concentration) isoflavones for 72 h. The BrdU antibody was used to label S-phase cells, BrdU-positive cells were counted, and positivity rates were determined (Fig 2). Cell treatment with 10 μM isoflavones significantly decreased BrdU incorporation compared to that of the control, whether SKOV-3 or MCF-7 cell line. These results indicate that isoflavone treatment inhibited cell proliferation and significantly decreased cell viability.

Isoflavone treatment mediated the mRNA expression levels of cell cycle factors

We designed a PCR primer and performed a real-time quantification PCR assay to detect the mRNA levels of PCNA, CDK4, cyclin D1, p21, and p27. The results indicate the role of isoflavones in cell cycle

regulation. PCNA is strongly correlated with DNA synthesis as it is usually used as an indicator of cell cycle processes. CDK4 and cyclin D1 are regulators of the cell cycle that act on the G1–S transition checkpoint. In contrast, p21 and p27 are negative regulators of the cell cycle. Real-time quantitative PCR results are shown in Figure 3. After 48 h of isoflavone treatment, PCNA, CDK4, and cyclin D1 mRNA levels were significantly reduced, while p21 and p27 mRNA levels were significantly increased. These results suggest that isoflavones can inhibit the G1–S transition in SKOV-3 (Fig 3A) and MCF-7 (Fig 3B) cells.

Alternation of expression levels of cell cycle factors by isoflavone treatment

To verify the above results, western blot analysis was carried out on the total protein isolated from SKOV-3 and MCF-7 cells treated with or without isoflavones for 48 h. β-actin was used as a housekeeping gene for optical density analysis and standardization of results. As shown in Figure 4, the expression levels of PCNA, CDK4, and cyclin D1 were significantly reduced, while p21 and p27 expression levels were significantly increased. These results demonstrated that isoflavone-mediated expression of critical factors of the cell cycle led to cell cycle arrest in breast cancer MCF-7 cells and ovarian cancer SKOV-3 cell lines.

Discussion

CDK4 is a serine/threonine kinase belonging to the cyclin-dependent kinase family. It is a catalytic subunit of the protein kinase complex controlled by cyclin D and p16INK4a (a CDK inhibitor), and it is limited to the G1–S phase of the cell cycle. The Ser/Thr kinase of the cyclin D-CDK4 complex promotes the phosphorylation of Rb family members (Rb-1). Phosphorylation of Rb-1 results in the separation of E2F from the Rb/E2F complex, which then initiates an E2F target gene transcription and promotes the G1 phase [16]. The cyclin D-CDK4 complex is the main integron of many mitotic and anti-pathogenic signals. It also inhibits Smad3 transcriptional activity by promoting Smad3 phosphorylation in a cell cycle-dependent manner [17]. In our study, we found that isoflavone significantly decreased cyclin D1 expression levels in ovarian and breast cancer cell lines. The results indicated that isoflavone might inhibit cell proliferation and regulate cyclin D1.

In contrast, PCNA, a DNA double-strand scaffold, promotes DNA polymerase and assists the transcription factor binding to DNA during synthesis [18]. Meanwhile, p21 is an effective cyclin-dependent kinase inhibitor that downregulates the G1–S transformation by binding with cyclins such as CDK4 and Cdk6. Similarly, p27 inhibits the activation of CDK2 and CDK4, thereby delaying cell cycle progression [17, 19]. In this study, we found that the PCNA and CDK4 expression levels decreased and p21 and p27 protein levels increased, suggesting that isoflavones could inhibit cell growth by modulating the cell cycle G1–S transition process.

It has been reported that there are some controversial results regarding isoflavones in many studies, and the effect of isoflavones on different tumor cell lines may be the opposite. For example, studies of many cell lines in prostate, colon, and breast cancers have shown that isoflavones inhibit cancer cell growth and proliferation [9, 20]. However, *Chen et al*. demonstrated that isoflavones may play an estrogen role in

the absence of estrogen and promote G1-S transformation through Akt activation and the NF-κB pathway [21]. In contrast, *Lucki et al.* reported that isoflavones can induce acid ceramide gene expression, thereby promoting MCF-7 cell proliferation [22].

