The effect and apoptosis mechanism of 6-methoxyflavone in HeLa cells

Chaohong Zhang\textsuperscript{a,b}, Yuchong Quan\textsuperscript{c}, Yingying Bai\textsuperscript{a,b}, Lijuan Yang\textsuperscript{a,b} and Yongxiu Yang\textsuperscript{a,b,d}

\textsuperscript{a}The First Clinical Medical College, Lanzhou University, Lanzhou, China; \textsuperscript{b}Key Laboratory of Gynecological Oncology of Gansu Province, Lanzhou, China; \textsuperscript{c}College of Basic Medicine, Dalian Medical University, Dalian, China; \textsuperscript{d}Department of Obstetrics and Gynecology, First Hospital of Lanzhou University, Lanzhou, China

ABSTRACT

Introduction: Tumour cell apoptosis is a crucial indicator for judging the antiproliferative effects of anti-cancer drugs. The detection of optical and macromolecular biomarkers is the most common method for assessing the level of apoptosis. We aimed to explore the anti-tumour mechanisms of 6-methoxyflavone.

Materials and methods: Three optical methods, including the percentage of apoptotic cells, cell morphology, and subcellular ultrastructure changes, were obtained using flow cytometry, inverted fluorescence microscopy, and transmission electron microscope imaging. The mRNA or protein expression of macromolecular biomarkers related to common apoptotic pathways was determined via polymerase chain reactions or western blot assays. The functional role of the core gene biomarker was investigated through overexpression, knockdown, and phosphorylation inhibitor (GSK2656157).

Results: Transcriptome sequencing and the optical biomarkers assays demonstrated that 6-methoxyflavone could induce apoptosis in HeLa cells. The expression of macromolecular biomarkers indicated that 6-methoxyflavone induced apoptosis through the PERK/EIF2\textsubscript{a}/ATF4/CHOP pathway. Phosphorylated PERK was identified as the core biomarker of this pathway. Both overexpression and GSK2656157 significantly altered the expression level of phosphorylated PERK in 6-methoxyflavone-treated HeLa cells.

Discussion and conclusion: Macromolecular biomarkers, such as phosphorylated PERK and phosphorylated EIF2\textsubscript{a} are of great significance for assessing the therapeutic effects of 6-methoxyflavone.

Introduction

Cervical cancer is a major threat to the health and well-being of adult women. The incidence (Bray \textit{et al}. 2018), mortality (Bray \textit{et al}. 2018), metastasis rate (Rong \textit{et al}. 2019), and recurrence rate (Holloway and Lea 2019) of cervical cancer are still relatively high. Concerning the treatment of cervical adenocarcinoma, there are still some problems, such as the high cost of targeted preparations (Liu \textit{et al}. 2021), drug and radiotherapy resistance (Suzuki \textit{et al}. 2021), strong side effects of radiotherapy and chemotherapy (Okonogi \textit{et al}. 2018), high probability of recurrence (Jung \textit{et al}. 2017), metastasis (Zhou \textit{et al}. 2021), lymphovascular infiltration (Saito \textit{et al}. 2020), and poor prognosis (Jung \textit{et al}. 2017). Therefore, the development of more effective treatment strategies for cervical cancer is critical. Cell proliferation assays of chemotherapeutic candidates have shown that 6-methoxyflavone has anti-proliferative activity against HeLa cells (Kinjo \textit{et al}. 2016). 6-Methoxyflavone can also significantly alleviate cisplatin-induced adverse effects (Shahid \textit{et al}. 2017). Concurrently, 6-methoxyflavone has been shown to have broad anti-inflammatory (Wang \textit{et al}. 2015) and immune-regulatory (So \textit{et al}. 2014) effects. The above results indicate that 6-methoxyflavone has anti-cancer activity against HeLa cells, although its mechanism of action remains uncertain.

Biomarkers are important prerequisites and strong cornerstones in the implementation of precision medicine (Chen \textit{et al}. 2020), stratified medicine (Newby and Ohman 2015), personalized medical care (Wang \textit{et al}. 2021), and have a pivotal position in existing early tumour screening (Walther-Antonio and Mariani 2019), diagnosis (Olovo \textit{et al}. 2021), treatment response (Asif and Teply 2021), recurrence monitoring (Tang \textit{et al}. 2017), targeted therapy (Karlsson \textit{et al}. 2021) and immunotherapy (Zhang \textit{et al}. 2021; Palmeri \textit{et al}. 2022) systems. Biomarkers are measurable biological characteristics associated with physiological and pathological processes, and therapeutic responses (Group 2016; Bravo-Merodio \textit{et al}. 2021). Biomarkers include small molecules (such as creatinine) (Carroll \textit{et al}. 2020), large molecules (such as nucleic acids and proteins) (Moranova \textit{et al}. 2022; Zeng \textit{et al}. 2022), imaging features (Vassileva \textit{et al}. 2019; Li \textit{et al}. 2020a), anatomical features (Gerendas \textit{et al}. 2018), and biological population features (Jiang \textit{et al}. 2019), etc. At present, the research strategy for biomarkers is mostly high-throughput screening strategies and feature candidate strategies. High-throughput screening strategies are based on genomics, transcriptomics, proteomics, and metabolomics techniques (Dean \textit{et al}. 2018;
Zhang et al. 2020; Senturk et al. 2021). Functional candidate strategies are based on key molecules (Martinou et al. 2021) or known physiological or pathological processes (Bachert et al. 2022).

Furthermore, previous studies have shown that cell apoptosis plays a key role in cancer therapy (Carneiro and El-Deiry 2020) and is closely associated with efficacy assessment (Schürmann et al. 2021), resistance reversal (Liu et al. 2020b), and sensitivity enhancement (Dey et al. 2021; Xie et al. 2021) of anticancer drugs. A retrospective single-center study of rectal cancer developed a novel apoptosis-based tumour regression grade to assess the efficacy of chemoradiotherapy (Ozaki et al. 2021). A multicolour fluorescence nanoprobe was used to observe the progression of tumour cell apoptosis to assess drug efficacy (Luan et al. 2018). Apoptosis-related phenomena were used as decisive factors in the evaluation of the efficacy of photodynamic therapy. Moreover, apoptosis can be used to assess disease severity (Chu et al. 2021).

In our study, we investigated the in vitro therapeutic effect of 6-methoxyflavone on cervical adenocarcinoma based on transcriptomics and relevant biomarkers of apoptosis. First, we identified the key biological processes of 6-methoxyflavone exerting anti-tumour effects by transcriptome sequencing. RNA-seq analysis indicated that 6-methoxyflavone was significantly related to apoptosis. Subsequently, we screened three commonly used optical biomarkers and a variety of significant molecular biomarkers to evaluate the level of apoptosis and efficacy after 6-methoxyflavone intervention. The biomarker analyses indicated that 6-methoxyflavone induced apoptosis through the PERK/EIF2α/ATF4/CHOP pathway. Subsequently, overexpression, knockdown, inhibition of phosphorylation activity, and co-immunoprecipitation experiments further confirmed that the Thr982 phosphorylation activity of PERK was identified as the core biomarker of the pathway. Finally, this study aimed to provide new ideas for the clinical treatment of cervical cancer.

Materials and methods

Chemicals and cell culture

6-Methoxyflavone (purity ≥ 98%) was purchased from Weikeqi Biological Technology Co., Ltd. (Chengdu, China). Selective PERK phosphorylation inhibitor GSK2656157 was obtained from MedChem Express (New Jersey, USA) and dissolved in dimethyl sulfoxide (DMSO). HeLa, C33A, SiHa, and HaCaT human cell lines were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Details of each cell line were presented in Table 1. There were no ethical issues involved in this study. C33A, SiHa, and HaCaT cells were cultures in modified Eagle’s medium (HyClone, Logan, UT, USA) with 10% foetal bovine serum (FBS). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT, USA) supplemented with 10% FBS. The cells were cultured at 37°C in a 5% CO2 atmosphere.

The potential targets of 6-methoxyflavone

The potential targets of 6-methoxyflavone were downloaded from four databases: Traditional Chinese Medicine Systems Pharmacology (TCMSP) (Ru et al. 2014), Traditional Chinese Medicine on Immuno-Oncology (TCMIO) (Liu et al. 2020a), HERB (Fang et al. 2021), and PubChem (Kim et al. 2016, 2021) databases. TCMSP predicts targets by browsing the chemical-related targets sub-database. TCMIO predicts targets of immuno-oncology by browsing the ingredients section. HERB predicts targets by browsing ingredients related to gene target sections. Simultaneously, we obtained potential targets of 6-methoxyflavone from two columns of the PubChem database: chemical-gene co-occurrences in literature and bioassay results. In the bioassay results column, we only outputted the human targets. Finally, 130 potential targets of 6-methoxyflavone were identified.

Kyoto encyclopedia of genes and genomes (KEGG) pathways enrichment analysis of targets

The 130 targets of 6-methoxyflavone were used for KEGG (Kanehisa and Goto 2000; Kanehisa et al. 2017) pathway enrichment analysis of the DAVID 6.8 online database (Jiao et al. 2012). Then, we set the threshold of the false discovery rate to <0.001 to filter the pathways predicted by DAVID. Finally, we used GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) to visualize the enrichment pathways of targets.

Proliferation-related biomarker (cell activity) assays

The Enhanced Cell Counting kit-8 (CCK-8) (Beyotime, Shanghai, China) was used to detect the viability of HeLa, C33A, SiHa, and HaCaT cells. Cells were seeded and cultured in 96-well plates for 24 h. Then, the cell lines were treated with five concentrations of 6-methoxyflavone (20, 40, 80, 120, and 160 μM) for 24, 48, and 72 h. The HeLa cells were treated with seven concentrations of GSK2656157 (0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μM) for 48 h. The control group of fresh CCK-8 working solution was added and the cells were cultured for 1 h at 37°C. The optical density (OD) values were measured at 450 nm using a multifunctional microplate reader (Thermo Fisher Scientific, Madison, WI, USA). Graphpad Prism version 8.0.1 for Windows was used to calculate the half-maximal inhibitory concentration (IC50).

Eukaryotic transcriptome sequencing

Human HeLa cells were seeded in 6-cm dishes for 24 h and cultured with 0.16% DMSO and 65 μM 6-methoxyflavone for

| Cells   | Morphology  | HPV     | Keratin | Mycoplasma | Chromosome |
|---------|-------------|---------|---------|------------|------------|
| HaCaT   | Epithelioid | HPV(−)  | (+)     | (−)        | 72–88      |
| SiHa    | Epithelioid | HPV(+)  | (+)     | (−)        | 51–72      |
| C33A    | Epithelioid | HPV(−)  | (−)     | (−)        | 46XX       |
| HeLa    | Epithelioid | HPV(+)  | (+)     | (−)        | 74–86      |

HPV: human papillomavirus; (+): positive; (−): negative.
48 h in an incubator. After 48 h, 1 ml RNAiso plus reagent (Takara, Dalian, China) was used to lyse cells and extract total RNA. The concentration and purity of RNA were confirmed using Qubit RNA assay kits and Qubit 2.0 Fluorometer (Life Technologies, Invitrogen division, Darmstadt, Germany). RNA integrity and genomic contamination were assessed using agarose gel electrophoresis. Transcriptome sequencing (RNA-seq) assays were performed by Sangon Biotech Co., Ltd. (Shanghai, China). After library construction and sample cluster generation, transcriptome sequencing was performed using an Illumina Hiseq™ platform (Illumina Inc, San Diego, California, USA). The relative expression of each gene was estimated using transcripts-per-million (TPM). The screening criteria for significantly differentially expressed genes (DEGs) were as follows: at least one of the two samples had a TPM value $\geq 5$, the absolute value of log2FoldChange was $>1$, and $q$-Value was $<0.05$. The $q$-values were estimated by the false discovery rate (FDR) correction method. We then performed gene ontology (GO) and pathway classification enrichment analyses using the DAVID and KEGG databases. The screening criteria for significantly different items were $p$-value $<0.05$. GraphPad Prism version 8.0.1 for Windows was used to visualize the significantly different items.