The difference in isoflavone function in tumor cells may be regulated by many factors or pathways. First, it has been reported that the activation of ER-α or ER-β can cause different cellular reactions. The final cell effect of isoflavones depends on the ratio of the two estrogen receptors in different cells. Second, isoflavones have a strong estrogenic effect when there is no estrogen or other estrogen analogs in the medium. However, there is a potent estrogen effect in a medium with high estrogen or other estrogen analogs. In this case, isoflavones compete with these potent estrogen compounds to bind to the endoplasmic reticulum and inhibit cell proliferation. Third, isoflavones may play their biological functions through the endoplasmic reticulum or other mechanisms mediated by epigenetics [11]. In addition, isoflavones may regulate histone modification, DNA methylation, and miRNA transcription through other unknown mechanisms [23, 24].

In our study, cells were cultured in a medium containing 10% FBS with high estrogen levels, partly explaining the biological function of isoflavones under estrogen conditions. *In vivo*, isoflavones can inhibit tumor growth in other ways, such as tumor immunology, microenvironment, and cell-cell interaction. Isoflavones can prevent the occurrence of ovarian cancer, although this is not automatically due to the direct inhibition of cancer cell growth. Therefore, it is necessary to establish animal models for more detailed experiments and more extensive *in vitro* studies to determine the specific biological functions and mechanisms of isoflavones in tumor cells.

Conclusion

We evaluated the effects of isoflavones (the main component of isoflavones in foods and herbs) on the growth of ovarian cancer cell SKOV-3 and breast cancer cell MCF-7. Isoflavones were found to significantly inhibited proliferation of cancer cells under high estrogen levels. In addition, isoflavones can alter the expression of various cell cycle factors. These results enrich our understanding of the biological activities of isoflavones, providing a theoretical basis for the clinical application of isoflavones in breast cancer treatment and ovarian cancer.

Abbreviations

PCNA: Proliferating Cell Nuclear Antigen

CDK4: Cyclin-Dependent Kinase 4

Declarations

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Author Contributions

ZS, TC and FG conceived and designed the study, analyzed the results, and wrote the paper. ZS, YQ and LW carried out the experiments. YQ and LW provided experimental support. All authors reviewed the results and approved the final version of the manuscript.

Corresponding authors

Correspondence to Tingting Cheng and Fengbiao Guo.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors agree to the publication clause.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable

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Table

Table 1. The real-time PCR primers.

NCBI accession No.	Gene	Direction	Primer sequence	Product size (bp)
NM_002592.2	PCNA	Forward	TCTGAGGGCTTCGACACCTA	95
		Reverse	TCATTGCCGGCGCATTTTAG	
NM_053056.2	Cyclin D1	Forward	CTGAGGAGCCCCAACAACTT	80
		Reverse	CAGTCCGGGTCACACTTGAT	
NM_000075.3	CDK4	Forward	GCCCTCAAGAGTGTGAGAGTC	70
		Reverse	CACGAACTGTGCTGATGGGA	
NM_000389.4	p21	Forward	TTGTCACCGAGACACCACTG	79
		Reverse	GGAAGGTAGAGCTTGGGCAG	
NM_004064.4	p27	Forward	TCACAAACCCCTAGAGGGCA	80
		Reverse	GCGGGGGTCTGTAGTAGAAC	

Figures

Figure 1

Isoflavones inhibits cell proliferation in ovarian and breast cancer cell lines. SKOV-3 and MCF-7 cells were seeded in a 96-well plate and treated with equal volumes of E2 (final concentration 1 nM) and 10 μM isoflavones for 24, 48, 72, and 96 h. The cells were collected by trypsinization and counted every 24 h. At 72-h and 96-h, the SKOV-3 (A) and MCF-7 (B) cell numbers were significantly increased in E2 or isoflavone treatment alone, and significantly decreased after treated with E2 and isoflavone compared to E2 or isoflavone treatment alone. The MTS reagent was added at every 24-h time points, and color comparison was performed. The two curves reflect the absorbance value at 490 nm for cells treated with or without isoflavone. Cell viability was significantly reduced at 72 h and 96 h after isoflavone treatment. *$P \leq 0.05$, **$P \leq 0.01$.

Figure 2

Results of the BrdU incorporation analysis. SKOV-3 and MCF-7 cells were seeded in a 2-well chamber and treated with 10 μM isoflavone for 48 h. A. Representative image of the control and isoflavone groups. B. Percentage of BrdU-positive cells in SKOV-3 and MCF-7 cells. The number of BrdU-positive cells in the isoflavone group are significantly lower than that of the control group. **$P \leq 0.01$.

Figure 3

Alternation of mRNA levels of cell cycle factors. mRNA was isolated from SKOV-3 (A) and MCF-7 (B) cells treated with (Isoflavone) or without (Ctrl) isoflavone for 48 h. After isoflavone treatment, the PCNA, CDK4, and cyclin D1 mRNA levels were significantly reduced, while the mRNA levels of p21 and p27 were significantly increased. **P ≤ 0.01.