The assessments of three optical biomarkers of apoptosis

The single-cell suspensions were seeded and cultured in 6-well plates for 24 h. After that, the cells were treated with five concentrations of 6-methoxyflavone (0.16% DMSO, 40, 65, 80, and 120 μM) for 48 h.

Bisbenzimide H 33342 labelled inverted fluorescence microscope imaging

The Hoechst 33342 staining dye solution kit (Beyotime Biotechnology, Jiangsu, China) was used to detect the ratio of apoptotic HeLa cells. Based on the manufacturer’s instructions, 500 μl of fresh Hoechst 33342 solution was added and incubated for 30 min at 37°C. An inverted fluorescence microscope (Nikon Corp., Tokyo, Japan) was used to observe the morphology of the apoptotic HeLa cells.

Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) labelled flow cytometry optical testing system

An annexin V-FITC/PI apoptosis detection kit (Meilunbio, Dalian, Liaoning, China) was used to measure the percentage of HeLa cells in the early and late apoptotic stages. The cells ($5 \times 10^5$) were carefully collected and suspended in a 200 μl binding buffer containing 10 μl of annexin V-FITC and 20 μl of PI. After 20 min of incubation, HeLa cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Transmissive electron microscope (TEM) imaging

HeLa cells treated with 0.16% DMSO or 65 μM 6-methoxyflavone were collected and centrifuged at 3000 rpm for 10 min at 25°C. Cold 2.5% glutaraldehyde was used to fix for 48 h, followed by 1% osmium tetroxide for 1.5 h, followed by dehydration with 50, 70, 80, 90, 100% ethanol, and 100% acetone. Next, the cells were incubated with Epon812 epoxy resin and an equal volume of pure acetone for 2 h, embedded in 100% Epon812 resin, and cured at 35, 45, and 68°C for 24 h. Finally, the ultrathin section was completed using an ultra-thin microtome (Leica EM UC7, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. The lead-stained sections were observed and photographed using a Transmission Electron Microscope (FEI Tecnai G2 Spirit Bio-TWIN, Hillsboro, USA).

The assessments of two types of macromolecular biomarkers of apoptosis

Real-time fluorescence quantitative polymerase chain reaction (PCR)

Single-cell suspensions were seeded into 6-well plates for 24 h. HeLa cells were then treated with either 6-methoxyflavone (65 μM) or 0.16% DMSO for 48 h. Total RNA was extracted from 6-methoxyflavone-treated HeLa cells using RNAiso Plus reagent (Takara, Dalian, China). The complementary DNA was synthesized by genomic DNAerase reaction and reverse transcription reaction using the PrimeScript™ RT reagent kit (Takara). Real-time PCR was performed using the TB Green® Premix Ex Taq™ II kit (Takara). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (Sangon Biotech, Shanghai, China) were used as housekeeping genes. The Livak method was used to analyze the relative quantitative real-time PCR data. The real-time PCR primer sequences are listed in Table 2.

Western blot analysis

HeLa cells were treated with either 6-methoxyflavone (65 μM) or 0.16% DMSO for 48 h, the cells were lysed for 20 min in cell lysis buffer (Beyotime) containing 1 mM phenylmethanesulfonyl fluoride (Beyotime). The lysate was centrifuged at 14,000 × g for 5 min at 4°C. Total protein samples were obtained from the supernatants of HeLa cell lysates. Protein concentrations were detected using an enhanced bicinchoninic acid protein assay kit (Beyotime). Subsequently, protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Burlington, MA, USA). After blocking with Tris-buffered saline-Tween 20 solutions containing 5% bovine serum albumin for 1 h at 25°C, the membranes were incubated with the primary antibodies overnight at 4°C. The primary antibodies used were: GAPDH, PERK (Sangon Biotech), phospho-PERK (Thr982), EIF2α, phospho-EIF2α (Ser51), ATF4, and CHOP rabbit antibodies (Beyotime). The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Sangon Biotech) for 1 h at 25°C. Finally, the protein bands were visually detected with an ultra-sensitive efficient chemiluminescence kit (Beyotime) using the Amersham Imager 680 system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).
The functional role of the core gene biomarkers

Cell transfection
HeLa cells were seeded in 6-well plates for 24 h. First, the complete medium was changed to Opti-MEM reduced serum medium (Gibco, Burlington, Canada). Then, three EIF2AK3/PERK small interfering RNAs (siRNA), GAPDH positive control siRNA, negative control siRNA (Genepharma, Shanghai, China), pcDNA empty vector (negative control), and human pcDNA3.1(C2) Flag-hEIF2AK3/PERK vector (NM_004836.7) (Hanbio, Shanghai, China) were transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After 6 h, the medium was replaced with a complete medium. After 48 h, a portion of the cells was digested with 0.25% trypsin buffer and collected by centrifugation at 1000 rpm for PCR analysis to determine transfection efficiency. Another part of the cells was re-plated for 24 h and treated with 0.16% DMSO and 1.25 M 6-methoxyflavone for 48 h. Then, the cells were collected for western blot analyses. The siRNA oligo sequences are listed in Table 3.

PERK phosphorylation inhibitor assay
HeLa cells were seeded in 6-well plates for 24 h. Then, the cells were pre-treated with 0.08% DMSO and 1.25 M GSK2656157 for 1 h. Subsequently, the cells were further treated with 0.08% DMSO and 65 μM 6-methoxyflavone for 48 h. The cells were digested and collected for western blot analyses.

Co-immunoprecipitation
HeLa cells were treated with 65 μM 6-methoxyflavone for 48 h. The cells were lysed using an immunoprecipitation cell lysis buffer (Beyotime). Protein A+G magnetic beads (Beyotime) were washed with 1× Tris-buffered saline in a magnetic separation rack three times and then incubated with primary antibody (anti-p-EIF2α) or normal rabbit IgG working solution (Beyotime) at 25°C for 1 h. Subsequently, cell lysates were incubated with magnetic beads conjugated with primary antibodies or normal rabbit IgG for 2 h at 25°C. After incubation, the magnetic beads were eluted in 1× SDS–polyacrylamide gel electrophoresis loading buffer (Beyotime) at 95°C for 8 min. Finally, the supernatant was collected for western blot analysis.

Statistical analysis
Three independent biological replicates were used for each experiment. Statistical analysis was carried out using GraphPad Prism version 8.0.1 for Windows. All results were represented as means ± standard deviation (SD) and analyzed by paired t-test, Wilcoxon test, or one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons tests. In inhibition assays of cell viability, IC50 values were calculated using non-linear regression (curve fit) analyses. Statistical significance was set at p < 0.05.

Results

KEGG pathways enrichment analysis and targets network construction
We collected 34, 6, 36, and 91 potential targets of 6-methoxyflavone from the TCMSP, TCMIO, HERB, and PubChem databases, respectively. After duplicate removal, 130 potential
targets of 6-methoxyflavone were identified. KEGG pathway enrichment analyses showed that the targets were remarkably associated with 12 pathways, including pathways in cancer, neuroactive ligand-receptor interaction, calcium signalling pathway, etc. \((p < 0.001)\) (Figure 1(A)). 6-Methoxyflavone was mainly related to pathways in cancer.

Figure 1. Function analyses of 6-methoxyflavone. (A) Functional enrichment analysis of 6-methoxyflavone targets in public databases. KEGG pathways enriched with 130 targets of 6-methoxyflavone \((p < 0.001)\). KEGG: Kyoto Encyclopaedia of Genes and Genomes; cGMP-PKG: cyclic guanosine monophosphate dependent protein kinase. (B–I) Transcriptome sequencing analyses of HeLa cells after 65 \(\mu\)M 6-methoxyflavone intervention. Control group: 0.16% dimethyl sulfoxide; Treated group: 65 \(\mu\)M 6-methoxyflavone. (B) Agarose gel electropherograms of six transcriptome-sequenced samples. (C,D) Histogram and minus-vs.-add plot of differentially expressed genes. (E) KEGG pathway analyses of annotated genes. The black numbers in the right Y-axis represent annotated genes. (F–I) Gene ontology functional enrichment analyses of differentially expressed genes. (F,G) Cell proliferation or apoptosis related biological processes \((p < 0.05)\). (H,I) Endoplasmic reticulum stress or protein modification related terms \((p < 0.05)\).
6-Methoxyflavone inhibits the cell viability of cervical cancer cell lines

We first tested cell viability using the CCK-8 assay. We treated HeLa, C33A, SiHa, and HaCaT cells with increasing concentrations of 6-methoxyflavone. We found that 6-methoxyflavone inhibited the viability of HeLa, C33A, SiHa, and HaCaT cells. Table 4 shows the IC50 values of 6-methoxyflavones in HeLa, C33A, SiHa, and HaCaT cells. The results revealed that HeLa cells are highly sensitive to 6-methoxyflavones. This result is consistent with that of a previous study on HeLa cells (Kinjo et al. 2016). Therefore, we chose HeLa cells for subsequent studies. For the subsequent experiments, HeLa cells were treated with 6-methoxyflavone (65 μM) for 48 h.

6-Methoxyflavone was significantly related to apoptotic biological processes

To reveal the anti-tumour mechanism of 6-methoxyflavone, we used RNA-seq to identify cancer-associated biological processes, molecular functions, KEGG pathways, and macromolecular biomarkers in HeLa cells treated with 6-methoxyflavone.

Agarose gel electrophoresis showed that all six samples met the requirements for library construction (Figure 1(B)). Compared with the three normal control groups (0.16% DMSO), there were a total of 1365 significantly differentially expressed genes in the three 65 μM 6-methoxyflavone-treated groups. Among these 1365 genes, 538 were upregulated and 827 were downregulated (Figure 1(C,D)).

Table 4. IC50 of 6-Methoxyflavones in various cells (mean ± standard error, μM).

| Cell    | 24 h   | 48 h   | 72 h   |
|---------|--------|--------|--------|
| HeLa    | 128.93 ± 24.51 | 65.20 ± 13.00 | 55.31 ± 9.14 |
| C33A    | 130.20 ± 3.72  | 115.17 ± 3.97  | 109.57 ± 4.05  |
| SiHa    | 344.90 ± 59.11  | 312.43 ± 50.67  | 208.53 ± 10.96  |
| HaCaT   | 331.07 ± 12.43  | 276.07 ± 39.6   | 226.53 ± 42.11  |