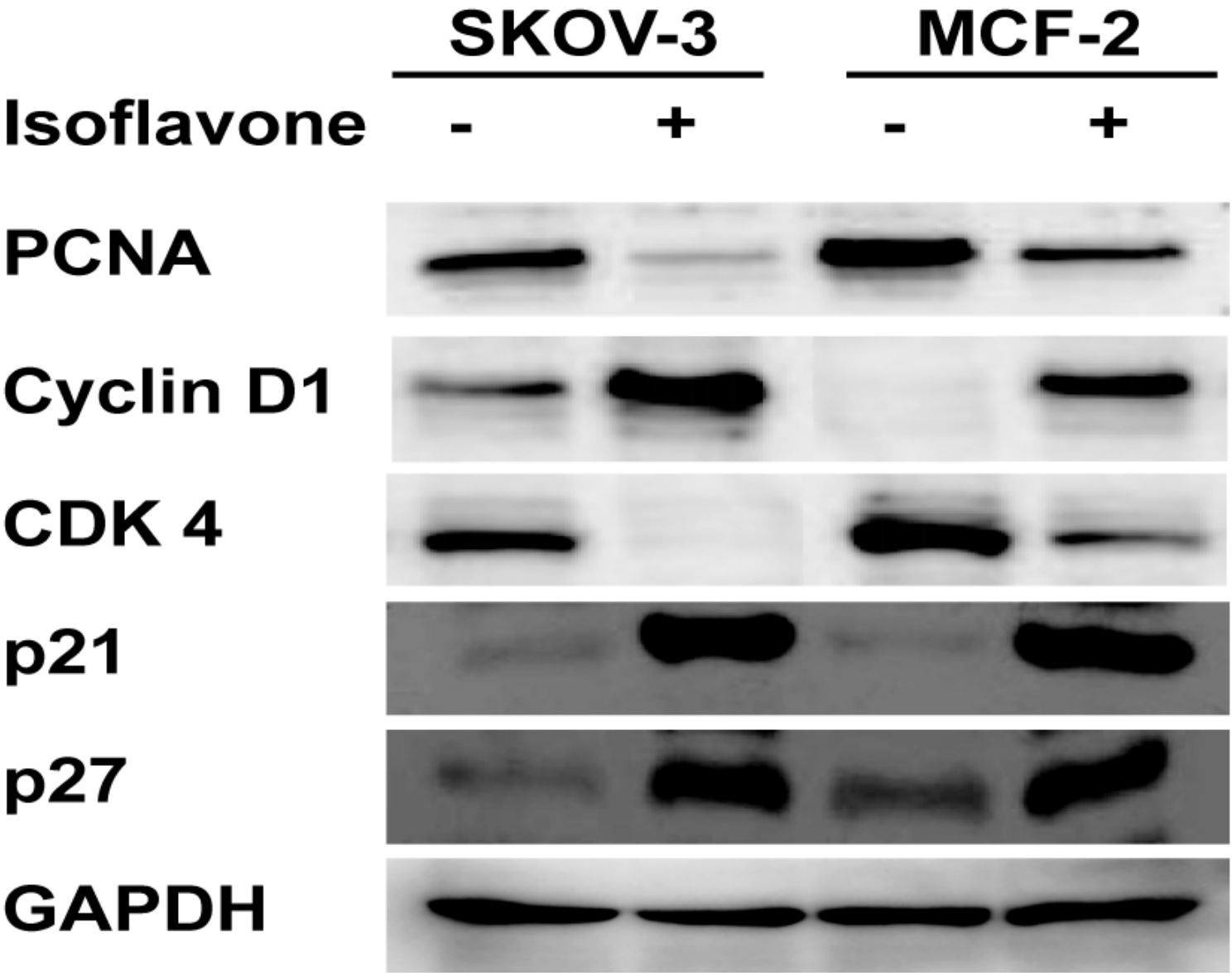

Figure 4

Alternation of protein levels of cell cycle factors. SKOV-3 and MCF-7 cells were treated with 10 μM isoflavone for 48 h, and their protein was extracted. Cell proteins are separated in 8%–12% SDS PAGE. The protein expressions of PCNA, CDK4, and cyclin D1 were significantly downregulated after the isoflavone treatment, while the protein expression levels of p21 and p27 were considerably increased.