Figure 2. The ratio of apoptotic HeLa cells was increased after 6-methoxyflavone treatment. (A) The fluorescence images of Hoechst 33342 staining of the nucleus. HeLa cells were intervened with four concentrations of 6-methoxyflavone (0, 40, 80, and 120 μM) in dimethyl sulfoxide for 48 h. (B) The histogram represents the ratio of apoptotic HeLa cells in Figure 3. A (30 fields in each group were randomly selected for counting). (C,D) HeLa cells were intervened with two concentrations of 6-methoxyflavone (0 and 65 μM) in dimethyl sulfoxide for 48 h. After annexin V-fluorescein isothiocyanate/propidium iodide double-staining, percentage of HeLa cells in early and late apoptotic stages was detected by flow cytometry. Each assay was repeatedly performed in three times. Statistical analysis was carried out using paired t-test and dunnett’s multiple comparisons test. *p < 0.05. DMSO: dimethyl sulfoxide.
genes are promising therapeutic targets and efficacy assessment biomarkers for 6-methoxyflavone. Subsequently, we performed GO and KEGG pathway analyses of these genes. We obtained 11 KEGG pathways, 157 biological processes, and 54 molecular functions. The KEGG enrichment results showed that 6-methoxyflavone was correlated with four cellular processes, three environmental information progresses, and four genetic information progresses (Figure 1(E)). The GO enrichment results indicated that 6-methoxyflavone was significantly related to eight apoptosis or death (Figure 1(F)), five cell proliferation or growth (Figure 1(G)), three endoplasmic reticulum stress (Figure 1(H)) biological processes, and five protein-binding and binding term (Figure 1(I)) (p < 0.05). Based on the above analyses, we focussed on three items, namely the apoptotic process, phosphorylation, and PERK-mediated unfolded protein response.

6-Methoxyflavone induces HeLa cell apoptosis

The percentage of apoptotic cells, cell nucleus morphology, and subcellular ultrastructural changes were three important non-invasive optical biomarkers of apoptosis. Moreover, the subcellular ultrastructural change under transmission electron microscopy is the most classical and reliable method for evaluating apoptosis, and it is considered to be the gold standard for determining apoptosis.

We observed the morphological changes in the nucleus and calculated the percentage of apoptotic cells in 6-methoxyflavone-treated HeLa cells using Hoechst 33342 staining dye solution. As the concentration of 6-methoxyflavone increased, the nucleus became bright, condensed, irregular, and fragmented (Figure 2(A)). 6-Methoxyflavone significantly increased the ratio of apoptotic HeLa cells in a dose-dependent manner (Figure 2(B)).

We also detected the apoptotic rates of HeLa cells using the Annexin V-FITC/PI apoptosis detection kit. Flow cytometry analysis further showed that the percentage of 6-methoxyflavone-treated HeLa cells in the early and late apoptotic stages was significantly higher than that in the 0.16% DMSO group (Figure 2(C,D)).

TEM, which is the best technique to further analyze subcellular ultrastructural changes, was used to verify apoptosis
Figure 4. 6-Methoxyflavone induces apoptosis in HeLa cells via the PERK/EIF2α/ATF4/CHOP pathway. HeLa cells were intervened with two concentrations of 6-methoxyflavone (0 and 65 μM) in dimethyl sulfoxide for 48 h to evaluated changes in mRNA and protein expression levels. Real-time quantitative polymerase chain reaction (PCR) and western blot were used to detect apoptotic mRNAs and proteins expression levels, respectively. (A) The relative expression of Bcl2, Bax, Apaf1, Caspase3, PARP, Caspase7, and Caspase9 mRNAs in the mitochondrion pathway of apoptosis. (B) The relative expression of Fas, TNFR, FADD, TRADD, TRAF, Caspase3, PARP, Caspase6, and Caspase7 mRNAs in the death receptor pathway of apoptosis. (C) The relative expression of IRE1, XBP1, and ATF6 mRNAs in the IRE1 and ATF6 endoplasmic reticulum pathway. (D) The relative expression of PERK, EIF2α, ATP4, and CHOP mRNAs in the PERK/EIF2α/ATF4/CHOP endoplasmic reticulum pathway. (E,F) The relative expression levels of the PERK/EIF2α/CHOP pathway related proteins. Each assay was repeatedly performed in three times. Statistical analysis was carried out using paired t-test or Wilcoxon test. The differential expression of Caspase6 was analyzed by Wilcoxon test, and the other genes were analyzed by paired t-test. *p < 0.05. n.s.: not significant; p-PERK: phosphorylational PERK; p-EIF2α: phosphorylational EIF2α.
in this study. Ultrastructural changes in HeLa cells treated with 6-methoxyflavone (65 μM) and 0.16% DMSO were compared using transmission electron microscopy imaging. The TEM images of HeLa cells in the normal control group (0.16% DMSO) showed that the morphology and structure of the cell membrane, cytoplasm, organelles, and nucleus were normal (Figure 3(A)). However, when HeLa cells were treated with 6-methoxyflavone (65 μM) for 48 h, typical apoptotic morphological changes at various stages were observed in these cells, such as cytoplasmic vacuolation, formation of annular bodies, apoptotic bodies, cell budding, and cytoplasmic blebbing (Figure 3(B–F)). The results revealed that 6-methoxyflavone significantly induced apoptosis in HeLa cells.

### 6-Methoxyflavone induces apoptosis through the PERK/EIF2α/ATF4/CHOP pathway

As our results showed that 6-methoxyflavone induced HeLa cell apoptosis, we next investigated whether 6-methoxyflavone altered the mRNA and protein expression levels of the apoptotic pathway.

The mRNA expression of caspase3 in 6-methoxyflavone-treated HeLa cells was significantly higher than that in the 0.16% DMSO group, while the mRNA expression of Aparf1, caspase9, Fas, TNFR, and TRAF was significantly lower. Nevertheless, the mRNA expression of Bcl2, Bax, PARP, caspase7, FADD, TRADD, and caspase6 was not significantly altered by treatment with 6-methoxyflavone (Figure 4(A,B)). Hence, we found that 6-methoxyflavone was unable to activate the mitochondrial or the death receptor pathway of apoptosis and only affected the mRNA expression of caspase3, Aparf1, caspase9, Fas, TNFR, and TRAF.

The mRNA expression of IRE1, XBPI, and ATF6 was not significantly altered by treatment with 6-methoxyflavone (Figure 4(C)). Accordingly, 6-methoxyflavone was unable to activate the IRE1 and ATF6 endoplasmic reticulum pathways.

HeLa cells treated with 6-methoxyflavone significantly upregulated the mRNA and protein expression levels of EIF2/EIF2α/phosphorylation EIF2α (p-EIF2α), ATF4, and DDIT3/CHOP. Moreover, the mRNA and protein expression levels of EIF2AK3/PERK in 6-methoxyflavone-treated HeLa cells were significantly lower than those in the 0.16% DMSO group, while the protein expression levels of phosphorylated PERK (p-PERK) were significantly higher (Figure 4(D–F)). These results are consistent with the transcriptome sequencing results (Table 5). Resultantly, we found that 6-methoxyflavone induced apoptosis in HeLa cells via the PERK/EIF2α/ATF4/CHOP pathway. EIF2AK3/PERK, especially the Thr982 phosphorylation of PERK, was the most significantly differentially expressed factor in this pathway.

### The functional role of the core gene biomarkers

In summary, we found that EIF2AK3/PERK was the core gene biomarker of 6-methoxyflavone-induced apoptosis in HeLa cells. To determine the specific functional role of EIF2AK3/PERK, we performed gain-of-function and loss-of-function genetic manipulation and phosphorylation activity inhibition experiments.

We determined the efficiency of EIF2AK3 overexpression and knockdown using PCR and western blot analysis (Figure 6(A–D)). Figure 5 shows the pcDNA3.1 3 × Flag-EIF2AK3/PERK vector atlas. The intervention efficiency of three EIF2AK3/PERK siRNAs and a human 3 × Flag-hEIF2AK3/PERK vector met the requirements for genetic manipulation. Western blot experiments showed that both overexpression and GSK2656157 significantly altered the expression level of phosphorylated PERK in 6-methoxyflavone-treated HeLa cells, but knockdown experiments did not (Figure 6(C–E)). The expression levels of p-PERK, p-EIF2α, ATF4, and CHOP were significantly altered by GSK2656157, an inhibitor of PERK phosphorylation activity (Figure 6(E)). Figure 5(G) shows the inhibitory effect of GSK2656157 on HeLa cells using the CCK-8 assay. The IC50 value of GSK2656157 was 30.12 μM at 48 h. 1.25 μM GSK2656157 had a little inhibitory effect on HeLa cells (Figure 6(E,G)).

Moreover, co-immunoprecipitation assays of phosphorylated EIF2α confirmed that there was an interaction between phosphorylated PERK and phosphorylated EIF2α (Figure 6(F)). Therefore, we believe that the Thr982 phosphorylation of PERK is the core mechanism by which 6-methoxyflavone exerts its pro-apoptotic effect. Thr982 phosphorylation activity of PERK is a core biomarker for evaluating the efficacy of 6-methoxyflavone. Other macromolecular biomarkers, such as p-EIF2α, ATF4, and CHOP are also important biomarkers for evaluating the efficacy of 6-methoxyflavone.

### Discussion

The Imperatae Rhizoma exhibits antioxidant (Zhou et al. 2013), anticancer (Li et al. 2020b), anti-complementary (Fu et al. 2010), anti-inflammatory (Zou et al. 2021), and immunological regulation (Lu and Huang 1996) activities. The compound 6-methoxyflavone extracted from Imperatae Rhizoma has exhibited anti-proliferative activity against HeLa cells (Kinjo et al. 2016). However, its anti-cancer effects and mechanisms in cervical cancer have not yet been clarified. Herein, we identified 130 potential targets of 6-methoxyflavone. KEGG pathway enrichment analyses of the targets showed that 6-methoxyflavone was mainly associated with pathways in cancer. Next, we tested the inhibitory effect of 6-methoxyflavone on HeLa, C33A, SiHa, and HaCaT cells using CCK-8 assays. The results revealed that 6-methoxyflavones are highly sensitive to HeLa cells. This result is consistent with that of a previous study on HeLa cells (Kinjo et al. 2016). Transcriptome sequencing identified that apoptosis was the key biological process of 6-methoxyflavone exerting antitumour effects. Subsequently, optical biomarker assays...
revealed that 6-methoxyflavone significantly induced apoptosis in HeLa cells.

The apoptotic signalling pathways contain mitochondrial control (Burke 2017), death receptor (Jo et al. 2020; Sato et al. 2020), and endoplasmic reticulum pathways (Chern et al. 2019; Wang et al. 2020). We investigated whether 6-methoxyflavone altered the mRNA and protein expression levels of significant molecular biomarkers of the apoptotic pathway.

At the mRNA expression level, 6-methoxyflavone was unable to activate the mitochondrial control pathway, death receptor pathway, and endoplasmic reticulum pathway of IRE1 and ATF6. However, the mRNA expression of EIF2AK3/PERK, EIF2S1/EIF2α, ATF4, and DDIT3/CHOP was significantly altered by treatment with 6-methoxyflavone. Moreover, the results of western blot assays confirmed that 6-methoxyflavone significantly altered the protein expression levels of PERK, phosphorylated PERK, EIF2α, phosphorylation of EIF2α, ATF4, and CHOP. In summary, significant molecular biomarker assays indicated that 6-methoxyflavone induced apoptosis in HeLa cells via the PERK/EIF2α/ATF4/CHOP pathway.

The PERK/EIF2α/ATF4/CHOP pathway is highly related to the apoptosis of cervical cancer cells (Chitnis et al. 2012; Hiramatsu et al. 2020). First, endoplasmic reticulum stress triggers the activation and phosphorylation of PERK (Chitnis et al. 2012). Phosphorylation of PERK causes the activation and phosphorylation of EIF2α. Phosphorylation of EIF2α then upregulates the expression levels of ATF4 and CHOP (Hiramatsu et al. 2020). Finally, the overexpression of CHOP initiates the apoptosis-promoting program (Lee et al. 2018).

In our study, 6-methoxyflavone triggered the activation and phosphorylation of PERK and EIF2α. Subsequently, phosphorylation of EIF2α upregulated the expression levels of ATF4 and CHOP. Namely, 6-methoxyflavone induced cell apoptosis via the PERK/EIF2α/ATF4/CHOP pathway. Overexpression, knockdown, inhibition of phosphorylation activity, and co-immunoprecipitation experiments further confirmed that the

Figure 5. The pcDNA3.1 3 x Flag-EIF2AK3/PERK overexpressed vector atlas.
Figure 6. The functional role of the core gene biomarkers (EIF2AK3/PERK). (A,B) After overexpression and knockdown, the relative mRNA expression levels of EIF2AK3/PERK were detected by quantitative polymerase chain reactions (PCR). (C,D) Relative protein expression of PERK and p-PERK after 6-methoxyflavone, EIF2AK3/PERK overexpressed vector and siRNA oligos treatment. E. Relative protein expression of p-PERK, p-EIF2α, ATF4, and CHOP after 6-methoxyflavone and GSK2656157 treatment. (F) The results of co-immunoprecipitation assays of phosphorylated EIF2α.G. HeLa cells were treated with GSK2656157 (0.3125–20 µM) for 48 h. Percentage of viable HeLa cells decreased with increasing concentration. The cell counting kit-8 was used to detected the inhibitory effect of GSK2656157. Each assay was performed from three independent experiments in sextuplicate (n = 18). Statistical analysis was carried out using one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons tests. *p < 0.05. n.s.: not significant; GSK: GSK2656157; 6MF: 6-methoxyflavone; p-PERK: phosphorylational PERK; p-EIF2α: phosphorylational EIF2α; DMSO: dimethyl sulfoxide.
Thr982 phosphorylation activity of PERK was identified as the core biomarker of the pathway. To summarize, this study suggests that 6-methoxylavone is a potential drug for the treatment of cervical adenocarcinoma. Macromolecular biomarkers, such as p-PERK, p-EIF2α, ATF4, and DDIT3/CHOP are important biomarkers for evaluating the efficacy of 6-methoxylavone.

Conclusion

In this study, 6-methoxylavone inhibited HeLa, C33A, SiHa, and HaCaT cell growth and induced HeLa cell apoptosis in a concentration-dependent manner. Moreover, 6-methoxylavone can also significantly alter the mRNA and protein expression of EIF2AK3/PERK, EIF2S1/EIF2α, ATF4, and DDIT3/CHOP. In conclusion, 6-methoxylavone induces apoptosis in HeLa cells via the PERK/EIF2α/ATF4/CHOP pathway. These macromolecular biomarkers are of great significance for assessing the therapeutic effects of 6-methoxylavone.

Disclosure statement
The authors confirm that no conflicts of interest exist.

Data availability statement
The authors confirm that the data supporting the findings of this study are available within the article [and/or] its Supplementary Materials.

References

Asif, S. and Teply, B.A., 2021. Biomarkers for treatment response in advanced prostate cancer. Cancers, 13 (22), 5723.

Bachert, C. et al., 2022. Association between dupilumab treatment effect on nasal polyp score and biomarkers of type 2 inflammation in patients with chronic rhinosinusitis with nasal polyps in the phase 3 SINUS-24 and SINUS-52 trials. *International forum of allergy & rhinology*, 1–5.

Bravo-Merodio, L. et al., 2021. Translational biomarkers in the era of precision medicine. *Advances in clinical chemistry*, 102, 191–232.

Bray, F., et al., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*, 68 (6), 394–424.

Burke, P.J., 2017. Mitochondria, bioenergetics and apoptosis in cancer. *Trends in cancer*, 3 (12), 857–870.

Carneiro, B.A. and El-Deiry, W.S., 2020. Targeting apoptosis in cancer therapy. *Nature reviews. Clinical oncology*, 17 (7), 395–417.

Carroll, D.M. et al., 2020. Relationships between race, gender, and spot urine levels of biomarkers of tobacco exposure vary based on how creatinine is handled in analyses. *Nicotine & tobacco research*, 22 (11), 2109–2113.

Chen, Y., et al., 2020. Predicting peritoneal dissemination of gastric cancer in the era of precision medicine: molecular characterization and biomarkers. *Cancers*, 12 (8), 2236.

Chen, Y.J. et al., 2019. The interaction between SPARC and GRP78 interferes with ER stress signaling and potentiates apoptosis via PERK/EIF2α and IRE1α/XBP-1 in colorectal cancer. *Cell death & disease*, 10 (7), 504.

Chitnis, N.S., et al., 2012. miR-211 is a prosurvival microRNA that regulates chop expression in a PERK-dependent manner. *Molecular cell*, 48 (3), 353–364.

Chu, H., et al., 2021. Targeting highly pathogenic coronavirus-induced apoptosis reduces viral pathogenesis and disease severity. *Science advances*, 7 (25), eabf5777.
Liu, Z.J., et al., 2020b. Quercetin induces apoptosis and enhances gemcitabine therapeutic efficacy against gemcitabine-resistant cancer cells. *Anti-cancer drugs*, 31 (7), 684–692.

Lu, S. and Huang, H., 1996. Immunological regulation of rhizoma Imperatae on IL-2 and T-lymphocyte subpopulation. *Zhongguo zhong yao za zhi*, 21 (8), 488–489, 511.

Luan, M., et al., 2018. Simultaneous fluorescence visualization of epithelial-mesenchymal transition and apoptosis processes in tumor cells for evaluating the impact of epithelial-mesenchymal transition on drug efficacy. *Analytical chemistry*, 90 (18), 10951–10957.

Martinou, E., et al., 2021. A systematic review on HOX genes as potential biomarkers in colorectal cancer: an emerging role of HOXB9. *International journal of molecular sciences*, 22 (24), 13429.

Moranova, L., et al., 2022. Electrochemical LAMP-based assay for detection of RNA biomarkers in prostate cancer. *Talanta*, 238 (Pt 2), 123064.

Newby, L.K. and Ohman, E.M., 2015. Biomarkers: troponin testing-risk stratification to stratified medicine. *Nature reviews. Cardiology*, 12 (11), 625–626.

Okonogi, N., et al., 2018. Clinical outcomes of carbon ion radiotherapy with concurrent chemotherapy for locally advanced uterine cervical adenocarcinoma in a phase 1/2 clinical trial (protocol 1001). *Cancer medicine*, 7 (2), 351–359.

Olovo, C.V., et al., 2021. Faecal microbial biomarkers in early diagnosis of colorectal cancer. *Journal of cellular and molecular medicine*, 25 (23), 10783–10797.

Ozaki, K., et al., 2021. Development of a novel apoptosis-based tumor regression grade to assess the efficacy of preoperative chemoradiotherapy for rectal cancer: a retrospective single-center study. *International journal of clinical oncology*, 26 (9), 1679–1688.

Palmeri, M., et al., 2022. Real-world application of tumor mutational burden-high (TMB-high) and microsatellite instability (MSI) confirms their utility as immunotherapy biomarkers. *ESMO open*, 7 (1), 100336.

Rong, G., et al., 2019. Plasma CADM1 promoter hypermethylation and D-dimer as novel metastasis predictors of cervical cancer. *The journal of obstetrics and gynaecology research*, 45 (7), 1251–1259.

Ru, J., et al., 2014. TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. *Journal of cheminformatics*, 6, 13.

Saito, Y., et al., 2020. Aldolase A promotes epithelial-mesenchymal transition to increase malignant potentials of cervical adenocarcinoma. *Cancer science*, 111 (8), 3071–3081.

Saito, Y., et al., 2020. Fas ligand enhances apoptosis of human lung cancer cells cotreated with RIG-I-like receptor agonist and radiation. *Current cancer drug targets*, 20 (5), 372–381.

Schürmann, L., et al., 2021. Inhibition of the DSB repair protein RADS1 potentiates the cytotoxic efficacy of doxorubicin via promoting apoptosis-related death pathways. *Cancer letters*, 520, 361–373.

Senturk, A., et al., 2021. Quantitative proteomics identifies secreted diagnostic biomarkers as well as tumor-dependent prognostic targets for clear cell renal cell carcinoma. *Molecular cancer research*, 19 (8), 1322–1337.

Shahid, M., et al., 2017. The flavonoid 6-methoxyflavone allies cisplatin-induced neuropathic allodynia and hypoalgesia. *Biomedicine & pharmacotherapy*, 95, 1725–1733.

So, J.S., et al., 2014. 6-Methoxyflavone inhibits NFAT translocation into the nucleus and suppresses T cell activation. *Journal of immunology*, 193 (6), 2772–2783.

Suzuki, K., et al., 2021. Survival impact of adjuvant concurrent chemoradiotherapy after radical hysterectomy in FIGO stage IIIIC1 cervical adenocarcinoma. *International journal of clinical oncology*, 26 (7), 1322–1329.

Tang, Y., et al., 2017. Biomarkers for early diagnosis, prognosis, prediction, and recurrence monitoring of non-small cell lung cancer. *Oncotargets and therapy*, 10, 4527–4534.

Vassileva, V., et al., 2019. Evaluation of apoptosis imaging biomarkers in a genetic model of cell death. *EJNMMI research*, 9 (1), 18.

Walther-Antonio, M.R.S. and Mariani, A., 2019. Are early screening biomarkers for endometrial cancer needed to reduce health disparities? *American journal of obstetrics and gynecology*, 220 (4), 408–409.

Wang, H., et al., 2021. Blood transcriptome profiling as potential biomarkers of suboptimal health status: potential utility of novel biomarkers for predictive, preventive, and personalized medicine strategy. *The EPMA journal*, 12 (2), 103–115.

Wang, Q., et al., 2020. CCDC170 affects breast cancer apoptosis through IRE1 pathway. *Aging*, 13 (1), 1332–1356.

Wang, X., et al., 2015. 6-Hydroxyflavone and derivatives exhibit potent anti-inflammatory activity among mono-, di- and polyhydroxylated flavones in kidney mesangial cells. *PLOS one*, 10 (3), e0116409.

Xie, X., et al., 2021. Birinanpt enhances gemcitabine’s antitumor efficacy in triple-negative breast cancer by inducing intrinsic pathway-dependent apoptosis. *Molecular cancer therapeutics*, 20 (2), 296–306.

Zeng, Y., et al., 2022. Colorimetric immunosensor constructed using 2D metal-organic framework nanosheets as enzyme mimics for the detection of protein biomarkers. *Journal of materials chemistry. B*, 10 (3), 450–455.

Zhang, R., et al., 2020. Independent validation of early-stage non-small cell lung cancer prognostic scores incorporating epigenetic and transcriptional biomarkers with gene-gene interactions and main effects. *Chest*, 158 (2), 808–819.

Zhang, X., et al., 2021. Pan-cancer analysis of PARP1 alterations as biomarkers in the prediction of immunotherapeutic effects and the association of its expression levels and immunotherapy signatures. *Frontiers in immunology*, 12, 721030.

Zhou, X.R., et al., 2013. A study of extraction process and *in vitro* antioxidiant activity of total phenols from Rhizoma Imperatae. *African journal of traditional, complementary, and alternative medicines*, 10 (4), 175–178.

Zhou, Y., et al., 2021. Comparison of outcomes and prognostic factors between early-stage cervical adenocarcinoma and adenosquamous carcinoma patients after radical surgery and postoperative adjuvant radiotherapy. *Cancer management and research*, 13, 7597–7605.

Zou, W., et al., 2021. Imperatae rhizoma-Hedyotis diffusa Willd. herbal pair alleviates nephrotic syndrome by integrating anti-inflammatory and hypolipidaemic effects. *Phytomedicine: international journal of phytotherapy and phytopharmacology*, 90, 153644.